Trypanosoma congolense invariant surface glycoprotein: a potential diagnostic antigen for animal African trypanosomiasis.

by

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PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, from February 2015 to December 2016, under the supervision of Professor THT Coetzer. The study represents the original work by the author and has not otherwise been submitted in any other form to another University. Where use has been made of the work of others, it has been duly acknowledged in the text.

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Melanie Naidoo

As the candidates Supervisor I agree to the submission of this dissertation.

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Prof. Theresa H. T. Coetzer

DECLARATION – PLAGIARISM

I, Melanie Naidoo, 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.

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    a. Their words have been re-written but the general information attributed to them has been referenced.
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Melanie Naidoo

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'With God, all things are possible'
(Matthew 19:26)

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ABSTRACT

Trypanosoma congolense is a pathogenic protozoan parasite causing animal African trypanosomiasis, also called nagana, in livestock in Sub-Saharan Africa. The disease results in economic losses with increased livestock mortality. Vaccine development is unlikely due to a lack of long term antibody response to trypanosomal antigens by the host and antigenic variation of the parasite. Effective control of the disease is thus required; however, trypanocidal drug treatments are inefficient in clearing the infection due to drug resistance. Current diagnostic tests lack specificity and use native trypanosomal proteins that require *in vivo* or *in vitro* cultivation of parasites which is expensive, time consuming and can lead to inconsistent results. Due to these challenges, recombinant trypanosomal proteins are being explored as alternative diagnostic antigens.

Invariant surface glycoproteins (ISG) are transmembrane proteins located on the surface of trypanosome bloodstream forms and does not undergo antigenic variation. The aim of the study was to therefore examine *T. congolense* invariant surface glycoprotein (*Tc*ISG) as a potential diagnostic antigen for animal African trypanosomiasis. The *TcISG* gene coding the signal peptide, extracellular domain, transmembrane domain and intracellular transmembrane domain was cloned and expressed as a histidine tagged fusion protein within inclusion bodies in *Escherichia coli* BL21 (DE3) cells. Recombinant *Tc*ISG was solubilised with anionic detergents, refolded and purified using nickel affinity chromatography. Purified *Tc*ISG and an N-terminal peptide corresponding to immunogenic epitopes were selected and used to generate antibodies in chickens. Both types of antibodies demonstrated increased specificity following affinity purification and recognised native ISG in *T. congolense* lysates. *Tc*ISG was able to distinguish between non-infected and *T. congolense* infected cattle sera in an indirect antibody detection ELISA and was not recognised by antibodies against *T. brucei* ISG65 and ISG75 as well as *T. b. gambiense* VSGs RoTat 1.2, LiTat 1.3 and LiTat 1.5. These results show promise for *Tc*ISG to serve as a species-specific diagnostic antigen while further testing may demonstrate its possible use in lateral flow devices.

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ABBREVIATIONS

2 x YT	2 x yeast extract, tryptone
AAT	animal African trypanosomiasis
ABTS	2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
BCA	bicinchoninic acid
BCM	buffy coat method.
BES	bloodstream form expression sites
BSA	bovine serum albumin
BSF	bloodstream form
CATT	card agglutination test
CDD	conserved domain database
CRAM	cysteine-rich acidic repetitive transmembrane protein
DDT	dichlorophenyl-trichloroethane
DEAE	diethyl amino-ethyl
dH_2O	distilled water
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
E64	L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
g	G-force (RCF)
GARP	glutamate- and alanine- rich protein
GPI	glycosylphosphatidyl inositol
h	hour
HAT	human African trypanosomiasis

HRPO	horse radish peroxidase
IFAT	indirect fluorescent antibody test
IgG	immunoglobulin G
IgY	Immunoglobulin Y
IL-10	interlukein-10
IPTG	isopropyl-β -D-thiogalactopyranoside
ISG	invariant surface glycoprotein
LAMP	loop-mediated isothermal amplification
m-AECT	miniature anion-exchange chromatography technique
MBS	maleimidobenzoyl-N-hydroxysuccinimide ester
MEC	molecular exclusion chromatography
min	minute
PARPs	procyclic acidic repetitive proteins
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PF	procyclic form
PRS	protease resistant surface molecule
PSG	phosphate saline glucose
QBC	quantitative buffy coat
RA	rabbit albumin
RAB	ras-related proteins in brain
rISG65-1	recombinant ISG65-1
RME-8	required for receptor-mediated endocytosis-8
RNA	ribonucleic acid
RT	room temperature
s	seconds
Sarkosyl	N-lauroylsarsocine sodium salt
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE	Tris-acetate-EDTA

TBS	Tris buffered saline
*Tc*ISG	*Trypanosoma congolense* ISG
TfR	transferrin receptor
TM	transmembrane domain
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
VAT	variable antigen type
VSG	variable surface glycoprotein
WHO	World Health Organisation
X-gal	isopropyl-β-D-thiogalactopyranoside

CHAPTER 1

LITERATURE REVIEW

1.1 African trypanosomiasis

Trypanosomes are unicellular protozoan parasites which cause trypanosomiasis in both animals and humans in Sub-Saharan Africa (Baral, 2010). Animal African trypanosomiasis (AAT) also locally known as 'nagana' is caused by *Trypanosoma brucei brucei, T. congolense and T. vivax* (Schofield and Kabayo, 2008). Human African trypanosomiasis (HAT) also known as 'sleeping sickness' is caused by two sub-species of *Trypanosoma brucei*, *T. brucei gambiense* and tsetse fly (*Glossina spp*). The location of the tsetse fly can be divided into three different groups: *morsitans* (savannah), *palpalis* (riverine) and *fusca* (forest) (Uilenberg and Boyt, 1998) according to their habitat to overlap with areas containing livestock and wild animals (Figure 1.1) (Weitz, 1963). The tsetse fly is exclusively located over an area of 10 million km^2 of Africa known as the 'tsetse belt' (Barrett *et al*, 2003). The tsetse fly vector facilitates the cyclic or mechanical transmission of the parasite between various mammalian hosts (Baral, 2010). Trypanosomes transmitted cyclically include: *T. brucei gambiense*, *T. congolense* and *T. vivax* (Desquesnes and Dia, 2003). However, mechanical transmission can also occur in *T. vivax and T. evansi* (Hoare, 1972; Desquesnes and Dia, 2004). *Trypanosoma brucei brucei*, a subspecies of *T. brucei* which infects animals, is dispersed across sub-Saharan Africa whereas human pathogens, *T. brucei gambiense* is found in West and Central Africa while *T. brucei rhodesiense* is found in Eastern and Southern Africa (Hoare, 1972; Balmer *et al*, 2011).

Of the two trypanosomes that infect humans, *T. brucei gambiense* causes a chronic disease resulting in death if untreated (Blum *et al*, 2012) whereas *T. b. rhodesiense* HAT causes an acute disease that develops swiftly leading to death within six months (Odiit *et al*, 1997 and Blum *et al*, 2012). According to the World Health Organisation (WHO), *T. brucei gambiense* is responsible for 98% of reported sleeping sickness cases (WHO, 2013).

Tsetse fly transmitted AAT affects cattle and small ruminants (goats and sheep) and has a devastating effect on the economy with direct meat and milk production losses and indirect losses (draught power, hides and manure) amounting to a $4.75 billion annual loss in agricultural gross domestic product (Swallow, 2000; Chanie *et al*, 2013). Approximately 40 million cattle are vulnerable to the disease of which 3 million die annually from AAT (Eyford *et al*, 2011). Animal

African trypanosomiasis caused by the dominant *T. congolense* species continues to seriously harm livestock and the farming industry (Abera *et al*, 2016).

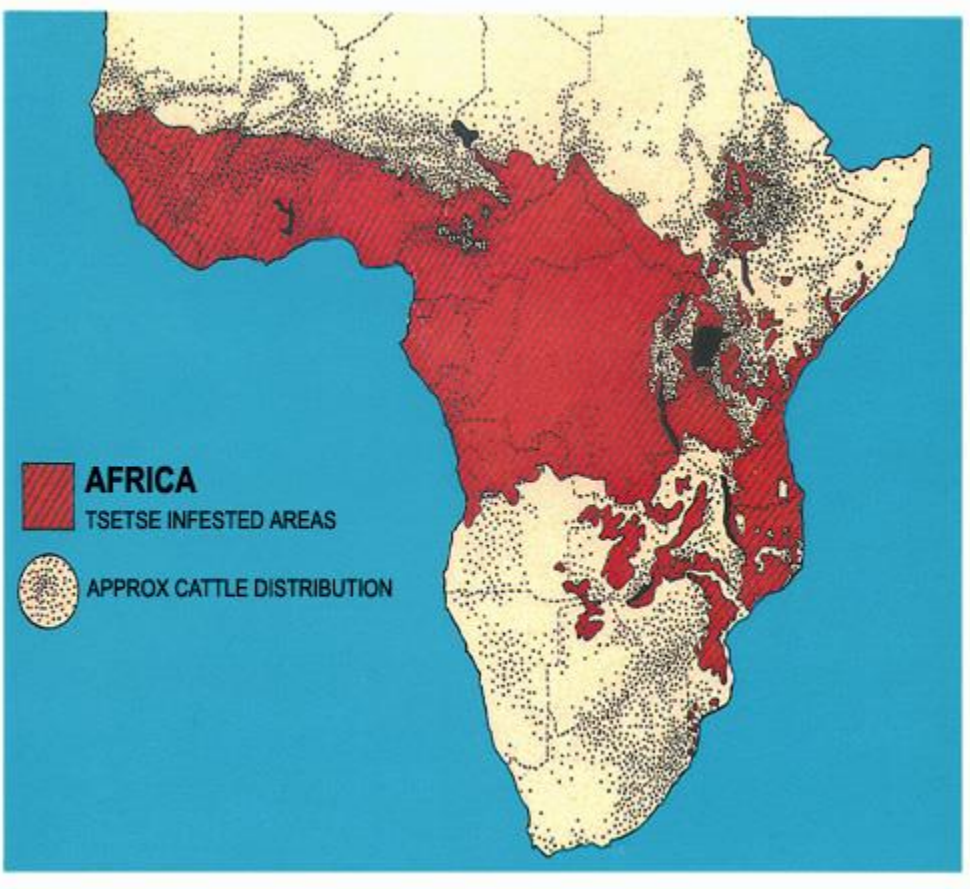

Figure 1.1: Map of the African continent illustrating the distribution of tsetse fly populations and cattle raising regions. (http://www.irinnews.org/news/2009/05/12/tsetse-fly-costs-agriculture-billions-every-year).

1.2 Classification of trypanosomes

Trypanosomes belong to the Kingdom protozoa and the family of Trypanosomatidae (Hoare, 1972) as displayed in Figure 1.2. Trypanosomes are assembled in the order Kinetoplastida due to the presence of a DNA containing kinetoplast (Hoare, 1972 & Baral, 2010). The genus '*Trypanosoma'* is further divided into two groups: stercoraria and salivaria (Hoare, 1972 & Baral, 2010). Stercoraria parasites develop within the intestinal tract of the vector and transmission occurs through the faeces of the parasite. The stercoraria group is further divided into three subgenera: *Schizotrypanum*, *Megatrypanum* and *Herpetomonas* (Hoare, 1972). Conversely,

salivarian parasites develop within the vector gut and the infective metacyclic forms are injected into the host bloodstream during a blood meal (Hoare, 1972). The salivarian group is further divided into four subgenera, *Trypanozoon*, *Duttonella*, *Nannomonas* and *Pycnomonas* (Hoare, 1972). All parasites belonging to the salivarian group are transmitted by the tsetse fly (*Glossina spp*) and are established in Africa i.e. *T. vivax*, *T. congolense* and *T. brucei* (Hoare, 1972 & Baral, 2010).

1.3 Morphology of trypanosomes

Trypanosoma congolense parasites located in the bloodstream of cattle possess a single elongated mitochondrion and a centrally positioned nucleus (Morrison *et al*, 1981; Stevens and Brisse, 2004). The medium sized kinetoplast containing DNA is situated towards the posterior end of the parasite (Figure 1.3). *Trypanosoma congolense* parasites are approximately 8-24 µm in length. The posterior ends of the small parasites appear rounded whereas in larger parasites the posterior ends are typically pointed (Stevens and Brisse, 2004). The parasite also possesses a surface coat which plays a significant role in instigating a relentless infection (Morrison *et al*, 1981). The surface coat consistently covers the plasma membrane and is composed of glycoproteins (Morrison *et al*, 1981).

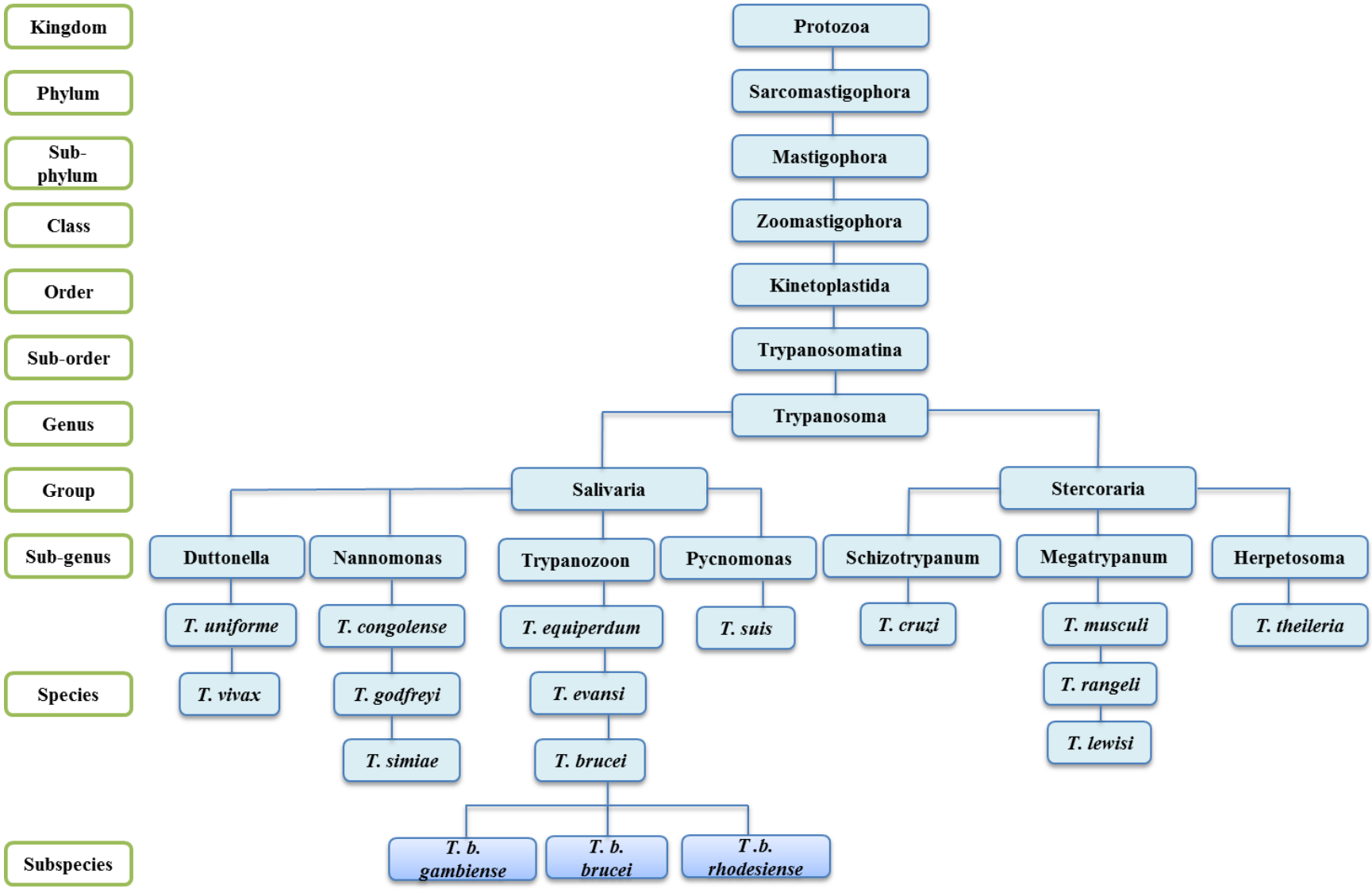

Figure 1.2: Classification of trypanosomes. (Adapted from Baral, 2010).

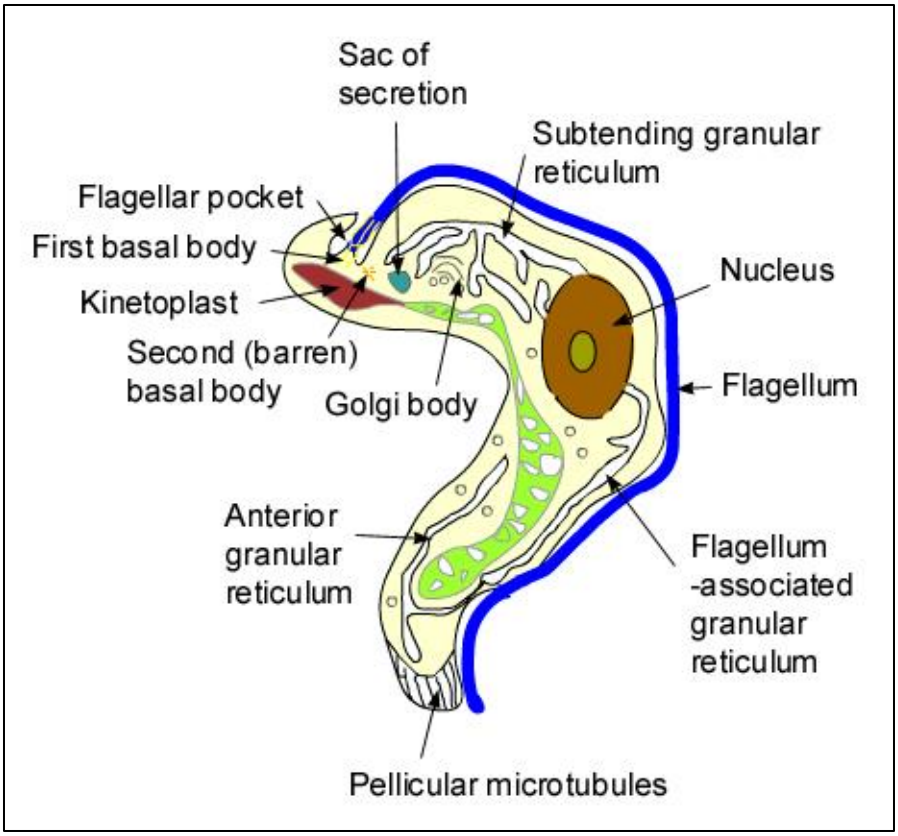

Figure 1.3: Morphological features of *Trypanosoma congolense* in its bloodstream form. (Vickerman, 1969a).

1.4 Life cycle of African trypanosomes

The life cycle of African trypanosomes begin when the tsetse fly feeds on the blood of the host by piercing the skin with its proboscis resulting in metacyclic trypomastigotes entering the host bloodstream (Figure 1.4) The metacyclic trypomastigotes then develop into the infective bloodstream trypomastigotes known as the blood stream form (BSF) at the infection site. Blood stream forms appear long and slender and proliferate by the process of binary fission and enter various organs via the bloodstream (Hoare, 1972).

Within the host bloodstream, BSF trypanosomes are heavily coated with approximately 10^7 copies per cell of variable surface glycoprotein (VSG) (Turner, 1985). There are up to 1000 VSG genes coding prospective surface coat proteins, however only one VSG gene is expressed at any given time (Gruszynski *et al*, 2006). At the peak of parasitemia, BSF trypanosomes adopt a non-proliferative short and stumpy appearance in response to stumpy induction factor (Vassella *et al*, 1997; Nolan *et al*, 2000). The BSFs are then ingested by the tsetse fly when it obtains a

blood meal. In the fly the BSFs rapidly shed their VSG surface coat (Cross, 1975; Roditi and Liniger, 2002). The removal of the VSG coat occurs through the action of a proteolytic enzyme, i.e a zinc metalloprotease in conjunction with glycosylphosphatidylinositol-specific phospholipase C (Ziegelbauer *et al*, 1993 and Gruszynski *et al*, 2006).

Thereafter, the BSFs differentiate into procyclic trypomastigotes also known as procyclic forms (procyclics) within the midgut of the tsetse fly (Vickerman, 1985). *Trypanosoma congolense* procyclics express a procyclin coat known as protease resistant surface molecule (PRS) (Bütikofer *et al*, 2002) or glycosylphosphatidylinositol (GPI) anchored procyclic acidic repetitive proteins (PARPs) in addition to GPI anchored glutamate- and alanine- rich protein (GARP) within the midgut of the tsetse fly (Bayne *et al*, 1993; Urwyler *et al*, 2007). The PRS are generously expressed by early procyclics and weakly expressed during the late procyclic stage. The GARP is weakly expressed and decreases in expression during the early stages of the procyclics (Bütikofer *et al*, 2002). *Trypanosoma brucei* procyclics expressing PARPS containing two forms known as EP-procyclins consisting of a Glu-Pro dipeptide and GPEET-procyclins comprising of a Glu-Pro-Glu-Glu-Thr pentapeptide repeat (Roditi *et al*, 1998). During the early stages of the procyclics, both procyclins, EP and GPEET are expressed (Vassella *et al*, 2000).

The procyclics then travel to the proventriculus of the tsetse fly, undergo asymmetric division and attach to the proboscis as epimastigote forms (epimastigotes) with elevated expression of GARP (Bütikofer *et al*, 2002; Coustou *et al*, 2010). Finally, the epimastigotes differentiate into infective metacyclic forms (metacyclics) which are coated in VSG molecules (Tetley *et al*, 1987; Urwyler *et al*, 2007). The infective metacyclics are then transmitted to a new host during a blood meal (Hoare, 1972). *Trypanosoma brucei* procyclics differentiate into epimastigotes within the salivary gland where they undergo proliferation and express GPI anchored *brucei* alanine-rich protein. The epimastigotes differentiate into infective metacyclics and reacquire a VSG coat (Tetley *et al*, 1987; Bütikofer *et al*, 2002; Matthews, 2005).

The life cycle of *T. vivax* begins by the ingestion of BSF trypanosomes from the host blood during a blood meal. Unlike *T. congolense* BSFs which travel to the midgut of the vector and differentiate into procyclics, *T. vivax* BSFs migrate directly to the proboscis of the vector and differentiate into epimastigotes thereby missing the procyclic stage in the vector gut (Hoare, 1972). The absence of a vector gut stage made it possible for *T. vivax* to spread to other continents where the tsetse fly is absent.

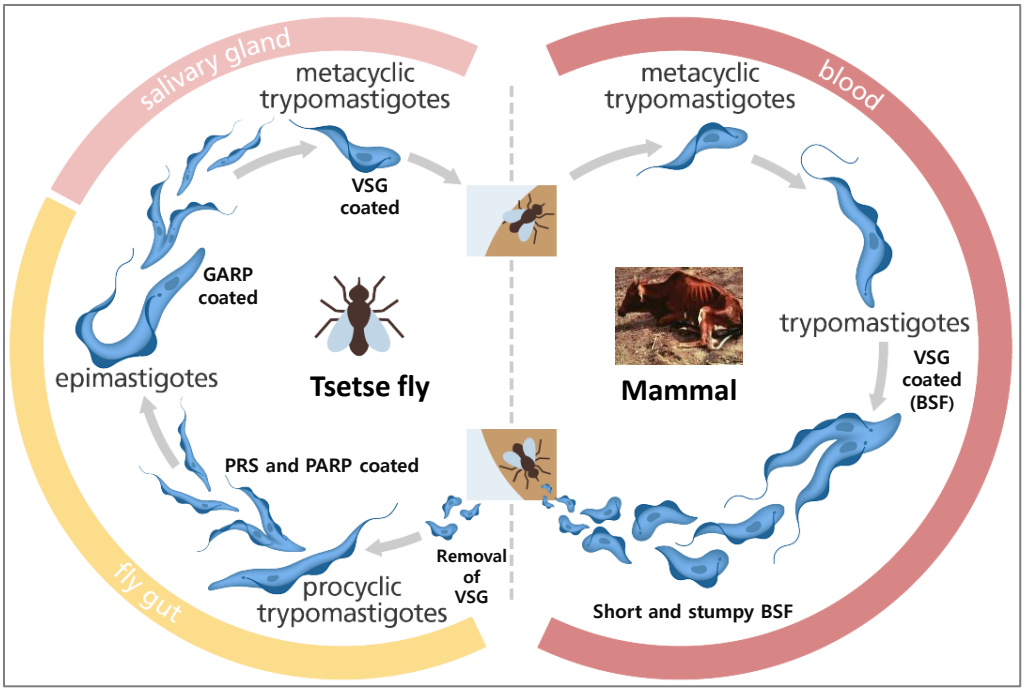

Figure 1.4: Diagrammatic representation of *T. congolense* life cycle. Image is courtesy of Genome Research Limited (Adapted from http://www.yourgenome.org/facts/what-is-african-sleeping-sickness).

1.5 Clinical features of animal African trypanosomiasis

When infective trypanosomes are injected into the host, a chancre develops, which is evident by swelling and inflammation after 2-3 weeks. At this site of infection, trypanosomes reproduce and differentiate into bloodstream forms which invade the lymph nodes and eventually the bloodstream of the host (Morrison *et al*, 1981; Uilenberg and Boyt, 1998).

Inflammatory responses experienced by the infected animal include oedema, vascular congestion and extravasation of polymorphonuclear leukocytes (Morrison *et al*, 1981). The infection begins with hyperthermia followed by host antibody production against parasite surface proteins (Uilenberg and Boyt, 1998). Although some parasites are destroyed by these antibodies, a few trypanosomes survive due to their ability to alter their surface coats which prevents the antibodies from destroying them (Uilenberg and Boyt, 1998; Barry and Carrington, 2004). The remaining active parasites reproduce and an increased parasitemia level along with hyperthermia generates a new set of antibodies against the new surface coat of the parasite in the host. This process is known as antigenic variation and continues until either the parasite exhausts its antigenic repertoire or when the host dies (Barry and Carrington, 2004).

The acute stage of AAT is characterised by a range of symptoms including weakness, intermittent fever, roughness of the coat, weight loss, abortion, enlargement of peripheral lymph nodes, pyrexia, and low milk yield (Conner, 1994). Lack of treatment can lead to death of the animal (Taylor and Authié, 2004). During chronic infection, anaemia can be prevalent for 3 to 4 months post infection (Morrison *et al*, 1981). During this stage, haemolytic anaemia results from escalated erythrocyte death by the process of phagocytosis in the lungs, bone marrow, spleen, liver and haemal nodes (Morrison *et al*, 1981). The erythrocytes are believed to be coated with the lysed parasites which allow the phagocytes to remove the foreign material causing a depletion of haemoglobin and consequently oxygenated blood leading to deterioration and wasting of the animal (Uilenberg and Boyt, 1998).

Progression of the disease results in nutritional deficiencies, immunosuppression and simultaneous infections (Morrison *et al*, 1981). During trypanosomal infections, many tissues and organs are damaged such as the heart resulting in heart failure (Morrison *et al*, 1981). Bodily systems affected by the infection include: skeletal muscle, endocrine organs and reproductive tract leading to premature births, abortions, testicular impairment and perinatal loss (Morrison *et al*, 1981).

1.6 Diagnosis of trypanosomiasis

Effective control of AAT can be provided by early detection of the disease followed by treatment. There are several diagnostic tests available for use however; each test varies in sensitivity specificity as well as cost and applicability for field use. Direct detection of parasites in infected sera provides increased specificity, whereas indirect tests detect more parasites (Uilenberg and Boyt, 1998; Büscher *et al*, 2009). Serological diagnostic tests such as indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and card agglutination test (CATT) detect parasite antigens or anti-trypanosomal protein antibodies in infected animal sera (Voller *et al*, 1975; Songa and Hamers, 1988; Nantulya and Lindwvist, 1989). Molecular diagnostic tests have been explored to provide species differentiation thereby providing increased test specificity (Desquesnes and Dávila, 2002).

1.6.1 Direct detection

Motile parasites are observed microscopically in wet blood films from infected animals (Uilenberg and Boyt, 1998; Eisler *et al*, 2004). This is a simple and inexpensive diagnostic method. However, species identification cannot be performed and test sensitivity is limited to approximately 10^4 parasites per ml of blood (Uilenberg and Boyt, 1998). Thick blood smears involve staining a sample of infected blood with Giemsa on a slide followed by visualisation of parasites under a microscopic lens (Figure 1.5, panel A). Thin blood smears entail spreading the blood sample on the slide followed by staining with Giemsa for identification of parasites (Figure 1.5, panel B), however, the test sensitivity is very low (Eisler *et al*, 2004). Although these methods are commonly used, they are not sensitive enough to identify parasites at low parasitemia levels in large animals or after cure. Collecting blood samples during the early hours of the morning and from the ear or underside of the animal may increase the chance of parasite detection (Eisler *et al*, 2004).

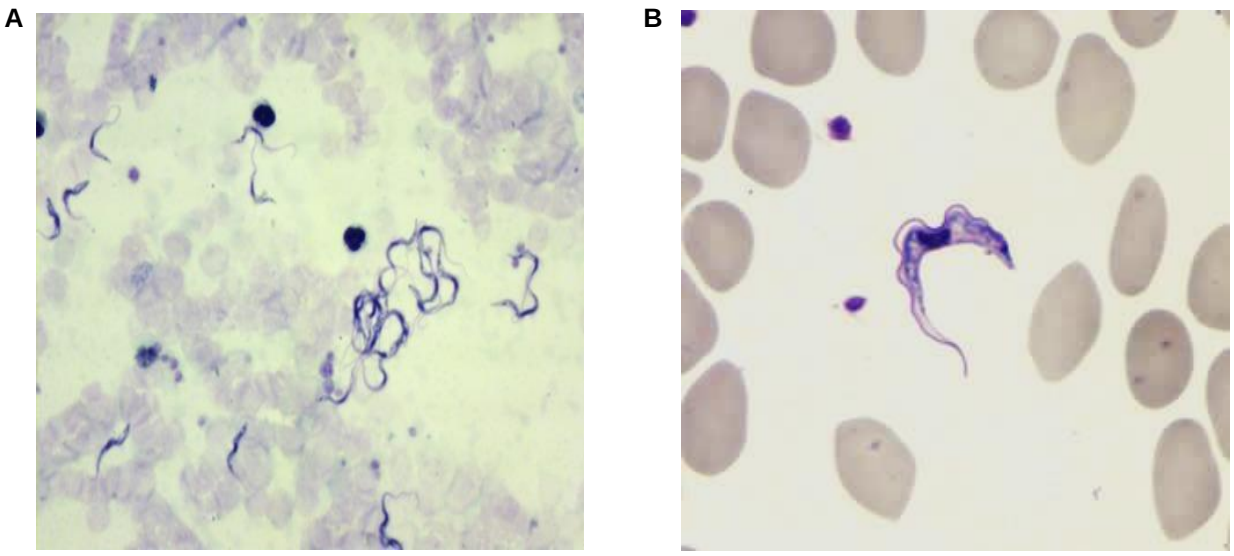

Figure 1.5: Trypanosomes stained with Giemsa in blood smears. **(A)** Thick and **(B)** thin blood smears for the detection of parasites. (Cottle *et al*, 2012; Centre of Disease Control and Prevention, https://www.cdc.gov/dpdx/trypanosomiasisAfrican/gallery.html).

The microhaematocrit centrifugation technique also known as the Woo method is based on the separation of the blood components according to their individual gravity (Woo, 1970). Blood samples are collected in heparinised capillary tubes and centrifuged. The buffy coat is studied through the capillary tube for the presence of motile trypanosomes using long microscopic focal lenses to enhance lens focusing during examination of the deep capillary tube (Woo, 1970).

This technique is considered highly sensitive and inexpensive in comparison to blood film methods and the diagnosis of anaemia can be performed. However, specialised equipment is required and species identification is not possible (Uilenberg and Boyt, 1998). The sensitivity of the method is 6525 parasites/ml (Paris and McOdimba, 1982).

The buffy coat (BCM) or Murray method is similar to the microhaematocrit centrifugation technique, however the capillary tube is cut and the buffy coat and erythrocyte layer are dispersed onto a microscope slide. Using dark-ground or phase-contrast microscopy, motile trypanosomes can be observed. *Trypanosoma congolense* parasites appear small, slow-moving and cling to erythrocytes through their anterior end (Murray *et al*, 1977). Similar to the Woo method, the BCM can also detect the presence of anaemia and both methods are considered highly sensitive (Desquesnes, 2004). Another technique used for rapid detection of trypanosomes is the quantitative buffy coat (QBC) (Bailey and Smith, 1992) which uses centrifugation to separate the components of infected blood and acridine orange is used to stain DNA (Bailey and Smith, 1994).

Sub-inoculation of infected blood into laboratory animals such as mice, rats, rabbits or guinea pigs can be employed to detect low levels of parasitemia (Morrison *et al*, 1981). Briefly, animals are injected intraperitoneally with the infected blood and parasitemia levels are monitored by tails pricks and microscopic analysis of the blood using the wet blood film method (Uilenberg and Boyt, 1998). Parasitemia levels are monitored for approximately two months after inoculation. This method is commonly avoided since results are not immediate and the animals have to be cared for which raises ethical concerns (Morrison *et al*, 1981; Uilenberg and Boyt, 1998).

1.6.2 Indirect detection

The miniature anion-exchange chromatography technique (m-AECT) is commonly used to diagnose HAT caused by *T. brucei rhodesiense* and *T. brucei gambiense*. Infected blood is collected and allowed to pass through a diethyl amino-ethyl (DEAE) cellulose matrix. Erythrocytes adhere to the resin since they are more negatively charged than the trypanosomes whilst the trypanosomes pass directly through the resin. The collected trypanosomes are centrifuged at a low speed and examined microscopically for motility in a transparent tube (Lumsden *et al*, 1979). This technique allows for the detection of fewer than 50 trypanosomes/ml since the volume of blood examined is large (Lumsden *et al*, 1979; Büscher *et*

al, 2009). Since 2006, the collection tubes, column racks and microscope viewing compartment have been redesigned to increase the robustness of the technique (Figure 1.6) (Büscher *et al*, 2009).

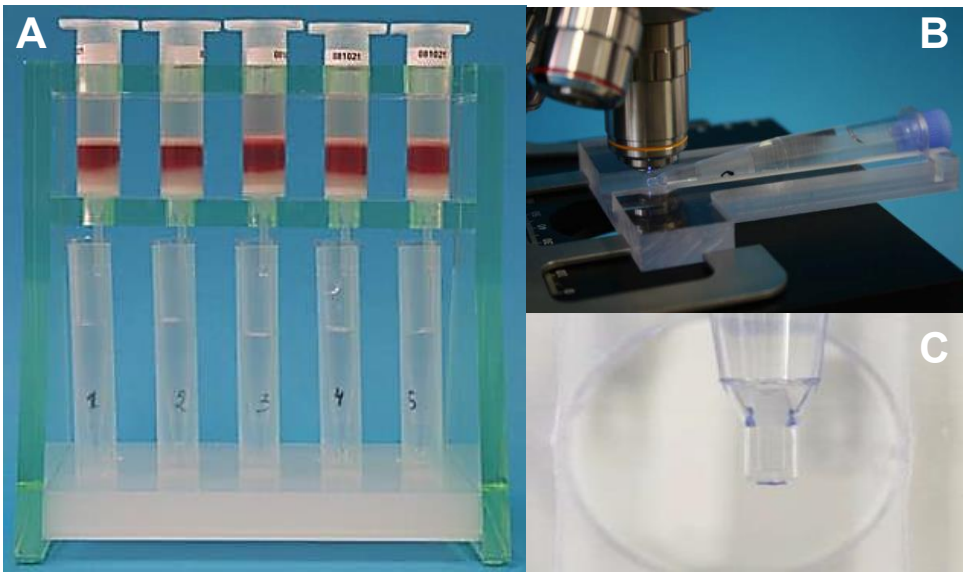

Figure 1.6: Illustration of miniature anion-exchange centrifugation technique. **(A)** Elution of parasites from blood into collector tubes using columns mounted onto a rack. **(B)** Visualisation of motile parasites by mounting the collector tube in the microscope viewing chamber. **(C)** Enlarged image of the collector tube tip (Büscher *et al*, 2009).

1.6.3 Serological testing

Serological testing uses antibody detection methods to detect antibodies produced by the host against trypanosomal proteins circulating in the bloodstream of the host (Uilenberg and Boyt, 1998). Antibody based techniques include IFAT and ELISA which were initially used for detection of antibodies against *T. brucei rhodesiense (*Voller *et al*, 1975). The detection of antibodies against the parasite does not demonstrate the presence of active infections as these antibodies can persist in the host bloodstream for long periods of time after cure (Uilenberg and Boyt, 1998).

The IFAT involves the binding of anti-trypanosome antibodies from infected sera to the antigen which is fixed onto a microplate. The antigen can be whole parasites or specific parasite proteins. Since each microplate typically contains 96 wells, several sera samples can be tested in a short space of time (Uilenberg and Boyt, 1998). Anti-bovine IgG antibody conjugated to a fluorescent tag or enzyme is added and the fluorescence is monitored. This test cannot

differentiate between different species and requires expensive specialised equipment and reagents, making it unsuitable for field use (Uilenberg and Boyt, 1998).

Antigen detection tests use a polyclonal or monoclonal antibody in an ELISA to capture trypanosomal antigens from the blood of the infected animal (Nantulya and Lindwvist, 1989). The antibody-antigen complex is detected using a secondary antibody conjugated to an enzyme (Uilenberg and Boyt, 1998). The test may be species specific and allows for large numbers of samples to be processed due to its automation. This test has been used extensively, yet its sensitivity was shown to be very low and cross reactivity with other *Trypanosoma* species occurred (Uilenberg and Boyt, 1998). In comparison to the microhematocrit centrifugation technique, this assay is more effective (Masake and Nantulya, 1991).

Agglutination tests such as CATT for trypanosomes uses whole, fixed or stained parasites as antigens (Songa and Hamers, 1988). The antigens cluster in the presence of anti-trypanosomal antibodies which indicate a positive reaction (Figure 1.7). Additionally, an antigen capture latex agglutination assay has been established and is based on the principle of coating the latex particle with the capture antibody. In the presence of the target antigen in the infected sera, the particles begin to agglutinate indicating a positive reaction (Songa and Hamers, 1988; Defontis *et al*, 2012).

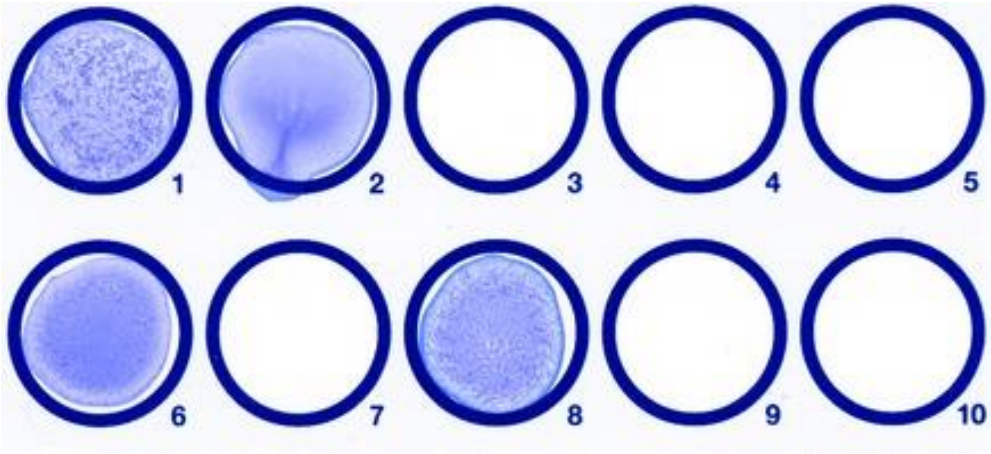

Figure 1.7: Illustration of the CATT/*T. evansi* test using infected cerebrospinal fluid and serum. The test reagent is fixed Coomassie blue stained *T. evansi* expressing VSG RoTat 1.2. The test reagent is mixed with the infected samples and an agglutination reaction occurs if antibodies against the test reagent are present within the infected samples and visualised by the blue dye. Well 1, positive control, well 2, negative control, well 6, cerebrospinal fluid (weak positive reaction), well 8, infected serum (strong positive reaction) and the rest of the wells were unused. (Defontis *et al*, 2012).

1.6.4 Molecular testing

Molecular testing in diagnostics refers to the detection of trypanosomal nucleotide sequences specific to a species, subgenus and strain (Desquesnes and Dávila, 2002). The earliest techniques established were DNA sequencing and DNA-probe synthesis followed by polymerase chain reaction (PCR) (Majiwa *et al*, 1994). Trypanosomes can be identified in the tsetse fly using PCR (Uilenberg and Boyt, 1998).

Polymerase chain reactions target specific trypanosomal genes present in a high copy number which elevates the chance of DNA amplification (Desquesnes and Dávila, 2002). Trypanosomal DNA can be obtained from whole or fragmented parasites in the host blood. Katakura and colleagues demonstrated the use of three specific repetitive DNA sequences that can be amplified from three subgroups of *T. congolense* parasites. Primer set TCN1 and TCN2 were designed for Savanna subgroup, TCF1 and TCF2 primer set for Forest subgroup and TCK1 and TCK2 primer set for Kenya Coast subgroup (Katakura *et al*, 1997). The technique proved to be highly sensitive and provided species identification and recognition from mixed infections. Another *T. congolense* DNA target for PCR includes mini-chromosomes which consist of satellite DNA (Masiga *et al*, 1992). In a separate study, a single PCR was able to distinguish between different *Trypanosoma* parasites affecting livestock (Desquesnes *et al*, 2001). However, the test was disregarded for field use due to the lack of sensitivity. This study was improved upon by using a single PCR in association with restriction fragment length polymorphism (Geysen *et al*, 2003). These molecular tests have proven superior to parasitological techniques since the infecting *Trypanosoma* species can be identified (Desquesnes and Dávila, 2002). Due to the presence of ‘ghost DNA’ i.e. DNA from dead trypanosomes, active infections cannot be identified (Desquesnes, 1997).

The loop-mediated isothermal amplification (LAMP) test amplifies DNA with elevated specificity and efficacy using an isothermal environment (Notomi *et al*, 2000). The technique uses four specifically designed primers instead of two primer sets in the case of PCR (Notomi *et al*, 2000). This technique allows for amplification of the target DNA with increased selectivity and specificity (Notomi *et al*, 2000; Kuboki *et al*, 2003). Moreover, LAMP is the superior technique since the reaction result can be determined visually by the reaction turbidity and expensive toxic fluorescent dyes are not required (Mori *et al*, 2001).

A fluorescence in situ hybridisation test using peptide nucleic acid PNA probes offer a rapid and simplified way of parasite detection. The technique involves incubating blood smears with

fluorescein-labelled probe which target 18S rRNA sequences not present in other protozoan parasites and is resistant to nucleases and proteases. Due to the specificity of the technique, a single parasite can be identified with a detection limit of 500 trypanosomes/ml of blood which proves superior to other direct detection tests (Radwanska *et al*, 2002).

1.7 Vector control

Trypanocidal drugs such as diminazene, isometamidium and homidium have been used to treat cattle for over 50 years (McDermott and Coleman, 2001). Numerous doses of these drugs are administered by livestock owners in an attempt to control trypanosomiasis (Geerts *et al*, 2001). However, due to the development of drug resistance, eradication of the vector appears to be the most suitable option as a long term solution (Van den Bossche and Delespaux, 2011).

Insecticides are frequently sprayed on vegetation where tsetse flies rest during the dry season. Dichlorophenyl-trichloroethane (DDT) was employed to control the vector population using aerial or ground spraying. However, such techniques had posed a health risk to the spraying team and the environment (Figure 1.8) (Holmes, 1997). Due to the high risks and increased cost factor, sequential aerosol spraying was introduced. Small volumes of non-residual insecticides for aerial spraying in combination with other control methods were used in a cost effective and efficient manner without harming the environment in Kenya, Zambia, South Africa, Zimbabwe, Uganda and Botswana (Grant, 2001; Vreysen *et al*, 2013).

The elimination of bushes removes the resting place of the savannah species of the vector (Hocking *et al*, 1963). This method of control is risky as tsetse fly populations may follow transported bushes which pass by animal herds susceptible to AAT hence this method is no longer performed (Uilenberg and Boyt, 1998). Dark blue or black screen cloths treated with insecticides such as pyrethroids have been used to destroy tsetse fly populations (Vale, 1993; Rayaisse *et al*, 2010). The technique can be enhanced by using attractive cloths and pleasant odour mixtures to lure the tsetse flies (Holmes, 2013). Additionally, ‘pour-on’ insecticides or live bait control can be used on animals in combination with an adjuvant ensuring the chemical is evenly distributed over the belly and legs (Thompson *et al*, 1991; Torr *et al*, 2007). Some cattle are also tagged with insecticide-impregnated ear collars (Leak *et al*, 1996). Disadvantages associated with live bait control include: increased insecticide cost, insecticide resistance and frequent treatment of the animals (Leak, 1999).

Figure 1.8: Vector control methods. (A) Ground spraying of DDT using a spraying machine in Zimbabwe. **(B)** Aerial spraying of insecticide using a rotary atomizer**. (C)** Biconical Trap used in Kenya to attract tsetse flies which are apprehended in the lower cone and travel towards the light upwards where they are retained in a cage or bag. **(D)** Blue and black cloths impregnated with pyrethroids in Botswana. (Allsopp, 2001).

The sterile insect technique uses radiation treated sterile tsetse flies in an attempt to reduce surviving vector populations after other control methods such as insecticides have been utilised (Dyck *et al*, 2005). The technique is based on releasing increased numbers of the sterilised male flies to out-compete the wildtype males. No offspring is produced upon mating of the sterile male fly with the wildtype female thereby reducing the prevalence of infective tsetse fly populations and transmission of trypanosomes. The major shortcoming of the technique is the high expense and logistical constraints (Holmes, 2013).

1.8 Trypanotolerance

By definition, trypanotolerance is the trait which allows an animal to tolerate trypanosome infection and remain productive (Murray *et al*, 1982; D'Leteren *et al*, 1998). Such animals are able to control the reproduction of the parasite and do not display any clinical symptoms of the disease. Trypanotolerance has been reported in cattle breeds located in West Africa such as N'Dama, Boran and Zebu as well as in goat and sheep breeds. These animals are typically in

continuous interaction with the infected tsetse flies which allow them to generate a stronger and swift immune response to VSG epitopes in comparison to trypanosusceptible breeds (Sileghem *et al*, 1993; Williams *et al*, 1996). Trypanotolerant animals are capable of reducing parasitemia levels, controlling anaemia and continue to remain productive (Murray *et al*, 1982).

In a separate study, F2 calves derived from N'Dama and Boran grandparents were utilised to identify the loci associated with trypanotolerance phenotypes (Hanotte *et al*, 2003). The study identified 18 quantitative trait loci of which the majority were associated with the control of anaemia (Hanotte *et al*, 2003). Naessens and colleagues demonstrated two mechanisms of trypanotolerance by cross breeding the Boran and N'Dama cattle resulting in Chimeric Boran/ N'Dama calves. This encompassed the improvement of anaemia control using the haematopoietic system and an innate mechanism, independent of the haematopoietic system which improved the control of parasites (Naessens *et al*, 2003).

1.9 Drug treatment for African trypanosomiasis

There are several drug treatments commercially available for the treatment of AAT which exhibit toxicity towards the parasite and reduce parasite reproduction, thus allowing the host immune system to overcome the infection (Uilenberg and Boyt, 1998). Trypanocidal drugs remain the primary treatment of AAT (Holmes and Torr, 1988) and can be distinguished as therapeutic and/or prophylactic. Key therapeutic drugs include diminazene aceturate, homidium bromide and homidium chloride (Figure 1.9). Prophylactic drugs comprise of isometamidium chloride, homidium bromide and chloride. When AAT is detected within a herd, the entire herd is usually treated for the disease since the infection may not be restricted to sick animals or AAT positively tested animals (Holmes and Torr, 1988).

Quinapyramine was successfully used as a prophylactic drug since the 1950s to treat infected cattle, however after just seven years of use; the drug was discontinued due to the rise of drug resistance (Curd and Davey, 1949). The drug was substituted with diminazene aceturate also known as Berenil®, Azidine®, Veriben®, Ganasegur® and Ganaseg® (Tsegaye *et al,* 2015). Berenil® is a therapeutic drug utilised in the treatment of animal trypanosomiasis since 1955 (Kuriakose and Uzonna, 2014). *Trypanosoma congolense* infected BALB/c mice treated with Berenil® had demonstrated the production of anti-trypanosomal antibodies and parasitemia was controlled (Uzonna *et al*, 1999). The shortcomings of the drug include drug resistance through the misuse of Berenil®, disease regress and increased cost (Kuriakose and Uzonna, 2014).

Phenanthridium compounds include Novidium® which is a mixture of homidium chloride and bromide and Ethidium®, a mixture of ethidium bromide and bromide salt (Tsegaye *et al,* 2015). Novidium® and Ethidium® have been available since 1952 for the treatment of *T. congolense* and *T. vivax* infections in cattle, horses, goats and sheep (Watkins and Woolfa, 1952; Tsegaye *et al,* 2015). Isometamidium chloride (Samorin® or Trypamidium®) is both a therapeutic and prophylactic phenanthridium compound available for use since 1961 (Tsegaye *et al,* 2015). Pyrithidium Bromide (Prothidium®) has been extensively used as a prophylactic drug since 1956 in East Africa to treat infected cattle. Trypanosomes had quickly established resistance to the drug and Berenil® was then employed to treat the Prothidium® resistant parasites (Melaku and Birasa, 2013).

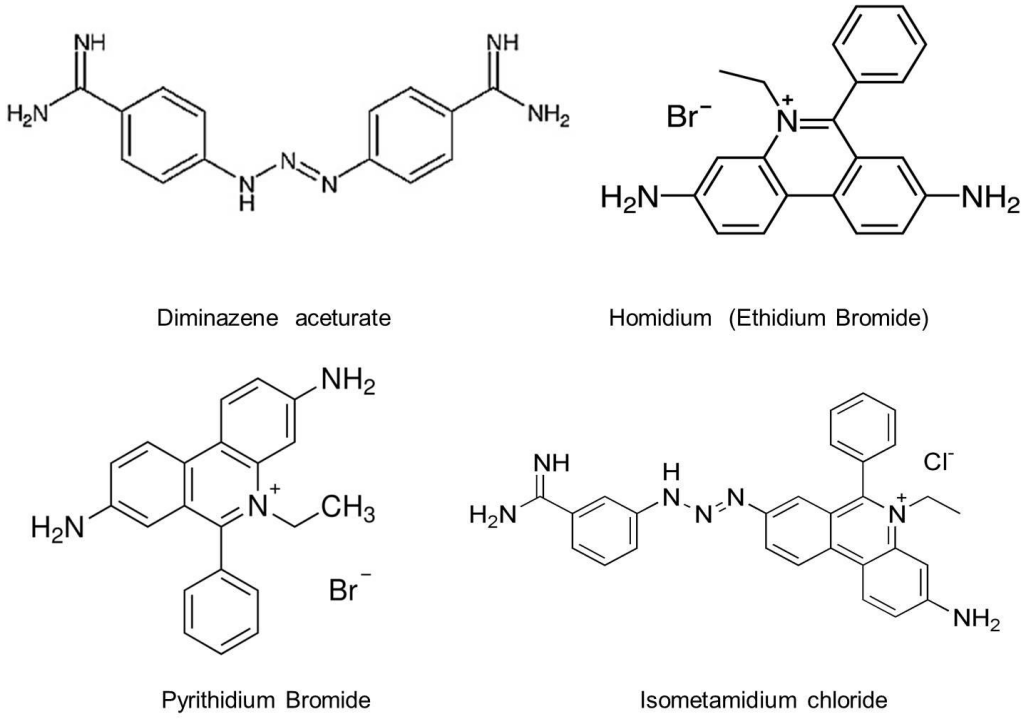

Figure 1.9: Structures of frequently used trypanocides in chemotherapy of AAT in Africa. (Adapted from Melaku and Birasu, 2013).

Unfortunately, all trypanocides depicted in Figure 1.9 are susceptible to trypanosome resistance. *Trypanosoma congolense* is the leading parasite in developing drug resistance during treatment. This is due to farmers underestimating the weight of their cattle, under dosing

chemotherapeutic drugs and misdiagnosis of the disease resulting in only a few parasites being destroyed. The surviving parasites then develop resistance towards the administered drug.

In an attempt to combat drug resistance, the anti-trypanocidal activity from *Dovyalis abyssinica* (Salicaceae) plant extracts was investigated. The extract was capable of reducing parasitemia levels in mice and enhanced the body weight and survival time of the animal in comparison to dimethylsulfoxide treatment (Tadesse *et al*, 2015). Similarly, extracts from the *Albizia schimperiana* (Fabaceae) leaf exhibited anti-trypanosomal activity by reducing parasitemia levels in mice (Tesfaye *et al*, 2015).

1.10 Vaccination

The ultimate goal of developing a vaccine for both HAT and AAT has not been accomplished to date. Early vaccine trials employed the use of VSG molecules since they exist in high abundance on the surface of trypanosomes. Unfortunately, due to antigenic variation displayed by the parasites and the short lived host antibody response, VSGs could not be used (Cornelissen *et al*, 1985; La Greca and Magez, 2011). Another potential source for vaccine candidates originated from the flagellar pocket of trypanosomes. The flagellar pocket is known to participate in endocytic processes and cell division and is a contributing factor to parasite virulence. Studies conducted showed that mice immunised with *T. brucei rhodesiense* or *T. brucei* flagellar pocket extract conferred partial protection to the mice (Mkunza *et al*, 1995; Radwanska *et al*, 2000). Unfortunately, protection was shown to be temporary and the idea of the flagellar pocket as a vaccine candidate was terminated. Cytoskeletal proteins such as actin and tubulin have also been investigated as vaccine candidates but met with little success. Using murine models, recombinant and non-recombinant forms of actin and tubulin were shown to provide partial protection against infection by induction of a host antibody response (Lubega *et al*, 2002; Li *et al*, 2007; 2009). Unsuccessful results were obtained when recombinant ISG65 and ISG75 from *T. brucei* were used to vaccinate mice (Ziegelbauer and Overath, 1993). In another approach, vaccination of cation pump ATPase's failed to induce long term protection (Ramey *et al*, 2009). Targeting the parasite itself showed limited success in developing a vaccine therefore an anti-disease vaccine was explored using the GPI anchor of VSG molecules. Mice treated with a liposome based GPI vaccine, increased IL-10 cytokine levels and alleviated clinical symptoms such as weight loss, anaemia, liver damage and motor skill damage but the vaccine could not provide complete and long term protection (Stijlemans *et al*, 2007). Keeping with the anti-disease vaccine strategy, vaccination with a *T. congolense*

cysteine protease known as congopain was postulated to reduce the severity of AAT symptoms in immunised cattle (Authié *et al*, 2001). Mice immunised with a *T. brucei* DNA vector coding a bloodstream stage ISG were partially protected against low dose infection while remaining live mice demonstrated increased IgG2a antibody levels (Lança *et al*, 2011).

Mice vaccinated with recombinantly expressed ISG75 extracellular domain resulted in increased levels of anti-ISG75 titers; however no protection was detected when challenged with *T. brucei* parasites. Further experiments indicated the lack of a B-cell memory response during an active infection suggesting suppression or elimination of specific antibody responses (Magez and Radwanska, 2014). In a separate study, mice vaccinated with DTPa vaccine Boostrix® were protected against bacterial infection however no protection was observed when the vaccinated mice where challenged with *T. brucei* and treated with Berenil®. These results indicated damage to the host B-cell compartment in the presence of trypanosomes (Radwanska *et al*, 2008). Each stage of the B-cell development process is associated with specific markers on the cell surface eg. Immunoglobulin M (IgM) is typically expressed on immature B-cells. Models depict the swift destruction of T1 B cells and marginal zone B cells which are known to lead to the production of IgM expressing cells. The destruction of T1 B cells prevents IgM B cell regeneration thus preventing protection from new variable antigens expressed by the parasite. Immunoglobulin M producing plasma cells are also destroyed after the first peak of parasitemia (Radwanska *et al*, 2008; Bockstal *et al*, 2011). It is clear that the success of forthcoming vaccine trials will depend on eliciting a long term IgM response through increased B cell memory and the complete eradication of parasites during infection (Magez and Radwanska, 2014)**.**

1.11 Trypanosomal membrane proteins

In this section, the most important trypanosomal membrane proteins that are either diagnostic or vaccine candidates will be discussed. These membrane proteins include variable surface glycoprotein, flagellum membrane proteins, p67 membrane glycoprotein and invariant surface glycoprotein.

1.11.1 Variant surface glycoprotein

The surface coat of trypanosomes is typically composed of VSGs (Ziegelbauer and Overath, 1992; Barry and Carrington, 2004). The genome of the parasite contains 10^7 VSG genes that undergo antigenic variation (Mehlert *et al*, 1998). The glycoproteins are homodimers which are covalently attached to the trypanosome surface via a carboxyl group present on the C-terminal

of the amino acid on a GPI anchor. The anchor is situated in the outer part of the plasma membrane. The VSG molecules pack densely to create a protective surface barrier (Overath *et al*, 1994b). Crystallography studies conducted on two *T. brucei* VSG variants, MiTat 1.2 and LiTat 1.24 concluded the presence of a conserved tertiary structure for all the VSG type 2 C-terminal domains (Figure 1.10). The C-terminal domain begins with a short alpha helix followed by a turn which is connected to an anti-parallel beta sheet via a short loop. The results also suggested that VSG dimers are formed via N-terminal interactions. The N-terminal domain is made up of seven alpha helices and constitutes approximately 47% of the structure (Freymann *et al*, 1990; Chattopadhyay *et al*, 2005).

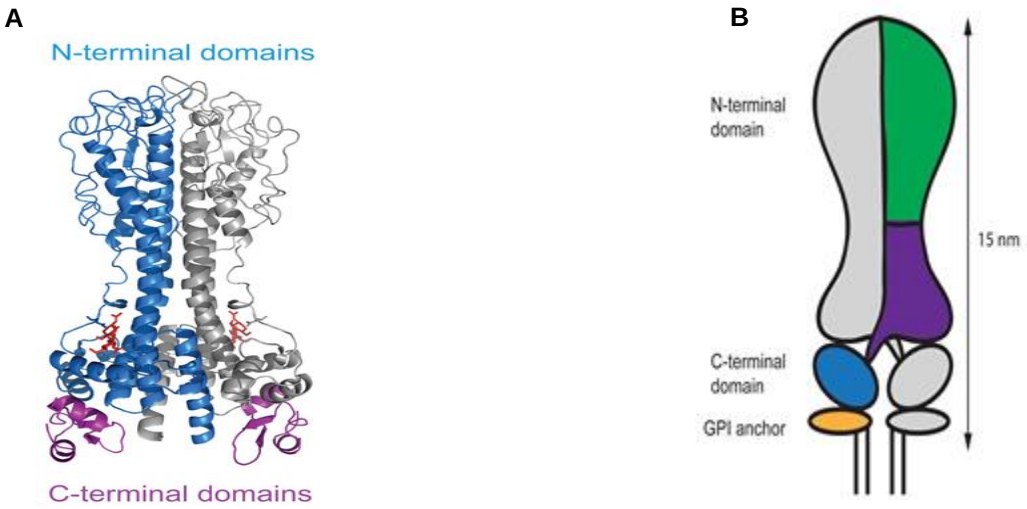

Figure 1.10: Diagrammatic representation of the MiTat 1.2 VSG dimer. (A) One monomer is represented in grey and the other in blue. The C-terminal regions are shown in purple and the N-linked oligosaccharide is represented in red (PDB: 1VSG and 1XU6). **(B)** One monomer is shown in grey; the C terminal is represented in blue and the GPI anchor in orange. The green indicates the presence of a loop connecting the helix structures and the purple represents the end of the N terminal region. Adapted from (Schwede and Carrington, 2010; Schwede *et al*, 2015).

The process of antigenic variation occurs when the parasite sequentially switches expression of one *VSG* gene for another. Each surface variant is identified as a variable antigen type which provides variation and prevents detection of the parasite from the host antibodies thereby protecting the parasite from death (Figure 1.11). Once the surface coat of the parasite has been altered, parasites expressing the new VSG surface coat reproduce and remain undetected from the previous host antibody immune response (Morrison *et al*, 2009). This phenomenon allows the parasite to escape the host immune response rendering the host defenceless against the parasite (Mehlert *et al*, 1998). An IgM host antibody response is initially produced in response to

the presence of VSG coated trypanosomes and is responsible for the elimination of parasites. Trypanosomes coated in VSG molecules are typically destroyed within a week before the production of a host IgG antibody response (Mugnier *et al*, 2016).

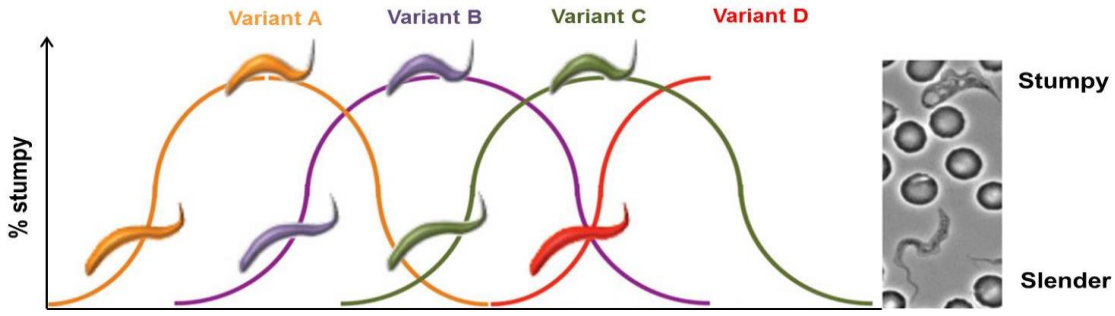

Figure 1.11: Diagrammatic representation of a trypanosome parasitemia profile. Each parasitemia wave represents the change of proliferative slender parasite forms into arrested stumpy forms expressing different VATs on the trypanosome surface. (Matthews *et al,* 2015).

The *VSG* genes are found on four different loci on mini chromosomes and located close to the telomeres. Variable surface glycoprotein genes are situated in the bloodstream form expression sites (BES) which contain a promoter and numerous expression site-associated genes (ESAGs) (Becker *et al*, 2004; Hertz-Fowler *et al,* 2008; Young *et al*, 2008). Approximately 25 genes are found in the metacyclic expression sites that are only active in metacyclic forms of the parasite, almost 1500 genes in the sub-telomeric arrays and roughly 200 genes in the diploid genome (Graham *et al*, 1999). From the many silent *VSG* genes, only 4% are complete genes, 21% are gene fragments and 65% are pseudogenes (Weirather *et al*, 2012). There are three different mechanisms of antigenic variation known as gene conversion, telomere exchange and transcriptional switching. Gene conversion involves replacing one *VSG* gene with a copy of another in the BES (Figure 1.12). In telomere exchange, there is crossing over of the *VSG* gene from the active BES to the silent BES and transcriptional switching involves the down regulation of the active BES followed by the activation of another bloodstream expression site (Borst and Fairlamb, 1998; Borst, 2002).

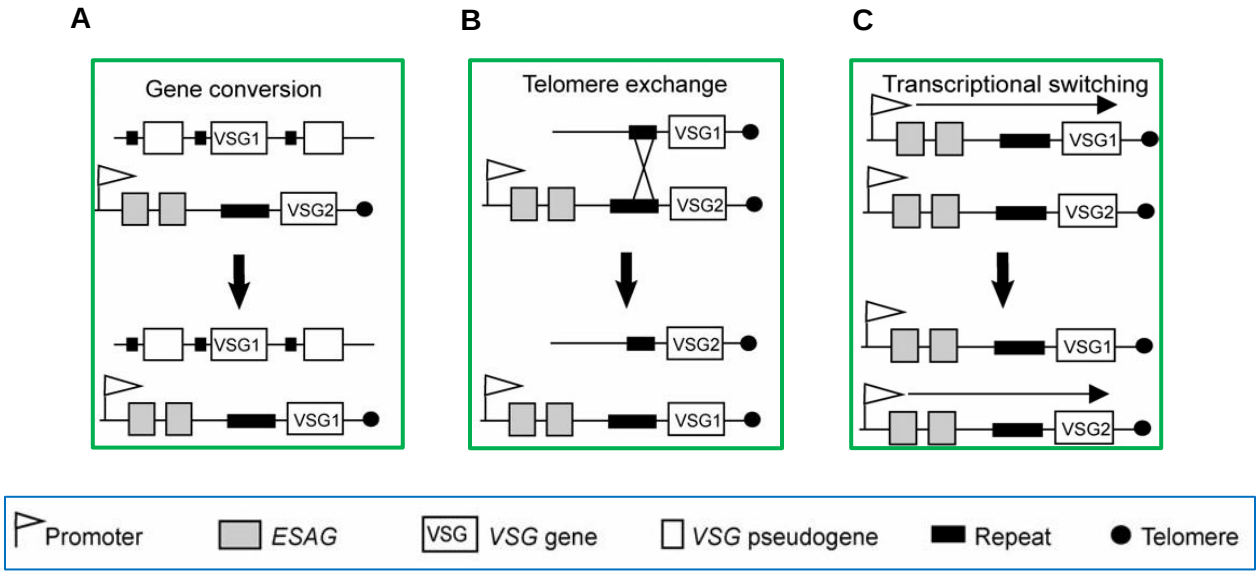

Figure 1.12: Mechanisms of antigenic variation. (A) In gene conversion, the active *VSG* gene is replaced by another *VSG* gene copy. **(B)** Telomere switching allows the active *VSG* gene to be transferred to a silent site while the silent *VSG* is transferred to the active site. **(C)** In transcriptional switching, the active VSG gene is down regulated whilst another *VSG* is activated. (Adapted from Schwede and Carrington, 2010).

Variable surface glycoproteins have a long half-life and undergo endocytic processing (Seyfang *et al*, 1990). The fundamental mechanism of *T. brucei* endocytosis occurs only in the region of the flagellar pocket and is mediated by clathrin (Allen *et al*, 2003). The VSGs journey from the endoplasmic reticulum (ER) through the Golgi apparatus and endosomes, and finally arrive at the cell surface (Gründfelder *et al*, 2002). Other components required for endocytosis include epsin (Ford *et al*, 2002), dynamin (Merrifield *et al*, 2002) and an adaptor complex (Boehm and Bonifacino, 2002). Variable surface glycoproteins are internalised by the trypanosome in large clathrin coated vesicles followed by the delivery of the cargo to early endosomes marked by ras-related proteins in brain (Rab) (Pal *et al*, 2002, 2003; Field and Carrington, 2009). Mediated by a Rab11-positive carrier in the lumen of the cisternal endosomes, VSGs are recycled to the cell surface upon fusion of the Rab11 carrier to the flagellar pocket membrane (Jeffries *et al*, 2001; Gründfelder *et al*, 2003). Endocytosis and recycling of VSGs are reported to take place within twelve minutes (Engstler *et al*, 2004).

Variable surface glycoproteins have contributed significantly to the improvement of diagnostic testing. These VSG based tests were utilised for the detection of *T. evansi* infections which cause surra in wild and domestic animals in Africa (Bauman and Zessin, 1992), Asia (Payne *et*

al, 1991) and South America (Franke *et al*, 1994). One such VSG, known as RoTat 1.2, has been used in the development of ELISA/*T. evansi* test for the detection of anti-RoTat 1.2 antibodies in infected sera (Büscher *et al*, 1995). A direct and indirect agglutination test, CATT/*T. evansi* (Songa and Hamers, 1988) and LATEX/*T. evansi* (Verloo *et al*, 1998) were also developed. The ELISA/*T. evansi* showed test specificity of 95% while CATT/*T. evansi* showed 98% and LATEX/*T. evansi* 82% specificity (Verloo *et al*, 2000). The ELISA/*T. evansi,* CATT/*T. evansi* and LATEX/*T. evansi* use native VSG RoTat 1.2 proteins. Recombinant VSG RoTat 1.2 has been evaluated as a potential diagnostic antigen and compared to CATT/*T. evansi* and LATEX/*T. evansi*. The results demonstrated no significant differences in test specificity and sensitivity suggesting the possible use of recombinant RoTat 1.2 instead of the native RoTat 1.2 antigen (Lejon *et al*, 2005; Sengupta *et al*, 2014).

In an attempt to eliminate the use of rodents for antigen production for serodiagnosis, recombinant *T. b. gambiense* VSG LiTat 1.3 and LiTat 1.5 were tested in an ELISA against sera from HAT infected patients and compared to native LiTat 1.3 and LiTat 1.5 (Rogé *et al*, 2014a). Individual native LiTat 1.3 and LiTat 1.5 proved superior to their recombinant forms. However, a combination of recombinant LiTat 1.3 and LiTat 1.5 produced promising results in replacing native LiTat antigens (Rogé *et al*, 2014a). Fragments of *T. evansi* VSG RoTat 1.2 were recombinantly expressed and could distinguish between the pre-immune and infected sera from goats experimentally infected with *T. evansi* (Rogé *et al*, 2013) and was used to develop a latex agglutination test for the diagnosis of *T. evansi* infections (Rogé *et al*, 2014b). The test is known as rLATEX/*T. evansi* and had demonstrated 82.4% sensitivity that was comparable to the CATT/*T. evansi* and had a higher specificity. The rLATEX/*T. evansi* test serves as an alternative diagnostic test with enhanced specificity using recombinantly produced antigens thus animals are not required for antigen production (Rogé *et al*, 2014b).

The diagnostic potential of VSG LiTat 1.5 peptide from *T. b. gambiense* was examined and compared to native LiTat and LiTat 1.5 in an indirect ELISA against sera from HAT patients. The synthesised VSG peptide demonstrated diagnostic potential almost identical to native VSG and may have the potential to replace the native antigen in diagnostic testing (Van Nieuwenhove *et al*, 2013). Two diagnostic tests were developed for *T. b. gambiense* infections using native LiTat 1.3 and LiTat 1.5 known as HAT Sero-strip and HAT Sero-K-set (Büscher *et al*, 1991; 2014).

1.11.2 Flagellum membrane proteins

The flagellum attachment zone is a domain through which the flagellum is attached to the cell body of the parasite. The flagellum plays a pivotal role in parasite motility, growth and the arranging of organelles which can be explored as therapeutic targets (Vickerman, 1969b; Sherwin and Gull, 1989; Kohl *et al*, 2003). The FLA1 protein is expressed by procyclic and BSF trypanosomes and localises in the flagellum as well as the flagellar pocket and is vital for flagellum attachment. Inhibition of FLA1 expression demonstrated a deficiency in flagellum attachment and inhibition of cytokinesis (LaCount *et al*, 2002). A putative calcium channel, FS179, located at the flagellum attachment zone plays a role in the attachment of the parasite flagellum (Oberholzer *et al*, 2011). Additionally, *T. brucei* FLA3 membrane protein associated with the flagellum attachment zone was recently identified by Woods and colleagues (Woods *et al*, 2013). The FLA3 membrane protein undergoes N-linked glycosylation and is only detected during the bloodstream stage of the parasite. The knockdown of the *FLA3* gene resulted in defective attachment of the flagellum and prevented cytokinesis in slender forms of the parasite (Woods *et al*, 2013).

Flagellar pocket associated receptor protein known as cysteine-rich acidic repetitive transmembrane protein (CRAM) is located on the surface of *T. brucei* procyclic flagellar pocket. The receptor comprises of a putative N-terminal peptide, an extracellular domain consisting of a 12 residue cysteine rich repeat, a transmembrane domain and finally a C-terminal hydrophilic cytoplasmic region containing 41 amino acids (Lee *et al,* 1990). The cytoplasmic region was hypothesised to contain two domains: one domain located near the C terminus provides effective transportation of CRAM and the second domain plays a role in restricting CRAM to the flagellar pocket (Yang *et al*, 2000). The protein has a predicted molecular weight of 200 kDa and undergoes glycosylation. This receptor has been implicated to function as a lipoprotein receptor in *T. brucei* procyclic forms (Liu *et al*, 2000) while mutagenesis studies revealed its significant role in effective transport from the ER to the flagellar pocket during receptor mediated endocytosis (Yang *et al*, 2000; Qiao *et al*, 2006).

The transferrin receptor (TfR) from *T. brucei* coded by ESAG6 and ESAG7 is exclusively expressed by BSF trypanosomes. The TfR is heterodimeric and located in the flagellar pocket with approximately 2 300 copies per cell (Salmon *et al*, 1994; Steverding *et al*, 1994, 1995). The receptor is GPI anchored to the flagellum membrane and attached to the ESAG6 gene product. Only ESAG6 is known to be GPI anchored and associates with ESAG7 via non-covalent

bonding. *Trypanosoma brucei* uses TfR to scavenge transferrin from the host (Steverding *et al*, 1998).

1.11.3 p67 membrane glycoprotein

Trypanosoma brucei p67, previously known as CB1-gp, is a lysosome-associated, type I membrane-like glycoprotein that undergoes excessive N-linked glycosylation (Brickman and Balber, 1993; Kelley *et al*, 1999). The transmembrane domain is composed of 19 residues and the cytoplasmic region consists of 24 residues (Kelley *et al*, 1999). In bloodstream forms, p67 exists as a 100 kDa protein known as gp100. Following processing in the Golgi apparatus, gp100 is converted into full length gp150. The full length gp150 is directed to the flagellar pocket where it is internalised and transported to the lysosome for degradation (Langreth and Balber, 1975; Lonsdale-Eccles and Grab, 1987; Brickman and Balber, 1994). Degradation products produced are gp75, gp42 and gp32. However, in procyclic forms, p67 does not undergo processing the Golgi apparatus and is transported directly to the lysosome (Kelley *et al*, 1995).

1.11.4 Invariant surface glycoprotein

Invariant surface glycoproteins are transmembrane proteins located on bloodstream form trypanosomes (Ziegelbauer and Overath, 1992). These glycoproteins are conserved among the subgenus *Trypanozoon* (*T. b. gambiense*, *T. b. rhodesiense*, *T. equiperderm* and *T. evansi*) and belong to either the ISG65 or ISG75 family (Tran *et al*, 2008). The identification and characterisation of *T. brucei* ISG65 and ISG75 showed that these molecules are only found on bloodstream forms and are sensitive to a trypsin-like proteolytic activity (Ziegelbauer and Overath, 1992; Overath *et al*, 1994a). There are approximately 50 000 and 70 000 copies of ISG75 and ISG65 respectively per cell (Tran *et al*, 2008). Other *T. brucei* ISG molecules discovered are ISG64, ISG70 (Jackson *et al*, 1993) and ISG100 (Nolan *et al*, 1997). Since the structure of the *T. congolense* homologue has not been solved, that of *T. brucei* ISG65 will be presented here.

Both ISG65 and ISG75 polypeptides consist of four domains (Ziegelbauer *et al*, 1995; Eyford *et al*, 2011). The N-terminal hydrophobic signal sequence (28 amino acids) is cleaved resulting in a mature protein. The hydrophilic extracellular region is followed by a single transmembrane domain containing hydrophobic amino acid residues and a hydrophilic cytoplasmic region. Members of the ISG65 family have identical transmembrane domains and a highly conserved

cytoplasmic domain (Chung *et al*, 2004; Schwede *et al*, 2015). There are three conserved lysine residues located in the cytoplasmic domain of the ISG65 family (Chung *et al*, 2004). The predicted structure of *T. brucei* ISG65 cytoplasmic tail reveals an open coiled conformation with Lys62 fully exposed for interaction with cytosolic proteins (Figure 1.13) (Chung *et al*, 2004).

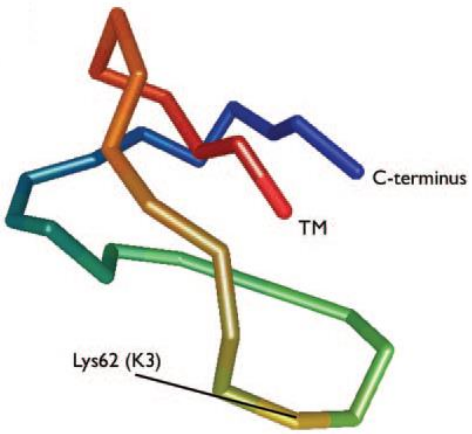

Figure 1.13: Predicted structure of ISG65 cytoplasmic tail. The C-terminus and transmembrane (TM) region is indicated with Lys62 exposed to the cytoplasm (Chung *et al*, 2004).

Membrane trafficking is a vital process in the progression of the cell cycle, infectivity and immune evasion mechanisms (Field *et al*, 2007). Experiments revealed the presence of ISG65 on the surface of endosomal membranes which is recycled faster than VSG molecules (Figure 1.14) (Chung *et al*, 2004). Invariant surface glycoproteins are synthesised in the Golgi apparatus and directly transported to the parasite surface membrane. Membrane trafficking begins at the flagellar pocket, adjacent to the flagellar pocket collar containing bilbo1, a structure known to be necessary for biosynthesis of the flagellar pocket collar (Bonhivers *et al*, 2008). Membrane bound ISG is internalised into clathrin coated early endosomes marked with Rab5 facilitated by EpsinR and transported to sorting endosomes assisted by Rab11 proteins. Upon arrival at the multivesicular body or late endosome, mediated by Rab4 and R11BP1 protein, ISG is recycled back to the parasite surface or transported to the lysosome for degradation. In association with endosomal sorting complex required for transport and factors TbVps23 and TbVps28, ISG is degraded in the lysosome (Leung *et al*, 2008; Field and Carrington, 2009; Henne *et al*, 2011; Koumandou *et al*, 2012).

Leung and colleagues depicted a model in which ISGs are internalised followed by ubiquitination and are targeted for endosomal degradation (Leung *et al*, 2011). The three lysine residues present on the ISG C-terminus was reported to play a significant role in the stability

and regulation of the trafficking process mediated by ubiquitin and endosomal sorting complex required for transport (Chung *et al*, 2008; Leung *et al*, 2011). Recently, the ISG75 subfamily was associated with the uptake of suramin used for HAT treatment (Alsford *et al*, 2012). The suramin compound binds to ISG75 and is targeted for degradation in the lysosome via endocytosis where cathepsin L protease releases suramin from ISG75. Several suramin sensitive genes in *T. brucei* were inspected in the trafficking route of ISG75 which appeared crucial to suramin uptake (Zoltner *et al*, 2015).

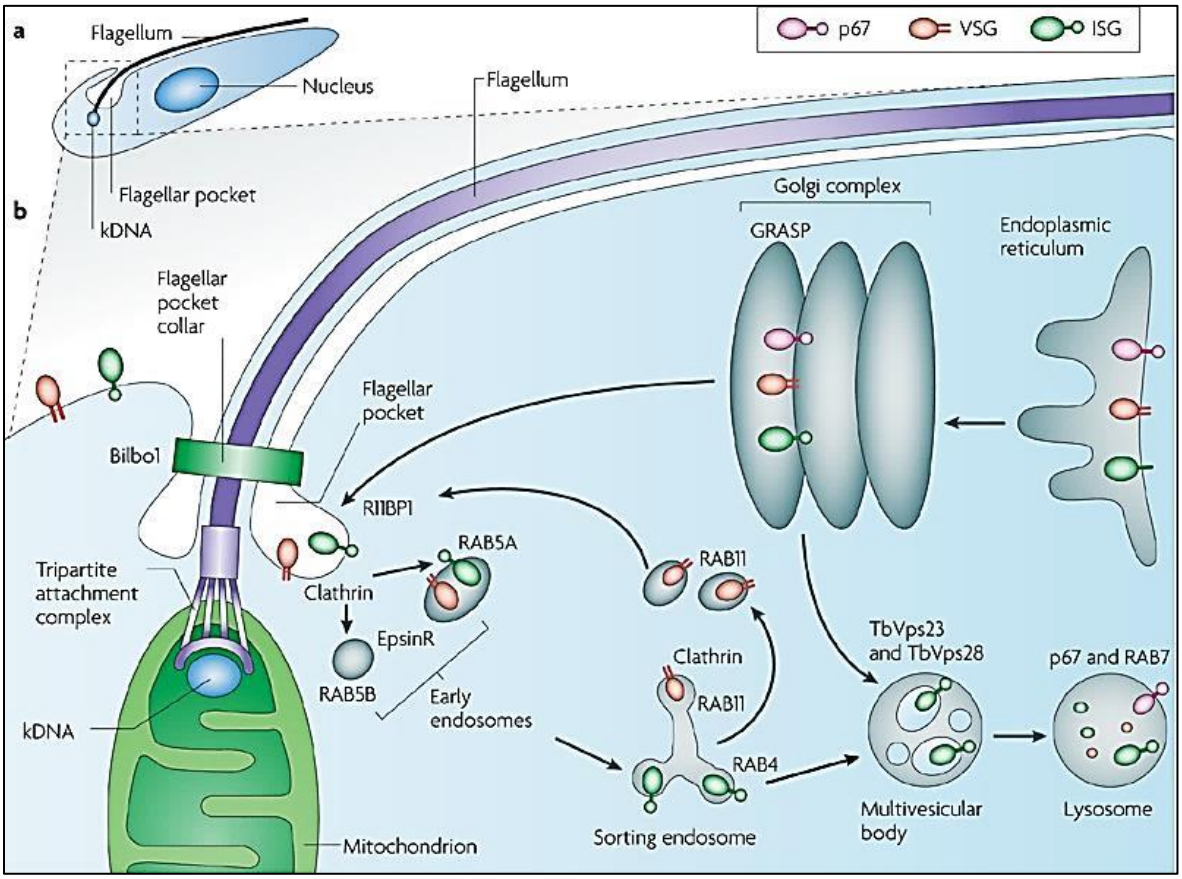

Figure 1.14: Membrane trafficking of *T. brucei* ISG and VSG in the flagellar pocket. (A) Trypanosome nucleus, kinetoplast and flagellum emerging from the flagellar pocket. **(B)** The endocytic pathway for ISG and VSG (see text for details; Field and Carrington, 2009).

1.12 Application of ISG in diagnosis of trypanosomiasis

Invariant surface glycoproteins do not undergo antigenic variation in comparison to VSG which makes them promising potential diagnostic antigens (Ziegelbauer and Overath, 1992; Tran *et al*, 2008). Over the years, several ISG molecules have been examined for their potential to serve as a vaccine or as a diagnostic antigen demonstrating its significance to the parasite.

Invariant surface glycoproteins were explored in relation to *T. evans*i infections in order to develop a new diagnostic reference test (Tran *et al*, 2009). An ELISA based test using *T. evansi* recombinant ISG75 demonstrated 100% specificity and 94.6% sensitivity against *T. evansi* infected camel sera. These results were consistent with the results obtained from RoTat 1.2-based assays: ELISA/*T. evansi*, CATT/*T. evansi* and the immune trypanolysis assay which was the gold standard of serological diagnosis (Tran *et al*, 2009). This assay could potentially be applied for diagnosis of all *Trypanozoon* subgenus members and could replace native antigen based tests (Tran *et al*, 2009). A 52 kDa invariant surface antigen from *T. evansi* was characterised and used to develop an assay for detection of the antigen in infected animal sera (El Hassan, 2014). The results showed early detection of the 52 kDa antigen in infected rabbits and rats and was depicted as a suitable candidate for *T. evansi* diagnosis (El Hassan, 2014).

The gene coding for *T. evansi* ISG75 has also been explored in a PCR based diagnostic assay by screening blood from experimentally infected animals for trypanosomal nucleic acids (Rudramurthy *et al*, 2013). This *ISG-75* gene based PCR assay identified *T. evansi* infections within 24 hours post infection and could prove useful in drug resistance and trypanocidal drug design (Rudramurthy *et al*, 2013). Additionally, the recombinant extracellular domain of *T. evansi* ISG75 has recently showed potential for use in serodiagnosis of AAT (Rudramurthy *et al*, 2015). The test results revealed 99.1% sensitivity and 98.47% specificity indicating once again the strong potential of ISG75 in AAT serodiagnosis.

Since ISG75 is conserved among the *Trypanozoon* subgenus i.e. *T. brucei*, *T. equiperdum* and *T. evansi* (Tran *et al*, 2006), the extracellular domain of *T. gambiense* ISG75 was recombinantly produced and evaluated as a possible diagnostic antigen. Sera from *T. brucei* infected goat recognised recombinant ISG75 in an ELISA. Additionally, antibodies made against recombinant ISG75 detected native ISG75 from *T. brucei, T. brucei gambiense, T. brucei rhodesiense*, *T. equiperdum, T. evansi* and *T. congolense* BSF parasite lysates (Tran *et al*, 2008).

The development of a prototype lateral flow device by Sullivan and colleagues revealed encouraging results using recombinant ISG65-1 (rISG65-1) as a possible diagnostic antigen for *T. gambiense* infections (Figure 1.15) (Sullivan *et al*, 2013). Using the ELISA technique, ISG64 and ISG65 proteins had identified *T. brucei gambiense* infections well in comparison to *T. brucei rhodesiense* infections. The ISG65-1 protein was used in the development of a lateral flow device which displayed sensitivity of 88% and specificity of 93%. The use of a lateral flow device in the field is favorable due to its simplicity and stability (Posthuma-Trumpie *et al*, 2009).

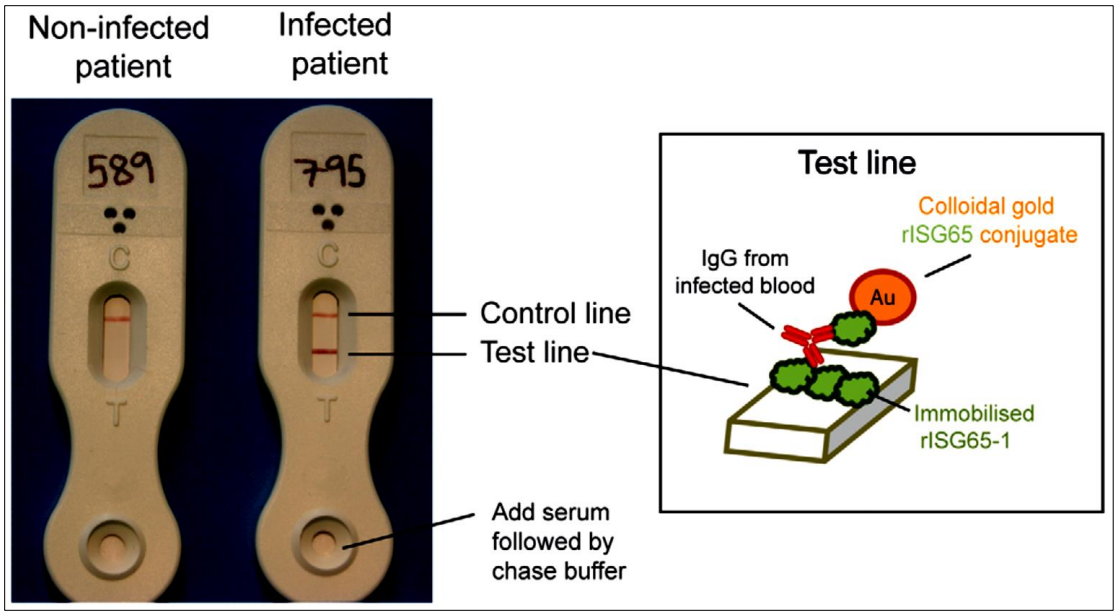

Figure 1.15: The prototype lateral flow device for the detection of *T. b. gambiense* infections. The rISG65-1 protein is immobilised on the nitrocellulose strip and the infected serum containing ant-ISG65 IgG antibody is the added followed by a chase buffer. The reaction is developed using by colloidal gold-coupled rISG65-1 (Sullivan *et al*, 2013).

In respect to *T. congolense* ISG molecules, Eyford and colleagues conducted a study which provided identification of protein expression throughout *T. congolense* life cycle stages (Eyford *et al*, 2011). Membrane proteins identified included VSG, ISG65, ISG75 and TcIL3000.0.38630 gene. Mochabo and co-workers have since identified TcIL3000.0.38630, as a stage specific gene that is abundantly expressed on the surface of *T. congolense* metacyclics and bloodstream forms. The study showed detection of anti-TcIL3000.0.38630 antibodies 7 days post infection in mice and could serve as an immunodiagnostic target (Mochabo *et al*, 2013).

Fleming and colleagues have recently identified antigens for the diagnosis of *T. congolense* infections. Three proteins from the family of ISGs were evaluated in an ELISA against infected sera. Results showed all three proteins performed well in the ELISA while Tc38630 antigen demonstrated 87.2% sensitivity and 97.4% specificity when tested against a blinded panel of experimentally infected cattle sera. The results also indicated that Tc38630 antigen could be useful in monitoring the efficacy of drug treatments and provides promise for the development of a lateral flow test using ISG antigens (Fleming *et al*, 2014).

Recently, ISGs from *T. vivax* were recombinantly expressed and purified for the manufacture of a prototype lateral flow test (Fleming *et al*, 2016). The conjugate pad contains the ISG antigen

known as p310-gold colloid conjugate and an unrelated antibody bound to gold colloid to serve as a control. When the infected serum is applied to the pad along with the chase buffer, the p310-gold colloid conjugate flows up the strip and onto the p310 antigen immobilised on the test line. The anti-p310 antibodies from the infected serum bind on the test line as well as the p310-gold conjugate from the conjugate pad resulting in a visible positive reaction (Figure 1.16). The lateral flow test revealed 92% sensitivity and 89.9% specificity and no cross reactivity was observed with *T. congolense* infected sera. These results show great promise in accurately diagnosing *T. vivax* infections.

A **B**

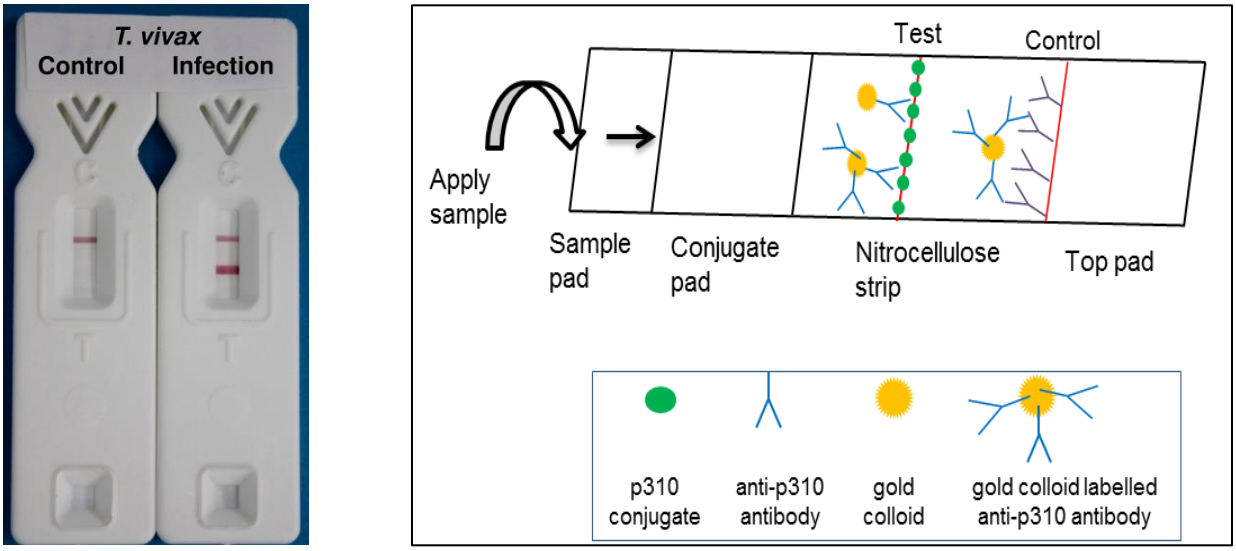

Figure 1.16: TvY486_0045500 prototype lateral flow device for the detection of *T. vivax* infections. **(A)** The top line represents the control line containing an unrelated immobilised protein whereas the bottom is the test line containing *T. vivax* immobilised protein. **(B)** Diagrammatic representation of prototype lateral flow test (Adapted from Fleming *et al*, 2016).

1.13 Objectives of the present study

The overall objective of the current study is to evaluate recombinant *T. congolense* (IL 3000) ISG (*Tc*ISG) as a potential immunodiagnostic antigen for animal African trypanosomiasis.

Specific aims:

1. Obtaining adequate quantities of *Tc*ISG protein though cloning, expression and purification.
2. Investigating the antigenicity of *Tc*ISG.
3. Identification of native ISG from *T. congolense* (IL 3000) bloodstream form lysates.
4. Evaluating the diagnostic potential of *Tc*ISG with non-infected and *T. congolense* infected sera.
5. Exploring the cross reactivity of *Tc*ISG antigen and anti-*T. brucei* ISG and anti-*T. b. gambiense* VSG antibodies.

CHAPTER TWO

CLONING AND RECOMBINANT EXPRESSION OF INVARIANT SURFACE GLYCOPROTEIN FROM *TRYPANOSOMA CONGOLENSE*

2.1 Introduction

Animal African trypanosomiasis is a devastating disease transmitted by trypanosome infected tsetse flies in Sub-Saharan Africa. Trypanosomes are kinetoplast Protozoa that cause direct and indirect losses in animal and crop farming particularly affecting the poorest rural communities (Swallow, 2000 & Eyford *et al*, 2011). The parasites evade host immune responses in a process known as antigenic variation through alteration of the surface coat expressing numerous variable antigen types (Morrison *et al*, 2009). Due to this process, the development of a vaccine is unlikely. Therapeutic and prophylactic drugs such as diminazene aceturate and phenanthridium have been employed to treat trypanosomiasis since the 1950s. However, drug resistance has become evident (Tsegaye *et al,* 2015). Improved diagnostic techniques are thus required for early detection of the parasite to provide accurate treatment. Most diagnostic tests are not species specific or compatible with field use (Uilenberg and Boyt, 1998). In response to these challenges, trypanosomal proteins are being investigated as possible diagnostic targets.

Invariant surface glycoproteins are transmembrane proteins located on the surface of trypanosome bloodstream forms. The ISGs are shielded by large VSG molecules which are also abundantly expressed on the parasite surface (Ziegelbauer and Overath, 1992; Overath *et al*, 1994b). Unlike VSGs, ISGs do not undergo antigenic variation and are exclusively expressed in bloodstream forms making them attractive stage specific diagnostic antigens. The ISGs are attached to the parasite cell membrane via a GPI anchor and are composed of four domains (Figure 2.1) (Ziegelbauer *et al*, 1995; Eyford *et al*, 2011). To date, *Tc*ISG has not been classified under ISG65 or ISG75 family however sequence analysis of *Tc*ISG revealed the presence of conserved domains from both families (Appendix B).

A

MGMQRIVCVGSLLVAVLLSGGATGNAGNSNGVCRLNENAAGLLCTIAKLVEKAKNITENH
DYKDIDETWGYVALHKEVVDHRVKNLPDIIETAKAKGTLTVKDAEHLTTLYLDAHNKNTQ
QHNKSKAAMDAHNKTHEDAKNSTALALGEGYVTGNCNMVSSLLGILQCYVKGEQPHSNLN
VETLCKEKNYNLDESQHTLLTNCNKIGNRKTYCNGTGAALKVALDKWNGMDKKKAADNGN
CEVKKDWEERTKKAQEHMSRLDEHVQIIHDAKLLTTAYFSIVDKIKTGVENGKPMKVIVA
NAREAGQKGAKVVVEKLSVNTENDPHNTTKPLVEEEEVNVNVQLDGLKFDEDENGPAHSK
ESFPKIYIYLLSILLPLFCLVMGITLYCLISKPRSSHPEKSIPVDGATMNKAGDTQAHF

B

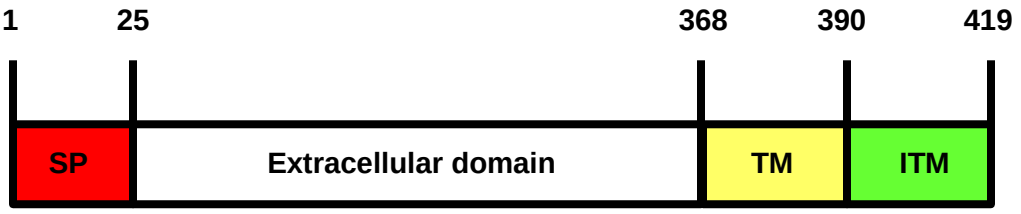

Figure 2.1: Predicted domains of *T. congolense* ISG (*Tc*ISG) polypeptide. **(A)** Amino acid sequence showing the signal peptide (SP) in bold red, the extracellular domain within a black dashed box followed by the transmembrane domain (TM) and intracellular transmembrane domain (ITM) in yellow and green respectively. Conserved ISG65-75 domains are indicated in blue with the predicted GPI anchor in pink. **(B)** Diagrammatic representation of *Tc*ISG predicted N-terminal signal peptide in red, followed by the extracellular domain, transmembrane domain in yellow and intracellular transmembrane domain in green. The numbers above the domains correspond to the amino acid residue in panel A. The ISG65-75 conserved domains were detected within the extracellular domain of the *Tc*ISG protein sequence in the NCBI database (Appendix A) (Marchler-Bauer *et al*, 2015) while predicted transmembrane domains were detected using web-based TMHMM server v. 2.0 (http://www.cbs.dtu.dk/ services/TMHMM-2.0/) (Appendix B).

The functions of ISGs are poorly understood and are yet to be accurately determined. Experiments conducted show rapid turnover and recycling of ISG molecules back to the parasite cell surface which associates ISGs with virulence. The remaining ISGs are internalised by endosomes and are targeted to lysosomes for degradation via ubiquitination (Chung *et al*, 2004 & Leung *et al*, 2011).

Over the years, recombinant DNA technology has played a significant role in therapeutics, vaccine development and diagnostics (Walsh, 2005; Stijlemans *et al*, 2007; Rogé *et al*, 2013). Thymine-Adenine (TA) cloning is frequently used due to its efficiency and simplicity originating from the presence of complementary 3'-thymine (T) vector overhangs which facilitates ligation to 5'-adenine (A) overhangs on a PCR product. This eliminates the need for restriction enzymes

thereby reducing cost (Zhou and Gomez-Sanchez, 2000). TA cloning is also beneficial due to the presence of the *lacZ* gene in the multiple cloning site coding for a galactosidase enzyme which cleaves galactosidic bonds and assists in the identification of recombinant colonies using blue/white screening. Ligation of the PCR product into the vector interrupts the *lacZ* gene preventing *E. coli* JM109 cells from expressing the galactosidase enzyme thereby prohibiting the cleavage of galactosidase bonds within the isopropyl-β-D-thiogalactopyranoside (X-gal) substrate. This results in the growth of recombinant white bacterial colonies. *Escherichia coli* JM109 cells lacking the recombinant vector possess the complete *lacZ* gene expressing galactosidase thereby allowing cleavage of the X-gal substrate resulting in non-recombinant blue bacterial colonies. This unique property permits easy and quick screening of recombinant bacterial colonies during cloning (Messing *et al*, 1977; Norrander *et al*, 1983).

Bacterial cells are a popular expression system because its genome is well characterised and can be subjected to genetic manipulation for increased protein yields (Hannig and Makrides, 1998). *Escherichia coli* BL21 (DE3) cells lack the *Lon* and *OmpT* protease genes and therefore prevent degradation of extracellular proteins. The bacterial cells are also deficient in *hsdS*$_B$ (*r*$_B^-$ *m*$_B^-$) and *dcm* gene mutations thus preventing DNA methylation and plasmid degradation (Gottesman, 1996; Grodberg and Dunn, 1998, Rosano and Ceccarelli, 2014). The advantages of using *E. coli* as an expression system include: rapid growth rate, increased cell culture density, affordability, simple growth media and increased protein yields (Makrides, 1996; Sezonov *et al*, 2007). Additionally, recombinantly produced parasite proteins eliminate the use of animals for parasite propagation, are off high purity and can be easily produced on a large scale in a cost effective manner. Promising results were obtained when native trypanosomal antigens were replaced with recombinant proteins in diagnostic tests (Lejon *et al*, 2005; Van Nieuwenhove *et al*, 2013; Sengupta *et al*, 2014; Rogé *et al*, 2014b).

In this chapter, the cloning, expression and purification of full length *Tc*ISG is reported. The gene coding for the signal peptide, extracellular domain, transmembrane domain and intracellular transmembrane domain was amplified using PCR from *T. congolense* (IL 3000) genomic DNA, cloned into pGEM®-T vector and consequently sub-cloned into pET-32a expression vector containing the thioredoxin and histidine affinity tags. The *Tc*ISG protein was recombinantly expressed within inclusion bodies using the *E. coli* BL21 (DE3) host expression system. *Tc*ISG was solubilised using anionic detergents and thereafter refolded and purified using nickel affinity chromatography.

2.2 Materials and Methods

2.2.1 Materials

Molecular biology: The DE-52 Whatman® anion exchange cellulose resin was purchased from Whatman (England). High fidelity PCR enzyme with buffer, 10 mM dNTPs, 25 mM $MgCl_2$, Taq polymerase, O'GeneRuler™ 1 kb DNA Ladder, T4 DNA ligase, BamH I, Xho I, isopropyl-β-D-thiogalactopyranoside (X-gal), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (IPTG), dithiothreitol (DTT), TransformAid™ Bacterial Transformation Kit, GeneJet™ Plasmid Miniprep Kit and shrimp alkaline phosphatase were purchased from Fermentas (Vilnius, Lithuania). *Escherichia coli* cells, JM109 and BL21 (DE3) strain were obtained from New England Biolabs (Ipswitch, MA, USA). The ZymoResearch Clean and Concentrator™ kit was obtained from Zymo Research (Orange, CA, USA), Seakem®LE agarose was acquired from Lonza (Rockland, ME, USA) and ampicillin sodium salt was purchased from USB Corporation (Cleveland, OH, USA). The pGEM®-T cloning vector was obtained from Promega (Madison, WI, USA) and the pET-32a expression vector was purchased from Novagen (Darmstadt, Germany). Ethidium bromide and crystal violet were purchased from Sigma (St. Louis, MO, USA) and tryptone, yeast extract and bacteriological agar were acquired from Merck Biolab (Darmstadt, Germany). All commonly used reagents were of highest purity and purchased from Merck (Germany) and Sigma (St. Louis, MO, USA).

Protein Purification and quantification: His-select® nickel affinity resin; 4-chloro-1-naphthol, lysozyme and Kodak BioMax light film were obtained from Sigma (St. Louis, MO, USA). PageRuler™ prestained protein ladder was acquired from Thermo Scientific (Lithuania). Amicon® Ultra-15 Centrifugal Filter device (M_r cut-off of 10 kDa) and BCA™ Protein Assay kit were purchased from Merck Millipore (Billerica, MA, USA). BioTrace™ nitrocellulose was obtained from PALL Corp (Ann Arbor, USA). Dialysis tubing with 10 kDa Mr cut-off was purchased from Pierce (Rockford, IL, USA) and Nunc-Immuno™ Maxisorp 96-well plates were obtained from Nunc Intermed (Roskilde, Denmark).

Antibodies: mouse anti-6xHis antibody conjugated to HRPO was purchased from Roche (Mannheim, Germany).

2.2.2 Molecular weight marker calibration curves

The molecular sizes of DNA and protein were determined by constructing calibration curves. The O'GeneRuler™ 1 kb DNA ladder was separated on a 1% (w/v) agarose gel and its migration measured and plotted (Figure 2.2 panel A). Commercially available PageRuler™ prestained protein ladder was electrophoresed on a 10% reducing SDS-PAGE gel and its migration was plotted against log of the molecular weight marker (Figure 2.2 panel B).

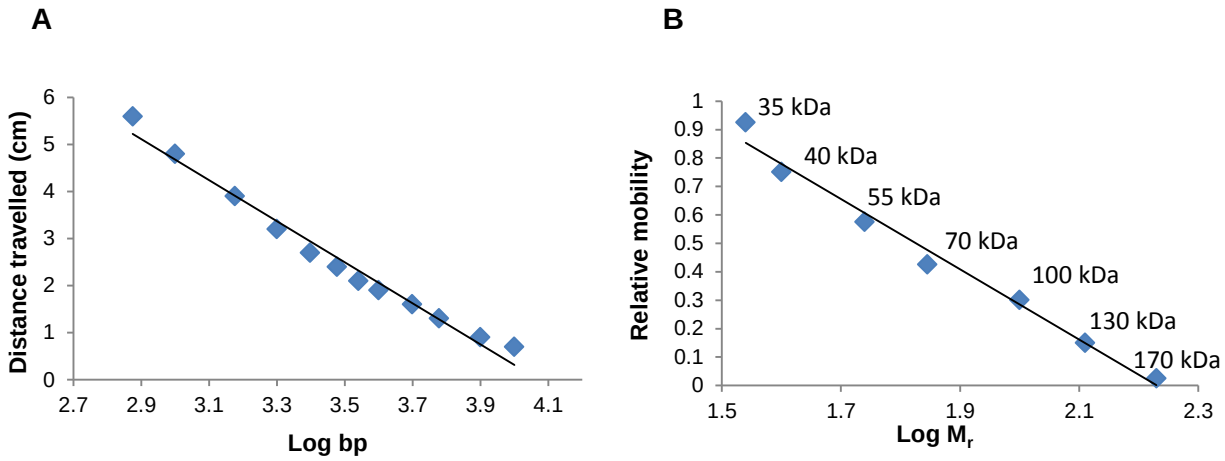

Figure 2.2: Calibration curves showing distance travelled and relative mobility versus log of base pairs and molecular weight markers on agarose and SDS-PAGE gels. **(A)** The migration of O'GeneRuler™ 1 kb DNA ladder on 1% (w/v) agarose gel was measured and the equation of the trendline given by y= -4.3658x + 17.778 with a correlation coefficient of 0.9796. **(B)** The PageRuler™ prestained protein ladder was separated on a 10% reducing SDS-PAGE gel and its migration plotted against log of the marker molecular weight. The equation of the trendline is y= -1.2344+ 2.7539 with a correlation coefficient of 0.9833.

2.2.3 BCA protein assay for protein quantification

Purified *Tc*ISG was quantified using the bicinchoninic acid (BCA) assay kit obtained from Pierce in Rockford, USA. The BCA assay is a biuret reaction based on the reduction of Cu^{+2} to Cu^{+} cuprous cation to produce a purple colored product in the presence of protein under alkaline conditions. The amount of Cu^{+} cuprous ions produced is directly proportional to the amount of protein present (Smith *et al*, 1985). The interaction of bicinchoninic acid from the BCA working reagent with peptide bonds and amino acids tyrosine, tryptophan and cysteine, produces the purple reaction product before quantification. Interference can be caused by compounds possessing similar functional groups to tyrosine, tryptophan and cysteine (Wiechelman *et al*, 1988). The bovine serum albumin (BSA) protein standards were serially diluted ranging from 2000 to 25 µg/ml. The BCA working reagent was made by combining reagents A and B according to a ratio of 50:1 (v/v). The BSA protein standard solutions (25 µl) and proteins of unknown concentration (25 µl) were added to a 96 well Nunc® plate and incubated with 200 µl of

the working reagent at 37°C for 30 min. Absorbancies were measured spectrophotometrically at 562 nm (Figure 2.3) using the VersaMax ELISA Microplate Reader (Molecular Devices CA, USA).

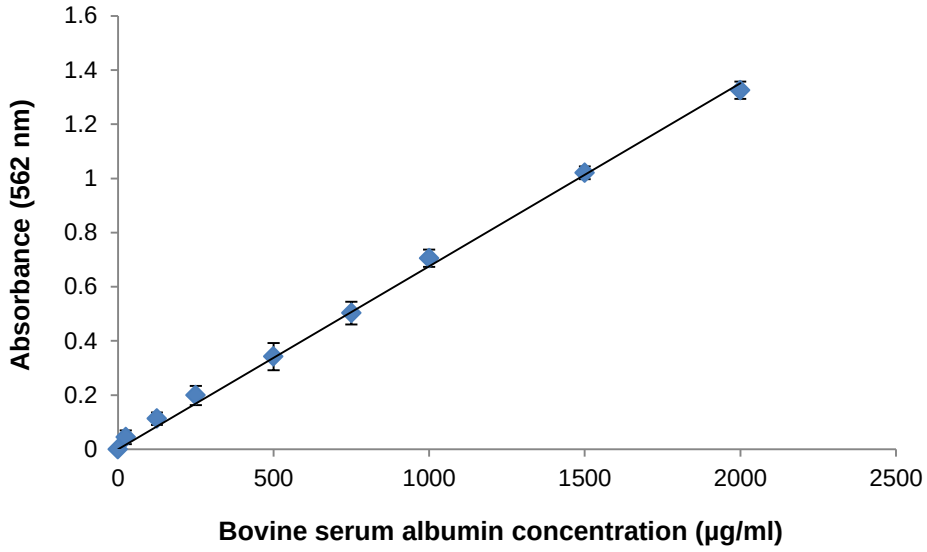

Figure 2.3: Calibration curve for the BCA protein assay kit for the quantification of *Tc*ISG. Bovine serum albumin solutions (25 to 2000 µg/ml) were combined with the BCA working reagent and incubated at 37°C for 30 min before measuring the absorbance at 562 nm using the Versmax microplate reader. The equation of the trendline is y= 0.0007x with a correlation coefficient of 0.9976. The assay was done in triplicate.

2.2.4 Preparation of trypanosomes

BALB/c mice were intraperitoneally infected with viable *T. congolense* (IL 3000) parasites and parasitemia was monitored by daily tail pricks. Blood from the tail pricks were diluted in phosphate buffered saline (PBS) (100 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.2) and viewed microscopically for motile parasites in between the erythrocytes. When infection status reached approximately 70%, the mice were sacrificed using cardiac puncture and the infected blood was collected in heparinised tubes. Trypanosomes were purified from the blood using a DE-52 Whatman® anion exchange cellulose resin according to Lanham and Godfrey (1970). Briefly, DE-52 resin was mixed with phosphate buffered saline (PBS) (100 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl and 137 mM NaCl, pH 8.0) and allowed to settle. The resin was subsequently washed with one column volume of PBS, allowed to settle and one column volume of phosphate saline glucose (PSG) (57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 42 mM NaCl, 50 mM glucose and 1 mM hypoxanthine). An equal volume of PSG and infected blood were mixed and incubated with the DE-52 resin for 30 min. Trypanosomes were eluted

from the resin using PSG (15 ml fractions), centrifuged (3 500 rpm for 15 min, 4°C) and resuspended in PSG containing 20% (v/v) glycerol. Trypanosomes were microscopically viewed, counted using a haemocytometer and stored at -80°C.

2.2.5 Isolation of Genomic DNA

Genomic DNA was isolated from *T. congolense* (IL 3000) bloodstream forms as described by Medina-Acosta and Cross (1993). Briefly, the pelleted trypanosomes were resuspended in TELT buffer [50 mM Tris-HCl pH 8.0, 62.5 mM Na_2EDTA, 2.5 mM LiCl (150 µl)] and incubated at room temperature (RT) for 5 min. Phenol-chloroform [1:1 (v/v)] (150 µl) was added, mixed using an end-over-end rotator for 5 min at RT and the emulsion was centrifuged (17000 x *g* for 10 min, 4°C). The top aqueous phase was recovered and twice the volume of 100% (v/v) ice cold ethanol was added and mixed using end-over-end rotation for 5 min at RT. Genomic DNA was pelleted by centrifugation (17000 x *g*, 10 min, 4°C), washed with 70% (v/v) ethanol and dried at 37° for 30 min. Genomic DNA was resuspended in TE buffer [100 mM Tris-HCl pH 7.5, 10 mM Na_2EDTA (30 µl)] containing RNAse (1 µg/ml) and incubated at 37°C for 45 min. Genomic DNA (3 µl) was combined with loading dye (1 µl) and electrophoresed on a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml) in Tris-Acetate-EDTA (TAE) buffer (40 mM Tris-HCl, 100 mM acetic acid, 1 mM Na_2EDTA, pH 8.0).

2.2.6 Cloning of *TcISG* gene into pGEM®-T vector

Trypanosoma congolense ISG nucleotide sequence (Accession CCD12004) was obtained from GenBank (http://www.ncbi.nlm.nih.gov) for primer design (Appendix C). The forward primer (5' AAGGATCC**ATG**GGGATGCAACGTATTGTT 3') contained a BamH I restriction site whereas the reverse (5' CAGGCACACTTT**TAA**CTTTATCCCTCGAGCT 3') contained a Xho I site as represented by the underline. The start and stop codon sequences are indicated in bold. Using genomic DNA as the template for PCR, the *TcISG* gene was amplified in a 20 µl PCR reaction comprising of 1 x High Fidelity Taq polymerase buffer, 0.4 mM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 3.13 mM $MgCl_2$ and 2 U High Fidelity Taq Polymerase. The PCR reaction was performed using the following conditions: initial denaturation at 95°C for 10 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 2 min. The final extension was carried out at 72°C for 7 min. The PCR product (5 µl) was combined with loading dye (2 µl) and analysed on a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/ ml) in TAE buffer.

TcISG was ligated into pGEM®-T cloning vector (Figure 2.4) using a vector to insert ratio of 3:1. The ligation mixture consisted of 1 x Ligation buffer, 50 ng pGEM®-T vector DNA, 13.202 ng/µl *Tc*ISG and T4 DNA Ligase (1 U) in a 10 µl reaction mix. The ligation mixture was mixed and incubated at 37°C for 1 h followed by incubation at RT for 16 h. The ligation mix was thereafter transformed into competent *E. coli* JM109 cells using the TransformAid™ Bacterial Transformation kit according to the manufacturer's instructions. Briefly, *E. coli* JM109 cells were 3 way streaked onto 2 x YT agar plates (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) bacteriological agar, pH 7.0) and allowed to grow at 37°C for 16 h. The freshly streaked *E. coli* JM109 were excised from the agar (4x4mm) and inoculated into pre-warmed C- medium (750 µl) and incubated at 37°C for 2 h with 220 rpm agitation. The cells were centrifuged (17000 x *g*, 1 min, RT), resuspended in T-solution (300 µl) and incubated on ice for 5 min. The cells were centrifuged (17000 x *g*, 1 min, RT) and the supernatant discarded. Pelleted cells were subsequently resuspended in T-solution (120 µl) and incubated on ice for 5 min. Competent *E. coli* JM109 cells were mixed with the ligation mixture (2.5 µl) and incubated on ice for 5 min. The cell mixture was immediately plated onto 2 x YT agar plates containing 100 µg/ml ampicillin, 20 mg/ml X-gal and 100 mM IPTG before incubation at 37°C for 16 h. Blue and white screening was conducted to identify recombinant colonies.

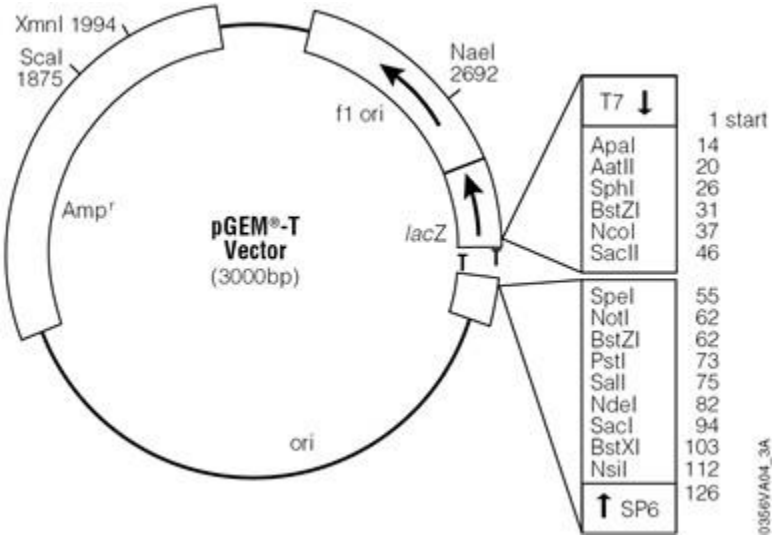

Figure 2.4: pGEM®-T vector map demonstrating the multiple cloning sites and antibiotic resistance. The vector has an origin of replication (ori), ampicillin resistance (Amp^r), multiple cloning site with T7 and SP6 promoters and a *lacZ* gene (Promega manual).

White colonies assumed to contain the recombinant vector were selected and grown in 2 x YT broth (5 ml) containing 100 µg/ml ampicillin and incubated at 37°C for 16 h with 220 rpm

agitation. Vector DNA was isolated using the GeneJet™ Plasmid Miniprep kit as per the manufacturer's instructions. Briefly, the cells were pelleted by centrifugation (6800 x *g*, 2 min, RT) and resuspended in resuspension solution (250 µl) containing RNAse A followed by the addition of lysis solution (250 µl). The resulting solution was mixed by inversion (6 times) and neutralisation solution (350 µl) was added and immediately mixed by inversion (6 times). The resuspension was centrifuged (17000 x *g*, 5 min, RT) and the supernatant transferred to the GeneJET™ spin column and centrifuged (17000 x *g*, 1 min, RT). The flow through was discarded and wash solution containing ethanol was subsequently added to the column followed by centrifugation (17000 x *g*, 1 min, RT). The wash was repeated and the flow through was discarded. The column was subjected to further centrifugation (17000 x *g*, 1 min, RT) to remove residual wash solution. The spin column was transferred to a sterile 1.5 ml microcentrifuge tube and elution buffer (50 µl) added directly onto the matrix and incubated for 2 min at RT. The DNA was eluted by centrifugation (17000 x *g*, 2 min, RT). The elution step was repeated to increase the overall yield. Recombinant pGEM®-T vector was visualised on a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Colony PCR was employed using isolated pGEM®-T vector DNA as template DNA in a 20 µl reaction. Amplification was conducted as described previously in Section 2.2.6 using gene and vector primers (T7 forward and SP6 reverse). The colony PCR products (5 µl) were combined with loading dye (2 µl) and analysed on a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Positive clones were sequenced at the Central Analytical Facility, Stellenbosch University.

2.2.7 Sub-cloning of *TcISG* into pET-32a expression vector

In a 50 µl reaction mixture, recombinant pGEM®-T vector was first digested with Xho I in its unique buffer at 37°C for 4 h followed by heat inactivation of the enzyme at 80°C for 20 min. The mixture was then cleaned and concentrated using the Zymo Research Clean & Concentrator™ kit according to the manufacturer's instructions. Briefly, twice the volume of DNA Binding buffer was added to the DNA mixture, briefly vortexed and transferred to a Zymo-Spin™ Column in a collection tube for centrifugation (16 000 x *g*, 30 s, RT). The flow-through was discarded. DNA wash buffer (200 µl) was added to the column and centrifuged (16 000 x *g*, 30 s, RT). The wash was repeated. DNA elution buffer (30 µl) was added directly onto the column matrix and incubated at RT for 1 min. The column was transferred to a sterile 1.5 ml microcentrifuge tube and centrifuged (16 000 x *g*, 30 s, RT). The cleaned and concentrated DNA was subsequently digested with BamH I in its unique buffer at 37°C for 4 h following heat

inactivation of the enzyme at 80°C for 20 min. The restriction digest was analysed on a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/ ml) in TAE buffer and consequently electrophoresed on a 1% (w/v) agarose gel in TAE buffer in which both the gel and buffer contained 10 µg/ ml crystal violet before excision of *Tc*ISG DNA. The gel was visualised on a light box and the *Tc*ISG DNA purified using the peqGOLD Gel Extraction kit as per the manufacturer's instructions. Briefly, *Tc*ISG DNA was excised from the agarose gel, added to an equal volume of Binding buffer and incubated at 60°C until the gel dissolved. *Tc*ISG DNA solution (750 µl) was added onto a DNA spin column and centrifuged (10 000 x *g*, 1 min, RT). The flow through was discarded and binding buffer (300 µl) and wash buffer containing ethanol (750 µl) was added to the spin column, incubated at RT for 2 min and centrifuged (10 000 x *g*, 1 min, RT). The flow through was discarded and the empty spin column centrifuged (10 000 x *g*, 1 min, RT) to remove residual ethanol. *Tc*ISG DNA was eluted by placing the spin column in a sterile 1.5 ml microcentrifuge tube and elution buffer (30 µl) was added onto the matrix and centrifuged (5000 x *g*, 1 min, RT). Purified *Tc*ISG DNA was analysed on a 1% (v/v) agarose gel containing ethidium bromide (0.5 µg/ ml) in TAE buffer.

A glycerol stock of *E. coli* BL21 (DE3) cells containing the pET-32a expression vector was streaked onto 2 x YT agar plates containing 100 µg/ ml ampicillin and incubated at 37°C for 16 h. A single colony was cultured in 2 x YT (5 ml) broth containing ampicillin (100 µg/ml) at 37°C for 16 h. The expression vector DNA was isolated using the GeneJet™ Plasmid Miniprep kit as per the manufacturer's instructions (Section 2.2.6). The vector was subjected to a restriction digestion (65 µl) with Xho I in its unique buffer for 4 h at 37°C following heat inactivation of the enzyme at 80°C for 20 min. The mixture was cleaned and concentrated using the Zymo Research Clean & Concentrator™ kit according to the manufacturer's instructions (Section 2.2.7). The cleaned and concentrated DNA (30 µl) was digested with BamH I in its unique buffer at 37°C for 4 h followed by heat inactivation of the enzyme at 80°C for 20 min and sequentially cleaned and concentrated using the Zymo Research Clean & Concentrator™. The restriction digest (3 µl) was analysed on a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/ ml) in TAE buffer. The vector was dephosphorylated using 1 U shrimp alkaline phosphatase at 37 °C for 1 h followed by heat inactivation of the enzyme at 65 °C for 15 min.

TcISG was ligated into the pET-32a expression vector using a vector to insert ratio of 3:1 and T4 DNA Ligase (1 U) in a 10 µl reaction mix. The ligation mixture was incubated at 37°C for 1 h followed by an additional 16 h incubation at RT. The ligation mix was thereafter transformed into competent *E. coli* BL21 (DE3) cells using $CaCl_2$ transformation (Cohen *et al*, 1972; Sambrook *et*

al, 2001). Briefly, *E. coli* BL21 (DE3) cells were streaked onto 2 x YT agar and incubated at 37°C for 16 h. A single colony was inoculated into 2 x YT (5 ml) broth and cultured at 37°C for 16 h with 200 rpm agitation. A 1:100 dilution of the overnight culture was prepared in 2 x YT broth (99 ml) and cultured until an optical density at 600 nm (OD_{600}) of 0.3-0.4 was reached. The cell culture was transferred to ice cold sterile centrifuge tubes, incubated on ice for 10 min and centrifuged (4500 x *g*, 10 min at 4°C). The pellet was resuspended in ice cold sterile 0.1 M $CaCl_2$ (40 ml) followed by centrifugation (4500 x *g*, 10 min at 4°C). The pellet was resuspended in $CaCl_2$ (6 ml). Competent *E. coli* BL21 (DE3) cells were combined with the ligation mix (4 µl), incubated on ice for 30 min and heat shocked at 42°C for 90 s. Thereafter, the cells were combined with super optimal cataboliser medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 20 mM glucose (800 µl)] and incubated at 37°C for 1 h with gentle agitation. The cells were plated onto pre-warmed 2 x YT agar plates containing 100 µg/ ml ampicillin and incubated at 37°C for 16 h.

Bacterial colonies were cultured in 2 x YT broth (5 ml) containing 100 µg/ ml ampicillin at 37°C for 16 h with 200 rpm agitation. The recombinant plasmid was isolated using the GeneJet™ Plasmid Miniprep kit as per the manufacturer's instructions (Section 2.2.6). Positive clones were verified using colony PCR which entailed the use of both the gene specific primers and pET-32a vector primers (T7 forward and T7 reverse). Colony PCR was conducted as described previously except Taq polymerase was used instead of High Fidelity Taq polymerase. The PCR products (5 µl) were combined with loading dye (2 µl) and analysed on a 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/ ml). Positive clones were sequenced at the Central Analytical Facility, Stellenbosch University.

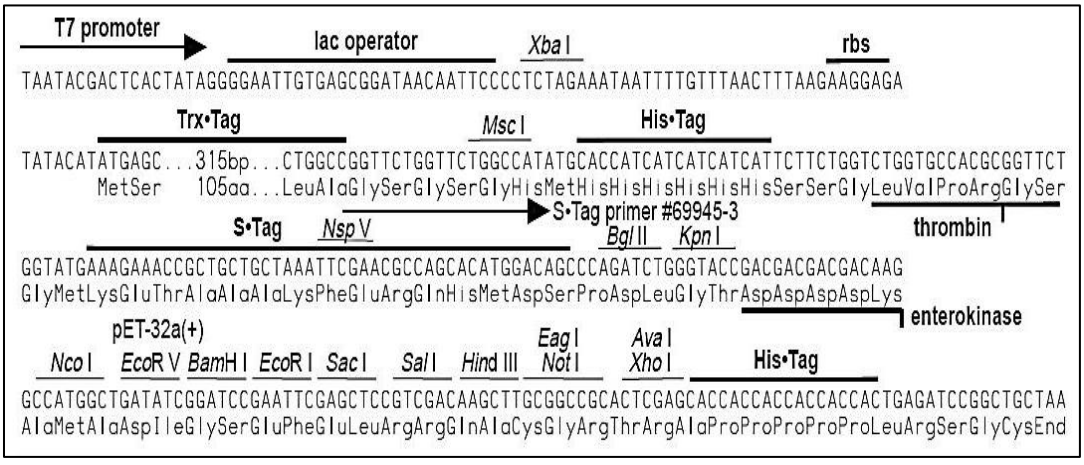

Figure 2.5: Map of the pET-32a expression vector showing the multiple cloning site. The vector consists of affinity tags and cleavage sites (Novagen manual).

2.2.8 Recombinant expression of *Tc*ISG

Protein expression of *Tc*ISG-pET-32a clone was first optimised in 2 x YT broth. The *Tc*ISG-pET-32a clone was streaked onto 2 x YT agar plates containing 100 µg/ ml ampicillin and grown at 37°C for 16 h. A single colony was selected and grown in 2 x YT broth (5 ml) containing 100 µg/ml ampicillin at 37°C for 16 h. The overnight culture was subsequently diluted [1:100] in 2 x YT broth (99 ml) comprising of ampicillin (100 µg/ml) and cultured at 37°C with 200 rpm agitation until an OD_{600} of 0.5-0.6 was obtained. Protein expression was consequently induced using IPTG concentrations of 0.1 mM, 0.3 mM, 0.5 mM, 0.7 mM and 1 mM at 37°C for 4 h with 200 rpm agitation. A final concentration of 100 µg/ ml ampicillin was added to the culture at the start of the induction period and 2 h post induction. Bacterial cells were centrifuged (5000 x *g*, 10 min, 4°C) and the pellet was resuspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2) containing 1% (v/v) Triton-x-100 and 1 mg/ml lysozyme. The cell suspension was incubated at 37°C for 1 h and frozen at -20°C for 16 h. The cells were thawed on ice and sonicated on ice (4 x 30 s) each at 10 s intervals and centrifuged (10 000 x *g*, 20 min, 4°C). The supernatant and pellet were subjected to SDS-PAGE analysis (Section 2.2.10).

2.2.9 Protein solubilisation, refolding and purification

Solubilisation was conducted as described by Schlager and colleagues (2012). The cells were thawed on ice and sonicated on ice (4 x 30 s) each at 10 s intervals and centrifuged (10 000 x *g*, 20 min at 4°C). The bacterial pellet was resuspended in lysis buffer (8 mM Na_2HPO_4, 286 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 1% (w/v) SDS, pH 7.4) containing 1 mM DTT. The suspension was sonicated on ice (2 x 2 min), incubated on ice for 30 min and centrifuged (10 000 x *g*, 20 min, 4°C). The supernatant containing solubilised *Tc*ISG was subsequently used for purification.

Purification was performed by placing His-select® nickel affinity resin (1 ml) in a 10 ml chromatography column. The column was washed with 2 column volumes of dH_2O and equilibrated with 5 column volumes of wash buffer (8 mM Na_2HPO_4, 286 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 0.1% (w/v) sarkosyl, pH 7.4). Solubilised *Tc*ISG (10 ml) was mixed and incubated with the nickel resin at 4°C for 3 h using end-over-end rotation. Thereafter, the unbound proteins were collected and the column washed with 50 ml of wash buffer until an absorbance value of zero was obtained at 280 nm. Bound proteins were eluted using 10 ml elution buffer (8 mM Na_2HPO_4, 286 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 0.1% (w/v)

sarkosyl, 50 mM imidazole, pH 7.4) and 1 ml fractions were collected. The column was subsequently washed with 2 column volumes of dH_2O, 5 column volumes of 6 M guanidine hydrochloride, 2 column volumes of dH_2O, 2 column volumes of 30% (v/v) ethanol and stored in 2 ml 30% (v/v) ethanol at 4°C. Solubilised *Tc*ISG, unbound proteins and eluted fractions were analysed on a 10% reducing SDS-PAGE gel and visualised using Coomassie Blue staining (Section 2.2.10).

2.2.10 SDS-PAGE

Protein analysis was conducted using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Briefly, a discontinuous buffer system consisting of running gel buffer (1.5 M Tris-HCl, pH 8.8) and stacking gel buffer (500 mM Tris-HCl, pH 6.8) was used. Protein samples were prepared using an equal volume of reducing treatment buffer (125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol) and boiled at 100°C for 2 min. Electrophoresis was conducted using the BioRad Mini protein III electrophoresis apparatus (BioRad, CA, USA) at 20 mA per gel in tank buffer (250 mM Tris-HCl buffer, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3). The SDS-PAGE gels were stained in Coomassie Blue staining solution (0.125% (w/v) Coomassie Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid) for 16 h. The gels were then incubated in destaining solution (50% (v/v) methanol, 10% acetic acid) on an orbital shaker and stored in distilled water. The gels were photographed using the Sys Gene® imaging system (Syngene®, USA).

2.2.11 Western Blotting

Proteins were first electrophoresed on a 10% reducing SDS-PAGE gel and transferred onto a nitrocellulose membrane (Towbin *et al*, 1979) using the Mini Trans-Blot® Cell apparatus (Biorad, CA, USA). The nitrocellulose was then stained using Ponceau S solution (0.1% (w/v) Ponceau S, 15% (v/v) acetic acid) to detect the transferred proteins and washed with distilled water containing a drop of 0.5 M NaOH solution. All remaining steps were performed on an orbital shaker. The nitrocellulose membrane was thereafter incubated in Tris buffered saline (TBS) (20 mM Tris-HCl buffer, 200 mM NaCl, pH 7.4) containing 5% (w/v) low fat milk powder at RT for 1 h. The membrane was washed with TBS (3 x 5 min) and incubated with mouse anti-6xHis antibody conjugated to HRPO [1:8 000 diluted in 0.5% (w/v) BSA-TBS] at 4°C for 4 h. The membrane was washed (2 x 5 min) and incubated in substrate solution (0.06% (w/v) 4-chloro-1-

naphthol, 0.1 % (v/v), methanol, 0.0015% (v/v) H_2O_2 in TBS) at RT in the dark and photographed using the Sys Gene® imaging system (Syngene®, USA).

2.2.12 Dialysis

*Tc*ISG protein from fractions 4 to 10 were pooled and dialysed against buffer (8 mM Na_2HPO_4, 286 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 0.1% (w/v) sarkosyl, pH 7.4) containing 5% (v/v) glycerol to prevent protein instability. The volume of dialysis buffer used was 5x the *Tc*ISG solution volume. Dialysis was carried out at 4°C for 3 h. The buffer was replaced and dialysed for a further 3 h at 4°C and subsequently replaced and left to dialyse at 4° for 16 h. The *Tc*ISG solution was then concentrated using an Amicon® Ultra-15 Centrifugal Filter device (M_r cut-off of 10 kDa) as per the manufacturer's instructions. Briefly, the protein solution was added to the filter and centrifuged (6 500 x *g*, 10 min, 4°C). *Tc*ISG was collected from the filter and stored at 4° until required.

2.3 Results

2.3.1 Cloning of *TcISG* into pGEM®-T cloning vector

Genomic DNA was isolated from *T. congolense* (IL 3000) bloodstream forms using phenol-chloroform extraction and visualised on a 1% (w/v) agarose gel (Figure 2.6, panel A). Genomic DNA was used as a template in the amplification of the *TcISG* gene using PCR. Amplification was achieved using the designed primers and High Fidelity Taq polymerase with proofreading ability. Amplification of *TcISG* gene resulted in a single amplicon with an anticipated size of 1260 bp (Figure 2.6, panel B).

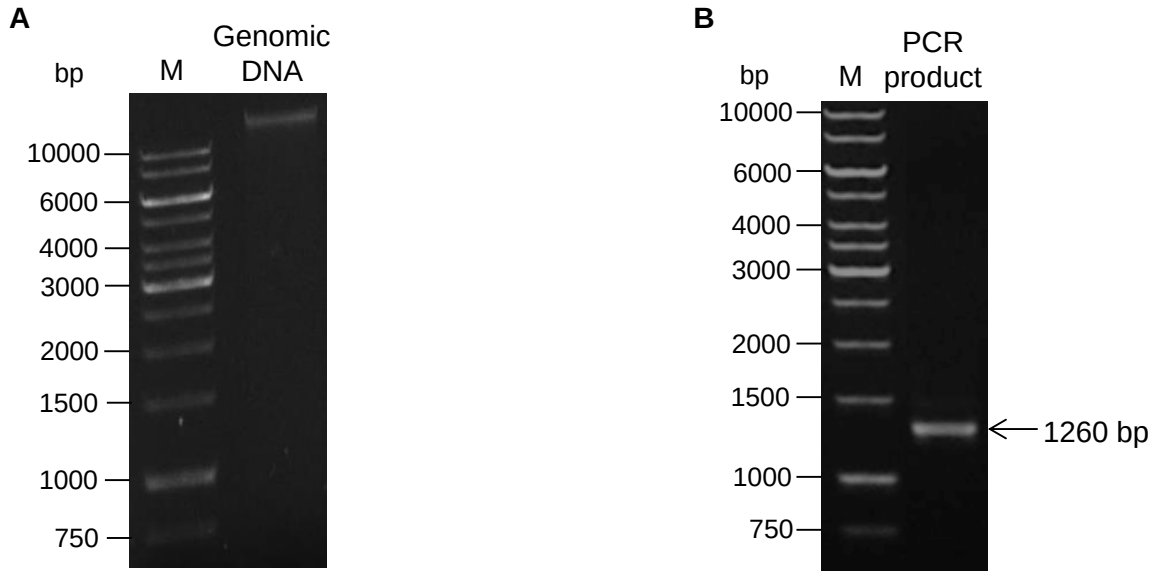

Figure 2.6: Isolation of *T. congolense* (IL 3000) genomic DNA and amplification of *TcISG* gene using PCR. **(A)** Isolated genomic DNA and **(B)** the PCR product were electrophoresed on 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide.

The PCR product was subsequently ligated into pGEM®-T cloning vector using TA cloning and transformed into competent *E. coli* JM109 cells. Recombinant colonies were identified using blue/white screening followed by isolation of *TcISG*-pGEM®-T recombinant vector which served as template DNA for colony PCR (Figure 2.7). Colony PCR was used to screen for the presence of the *TcISG* gene within the recombinant cloning vector. Using gene specific primers, amplification of the vector DNA revealed the presence of a 1260 bp *TcISG* gene while the use of T7 and SP6 promotor vector primers revealed the presence of a 1500 bp PCR product for colonies 1, 3 and 5. These colonies therefore contained the *TcISG*-pGEM®-T recombinant vector and its DNA was isolated and sequenced using gene specific primers. The sequencing results of colony 5 (Appendix D) demonstrated 99% nucleotide sequence identity to the Genbank® sequence (TcIL3000.0.29290) and was used further.

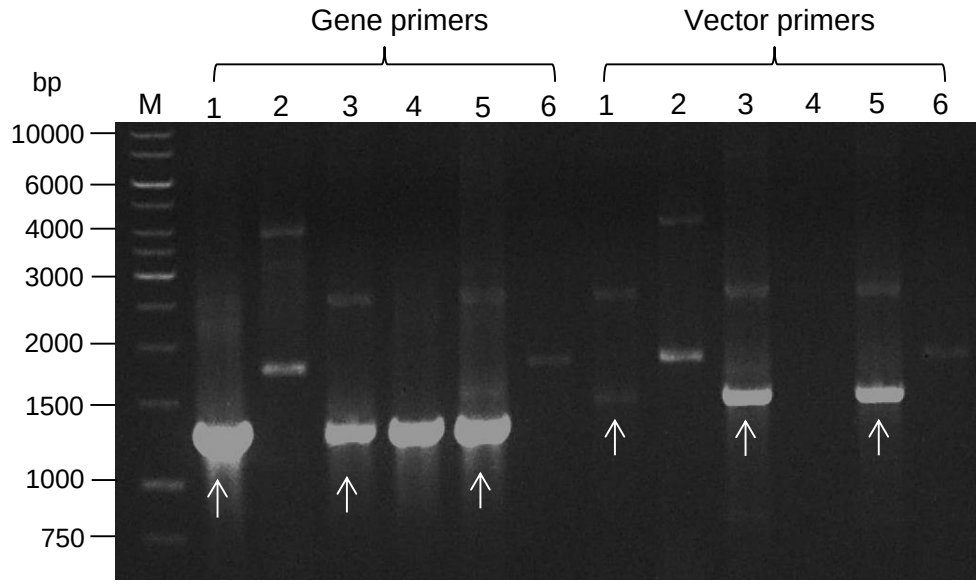

Figure 2.7: Colony PCR for screening of recombinant *TcISG*-pGEM®-T vector after transformation. The *TcISG* gene was ligated into pGEM®-T vector and transformed into competent *E. coli* JM109 cells. DNA was extracted from selected positive white colonies and used for colony PCR. The PCR products were amplified using the gene forward and reverse primers and the T7 promoter and SP6 vector primers. The DNA was analysed on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. The arrows indicate positive clones.

2.3.2 Sub-cloning of *TcISG* into pET-32a expression vector

Using colony 5, a large scale restriction digest was conducted on the recombinant vector to isolate the *TcISG* gene from the pGEM®-T cloning vector. The first restriction digest using Xho I resulted in a single linearilised vector at a size of 5000 bp (Figure 2.8 panel A, lane 1). Following the second digest using BamH I, two bands consistent with the expected vector and *TcISG* gene sizes were observed with sizes of 3000 bp and 1260 bp respectively (Figure 2.8 panel A, lane 2). The *TcISG* gene was analysed on a 1% (w/v) agarose gel containing crystal violet, excised from the agarose gel, purified using the peqGOLD Gel Extraction Kit and subsequently analysed on a 1% (w/v) agarose gel containing ethidium bromide (Figure 2.8 panel B). Double restriction digestion was performed on pET-32a expression vector in preparation for sub-cloning. The digest was performed using BamH I and Xho I to produce compatible sticky ends for ligation of the *TcISG* gene. Following restriction digestion, the pET-32a vector was dephosphorylated using alkaline phosphatase to prevent vector re-ligation following restriction digestion.

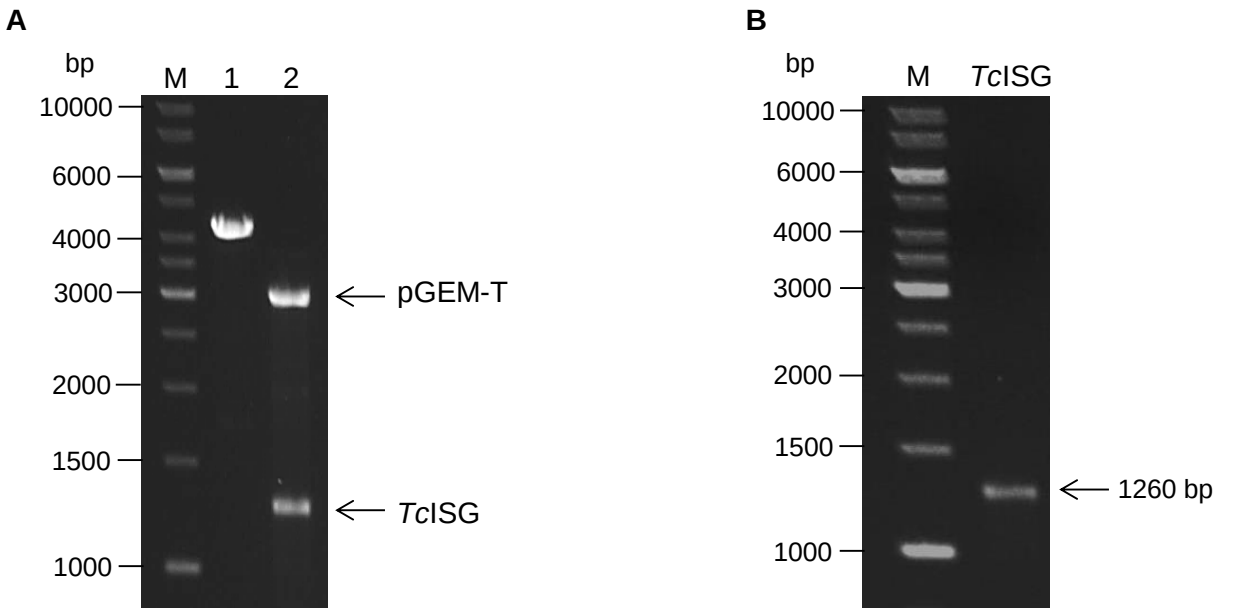

Figure 2.8: Analysis of restriction digestion and purification of the *Tc*ISG-pGEM-T® clone. (A) Lane 1, the *TcISG*- pGEM®-T vector DNA was isolated from colony 5 and digested using Xho I for 4 h at 37°C. Lane 2, the DNA was cleaned and concentrated and digested with BamH I for 4 h at 37°C. Following each restriction digest, the enzymes were inactivated for 20 min at 80°C. The DNA was electrophoresed on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. **(B)** Using crystal violet, *Tc*ISG was visualised and excised from the gel, purified and analysed on 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide.

The *TcISG* gene was ligated into the pET-32a expression vector and transformed into *E. coli* BL21 (DE3) cells. The DNA from *TcISG*-pET-32a recombinant colonies was isolated and used as template DNA for colony PCR. Using gene specific primers, colonies 2 and 3 (Figure 2.9) contained the 1260 bp *TcISG* gene and amplification using the T7 forward and T7 reverse vector primers revealed the presence of a 1900 bp band (Figure 2.9). This size of 1900 bp was expected due to the amplification of vector DNA adjacent to the *TcISG* insert on either ends. The DNA from colonies 2 and 3 was isolated and sequenced using T7 forward and T7 reverse vector primers. Highest sequence identity was obtained from colony 3 showing 99% with the TcIL3000.0.29290 nucleotide sequence (Appendix E) and 98.1% with the amino acid sequence (Appendix F) sequence on Genbank® and was thus used for protein expression.

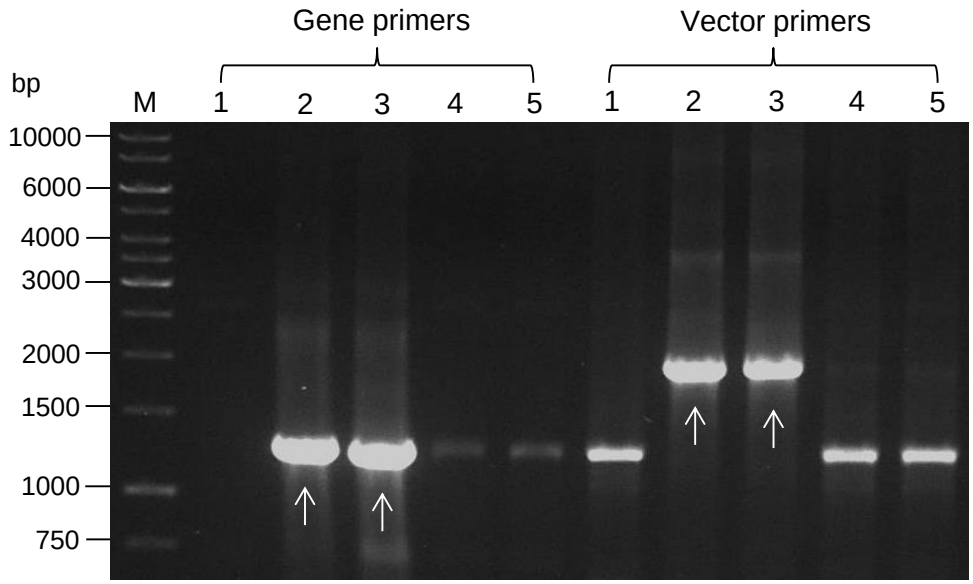

Figure 2.9: Analysis of colony PCR for screening of recombinant pET-32a vector containing the ligated *TcISG* gene after transformation. The *TcISG* gene was ligated into the pET-32a expression vector and transformed into competent *E. coli* BL21 (DE3) cells. DNA was extracted from selected positive white colonies and used for colony PCR. The PCR products were amplified using the forward and reverse gene primers and the T7 forward and T7 reverse vector primers. The DNA was analysed on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. The arrows indicate positive clones.

2.3.3 Recombinant expression, solubilisation, refolding and purification

2.3.3.1 Optimisation of recombinant *Tc*ISG expression

A bacterial system was used to recombinantly express *Tc*ISG at 37°C using IPTG induction. Expression was monitored hourly before and after IPTG induction and analysed on a 10% reducing SDS-PAGE gel. Protein expression was optimised using a range of IPTG concentrations to obtain increased *Tc*ISG yields. *Tc*ISG was identified as a histidine-tagged fusion protein on an SDS-PAGE gel at a size of approximately 66 kDa during the induction period and was expressed in the pellet within inclusion bodies (Figure 2.10 panels A, C, E and G). The SDS-PAGE results were further authenticated by western blots which detected the hourly expression of *Tc*ISG using a mouse anti-6xHis-HRPO conjugate antibody (Figure 2.10 panels B, D, F and H). A concentration of 1 mM IPTG was selected for large scale *Tc*ISG expression since *Tc*ISG could be detected well during 4 hours of protein expression in comparison to the other IPTG concentrations used.

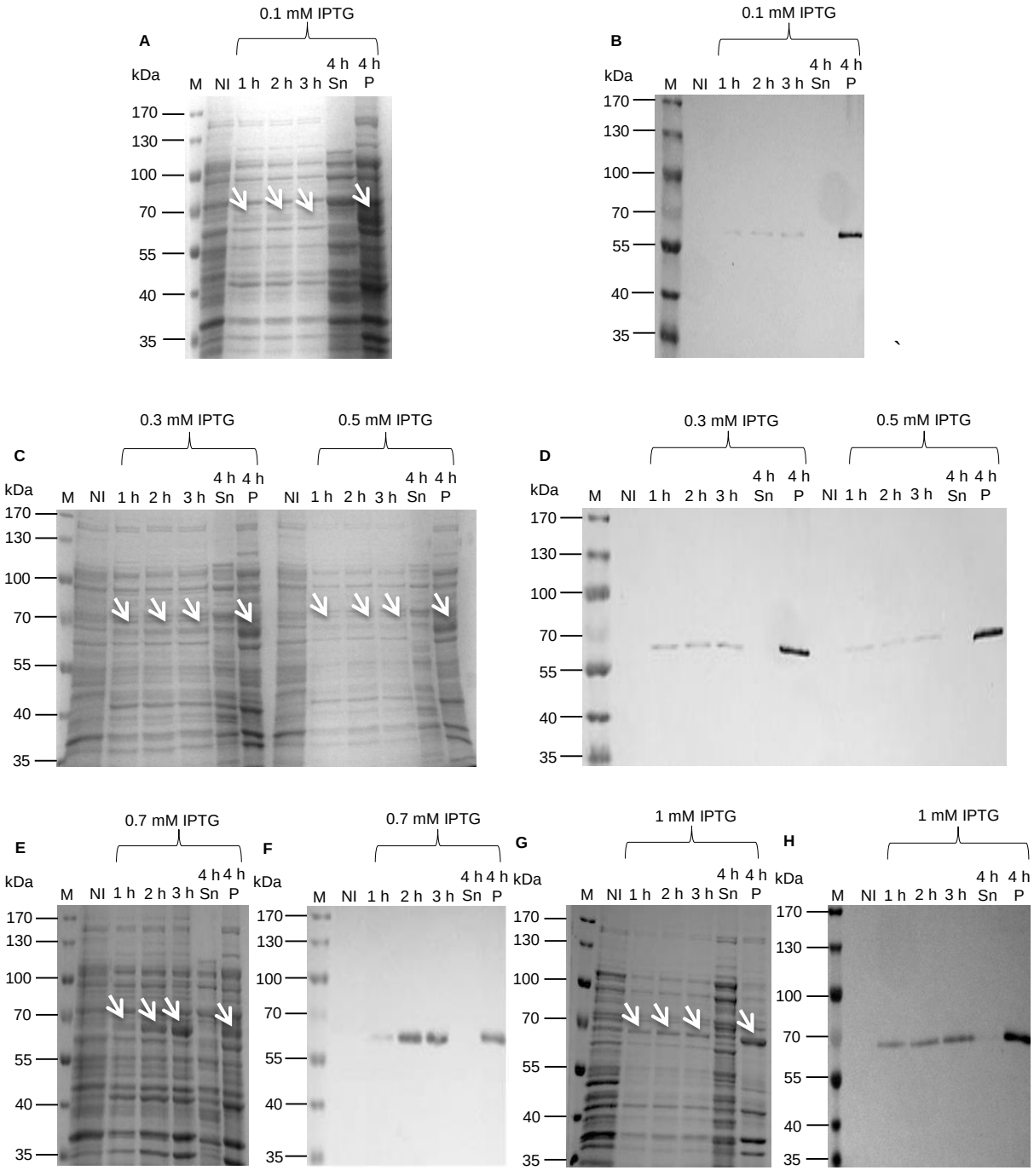

Figure 2.10: Recombinant expression of *Tc*ISG in a pET-32a expression vector. *Tc*ISG was expressed in 2 x YT medium at 37°C for 4 h. Protein expression and solubility was monitored hourly following induction with 0.1, 0.3, 0.5, 0.7 and 1 mM IPTG **(A, C, E and G)** on a 10% reducing SDS-PAGE gel and visualised with Coomassie blue staining. Western blot analysis **(B, D, F and H)** was conducted with mouse anti-6xHis-HRPO conjugate antibody [1:8000] and incubated with 4-chloro-1-naphthol-H_2O_2 chromogen-substrate solution. M: PageRuler™ prestained protein marker, NI: non-induced, SN: supernatant, P: pellet.

2.3.3.2 Solubilisation, refolding and purification of *Tc*ISG

The pellet containing the *Tc*ISG fusion protein was solubilised using SDS and sarkosyl followed by refolding and purification on a nickel affinity resin. Protein purification was analysed on a 10% reducing SDS-PAGE gel and visualised with Coomassie blue staining. In comparison to the solubilised pellet which was incubated with the nickel resin, the unbound fraction contained increased amounts of *Tc*ISG. Following washing of the resin, bacterial host proteins were present in fractions 1, 2 and 3 with little to no bacterial proteins present from fractions 4 to 10 (Figure 2.11 panel A). A western blot probed with mouse anti-6xHis-HRPO conjugate antibody detected purified His-tagged 66 kDa *Tc*ISG protein in fractions 1-10 (Figure 2.11 panel B). A single protein band of approximately 55 kDa was detected in the solubilised pellet, unbound fraction and fractions 1 and 2. Fraction 2 contained *E. coli* host proteins which were detected by the mouse anti-6xHis-HRPO conjugate antibody. No bands were detected in the non-induced bacterial lysate. Purified *Tc*ISG was used for antibody production as described in Chapter 3.

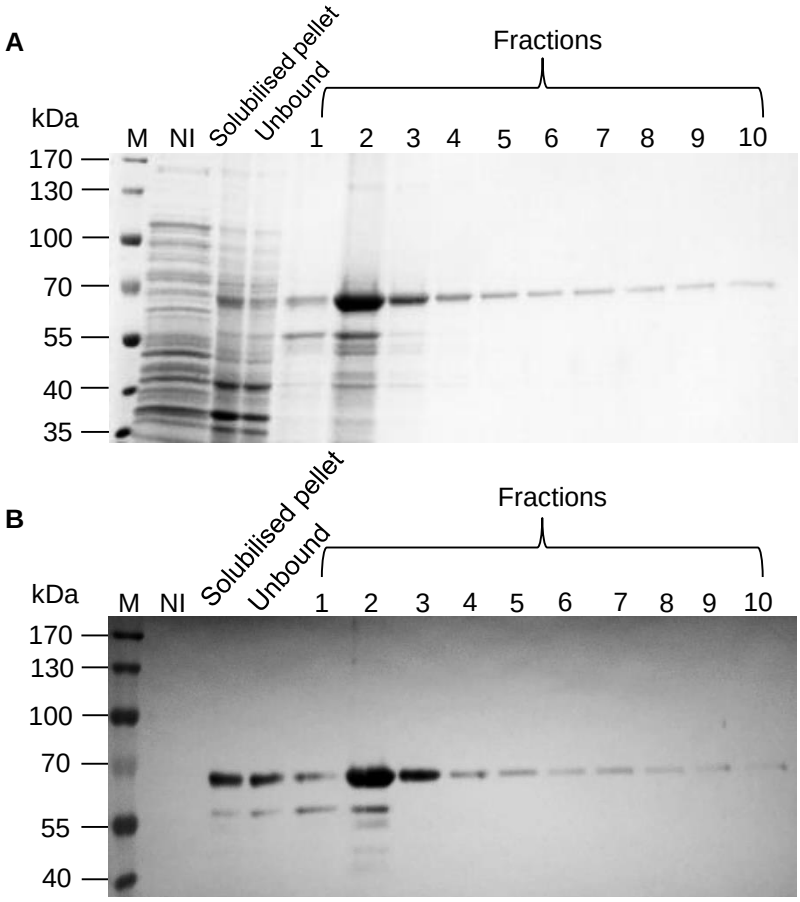

Figure 2.11: Purification of *Tc*ISG using nickel affinity chromatography. (A) *Tc*ISG was solubilised, purified and analysed by 10% reducing SDS-PAGE stained with Coomassie blue and by western blot **(B)**, probed with mouse anti-6xHis-HRPO conjugate antibody [1:8000] and incubated with 4-chloro-1-naphthol-H_2O_2 chromogen-substrate solution. M: PageRuler™ prestained protein marker, NI: non-induced.

2.4 Discussion

Trypanosoma congolense ISG (TcIL3000.0.29290) was first identified by Fleming and co-workers (2014) and only the extracellular domain was recombinantly expressed in the pET-15b vector using IPTG induction, purified using nickel affinity chromatography and tested as a diagnostic antigen in an ELISA. The recombinant ISG was able to distinguish between infected and non-infected cattle sera from experimentally infected cattle (Fleming *et al*, 2014). In the present study, the gene coding for the entire sequence of *Trypanosoma congolense* ISG (*Tc*ISG) (TcIL3000.0.29290) including all 4 domains (Figure 2.1) was amplified and cloned into a pGEM®-T cloning vector in order to produce a high copy number of *Tc*ISG-pGEM®-T vector replicates. Large scale protein expression was performed at 37°C for 4 hours using 1 mM IPTG for inducing *Tc*ISG expression in pET-32a expression vector in *E. coli* BL21 (DE3) cells. The use of lactose for protein expression presents a low-cost advantage to IPTG and can also function as a carbon source in addition to an inducer (Neubauer and Hofmann, 1994). However, lactose is more suited for large scale industrial produced recombinant proteins (Zhang *et al*, 2000). Soluble *Tc*ISG was attained for antibody production in chickens. Heterologous protein expression is commonly performed in the pET system using IPTG induction (Donovan *et al*, 1996) and has been used previously for ISG expression: *Trypanosoma brucei gambiense* VSG LiTat 1.3 was expressed as a 52 kDa histidine-tagged fusion protein (Tran *et al*, 2008), *Trypanosoma brucei* ISG (Lança *et al*, 2011), Tc38630 ISG (Fleming *et al*, 2014), *Trypanosoma evansi* ISG75 with a size of 55 kDa (Rudramurthy *et al*, 2015) and several ISG domains from *T. brucei gambiense* ISG65, ISG75 and ISG64 (Sullivan *et al*, 2013).

*Tc*ISG encodes a 419 amino acid protein with a predicted molecular weight of 46 kDa according to Compute pI/Mw on the Expasy server (https://www.expasy.org) (Gasteiger *et al*, 2005). *Tc*ISG was expressed as a histidine-tagged fusion protein approximately 66 kDa in size within inclusion bodies using a pET-32a expression vector in *E. coli* BL21 (DE3) cells. This size was much larger than the predicted molecular weight of 46 kDa. This may be attributed to the presence of the thioredoxin tag (LaVallie *et al*, 2000) and 6xHis tag (Murphy and Doyle, 2005; Correa and Oppezzo, 2015). Although, the tendency of ISG65 and ISG75 to migrate slower than expected on an SDS-PAGE gel resulting in larger sized proteins has been reported (Ziegelbauer *et al*, 1992; Jackson *et al*, 1993; Tran *et al*, 2008; Baiyegunhi, 2013). Ziegelbauer and colleagues suggested that this tendency is due to the presence of several negatively charged amino acids present in ISG proteins thus reducing the binding of ISGs to SDS. This would lead to slow migration during electrophoresis allowing ISGs to appear larger than

predicted (Ziegelbauer *et al*, 1992). Attempts were made to sub-clone *Tc*ISG into the pET-28a and pGEX-4T-1 expression vectors to compare protein expression profiles however; recombinant colonies were only obtained by using the pET-32a vector.

Protein expression performed at lower temperatures reduces protein aggregation and favours protein solubility (Schein, 1989; Sahdev *et al*, 2008) therefore protein expression was also carried out at 30°C, 25°C and 20°C in an attempt to obtain soluble *Tc*ISG, however, the protein was still expressed within inclusion bodies. Inclusion bodies are formed as a result of protein misfolding through the interaction of hydrophobic residues during overexpression of the protein leading to protein aggregation (Wetzel, 1994; Flink, 1998). *Tc*ISG is characterised as a transmembrane protein hence it possesses several hydrophobic residues (Ziegelbauer *et al*, 1995) that could contribute to the formation of protein aggregates (Mitraki *et al*, 1991). Recombinant protein expression in inclusion bodies is undesirable as it requires solubilisation and correct refolding to produce functional proteins. Furthermore, solubilisation techniques need to be adapted for each protein (Zhu *et al*, 2013). Although undesirable, protein expression in inclusion bodies can be advantageous as they are not susceptible to proteolytic degradation and are easily isolated by centrifugation as was found in this study (Idicula-Thomas and Balaji, 2007).

*Tc*ISG was solubilised using anionic SDS and sarkosyl detergents according to Schlager and co-workers (2012). The technique is based on using SDS and sonication for solubilisation of inclusion bodies. *Tc*ISG was subsequently purified on a nickel affinity resin using 50 mM imidazole in the presence of sarkosyl. Sarkosyl is a mild detergent used during protein solubilisation and allows refolding of proteins that are present in high concentrations (Burgess, 1996; Kudou *et al*, 2011). The method of protein solubilisation and refolding is similar to cloud-point extraction, a technique typically used to purify and concentrate membrane proteins (Arnold and Linke, 2007).

The presence of significant amounts of *Tc*ISG in the unbound fraction during nickel affinity purification is attributed to the reduced binding efficiency of *Tc*ISG His-tagged fusion protein to the nickel resin caused by SDS and sarkosyl detergents in addition to the reducing agent, DTT, employed during the solubilisation process. Repeated use of the affinity resin in the presence of detergents and reducing agents is known to remove nickel ions from the affinity matrix over time (Palmer and Wingfield, 2004). The affinity matrix was frequently recharged with nickel ions in an effort to prevent further reduced binding efficiency. Poly-His tagged membrane proteins can also

be purified using Co^{2+}–carboxylmethyl-aspartate affinity resin (Bornhorst and Falke, 2000) however, purification of *Tc*ISG was unsuccessful using the His-Select® Cobalt affinity resin (Sigma). Nevertheless, purification using SDS and sarkosyl detergents proved successful.

Affinity tags are typically used to avert the production of protein aggregation and improve protein purification (Sørensen and Mortensen, 2005) however, overexpression of the histidine tag leads to the production of inclusion bodies due to the formation of a free loop area (Zhu *et al*, 2013). The use of the histidine affinity tag allows for successful solubilisation and purification of insoluble proteins under denaturing conditions as displayed in this study and interference during downstream applications is less probable due to its small size (Bornhorst and Falke, 2000, Schlager *et al*, 2012). Studies have demonstrated increased protein solubility by the use of maltose binding protein, N-utilisation substance protein A and thioredoxin tag while the use of glutathione-S-transferase resulted in reduced protein solubility. Such tags are much larger in size and may not always produce soluble proteins once it is removed thus smaller affinity tags such as the one used in this study are preferred (Rosano and Ceccarelli, 2014).

Several bacterial host proteins or degradation products were present in the first three fractions obtained from imidazole elution from the nickel affinity resin similar to the results obtained by Schlager and colleagues (Schlager *et al*, 2012). Rogé and co-workers have reported the presence of degradation products following expression of *T. brucei gambiense* ISG75 using the *Pichia pastoris* yeast expression system followed by purification using anti-E tag affinity matrix. Attempts to eliminate the degradation products were unsuccessful (Rogé *et al*, 2013). Furthermore, degradation products were also evident subsequent to expression of recombinant VSG LiTat 1.3 in *Pichia pastoris* and purification using a nickel charged nitrilotriacetic acid agarose resin identical to the resin used in this study (Rogé *et al*, 2014a). In a separate study conducted by Tran and co-workers, the extracellular domain of *T. brucei gambiense* ISG75 was recombinantly expressed using the pET system (Tran *et al*, 2000). Highly pure ISG75 was difficult to obtain using the nickel affinity matrix in combination with ion exchange chromatography, hydrophobic interaction and molecular exclusion chromatography (MEC). A streptavidin-tag and nickel affinity chromatography was then used to enhance purification (Tran *et al*, 2008). Despite the fact that degradation products were detected in this study, it was only found in three fractions therefore it was possible to obtain pure protein from fractions 4-10 and the use of more than one purification technique was not required in comparison to the above mentioned studies. Molecular exclusion chromatography was conducted using a Sephacryl

S200 matrix in an attempt to separate the 66 kDa purified *Tc*ISG from the 55 kDa lower molecular weight band however, separation was unsuccessful.

Due to the presence of the unidentified 55 kDa protein in fractions 1-3, only fractions 4-10 were pooled and dialysed against imidazole deficient buffer containing 5% (v/v) glycerol. This was done to prevent protein precipitation during the concentration steps and promote long term storage of recombinantly expressed proteins (Sawanoa *et al*, 1992; Kim *et al*, 2006; Vagenende *et al*, 2009).

The expression of *Tc*ISG could be improved by using eukaryotic expression systems such as yeast. *Pichia pastoris* yeast vectors were not considered for this study since the intellectual property restrictions placed on the vectors would conflict with the possibility of developing *Tc*ISG for diagnostics therefore *E. coli* BL21 (DE3) cells were used instead. Yeast expression systems such as *Pichia pastoris* (Cregg *et al*, 2000; Zhou *et al*, 2006), *Saccharomyces cerevisiae* (Hitzeman *et al*, 1981) and *Schizosaccharomyces pombe* (Giga-Hama and Kumagai, 1999; Kumar and Singh, 2004) are suitable for heterologous expression since it shares several features with higher eukaryotes. The advantages of yeast expression systems include: increased growth rate and simple and inexpensive growth media comparable to the *E. coli* expression system. However, the yeast system produces correctly folded soluble proteins that undergo post translational modifications and are secreted into the culture media in comparison to bacterial host systems (Daly and Hearn, 2005). The *Leishmania tarentolae* expression system has recently provided great promise in improving the expression of trypanosomal proteins and potentially replacing native antigens in rapid serodiagnostic testing. The system is based on electroporation of the LEXSYS vector hyg2 containing the desired gene into LEXSYS host strain p10. Recombinant proteins can be processed as close to the native type by undergoing post translational modifications in an organism related to the *Trypanosoma* species. Soluble recombinant proteins that are similar to the native proteins are produced using a simple culture media without the use of animals and expensive equipment thereby reducing the cost factor and promoting safer working conditions (Rooney *et al*, 2015).

In this study, the *Tc*ISG transmembrane protein containing all four domains was cloned and recombinantly expressed as a histidine-tagged fusion protein in *E. coli* BL21 (DE3) cells. Soluble *Tc*ISG was obtained using SDS and sarkosyl detergents and was refolded and purified using a nickel chelate affinity resin. Purified *Tc*ISG was used for antibody production and detection of native ISG from *T. congolense* (IL3000) bloodstream form lysates. *Tc*ISG also

served as an antigen for testing whether anti-*Tc*ISG N-terminal peptide antibodies recognise the whole protein in an ELISA and western blot. Furthermore, *Tc*ISG was tested as a diagnostic antigen to discriminate between *T. congolense* infected and non-infected cattle sera. These studies will be described in the next Chapter.

CHAPTER 3

PRODUCTION AND AFFINITY PURIFICATION OF ANTI-*Tc*ISG CHICKEN ANTIBODIES, IMMUNODETECTION OF NATIVE ISG AND INDIRECT ANTIBODY DETECTION ELISA

3.1 Introduction

Animal African trypanosomiasis (AAT) is considered a neglected disease that affects various animals resulting in thousands of deaths each year and leads to financial loss hence accurate diagnosis and effective treatment are critical (Eyford *et al*, 2011). Direct and indirect diagnostic techniques are used for the detection of motile parasites in infected blood samples. Such techniques cannot differentiate between parasite species and inconsistent results can be obtained due to the lack of sensitivity (Chappuis *et al*, 2005). Serodiagnostic testing such as indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and agglutination tests such as card agglutination test (CATT) offer detection of trypanosome-induced antibodies present in infected sera in a cost effective manner. These techniques can also screen numerous serums using an uncomplicated procedure (Voller *et al*, 1975; Luckins, 1977; Songa and Hamers, 1988; Nantulya and Lindwvist, 1989). A major drawback to some ELISA based tests is the requirement for trypanosome lysate obtained through sub-inoculation of laboratory animals which can result in non-reproducible results and especially for human-infective forms, poses a risk to laboratory personnel (Greiner *et al*, 1997; Hopkins *et al*, 1998; Rebeski *et al*, 2000; Desquesnes *et al*, 2009).

Recently, recombinant trypanosomal proteins have been used in rapid serodiagnostic testing which obviates the use of animals and infectious parasites thereby reducing health risks and expenses. Recombinantly produced invariant surface glycoprotein (ISG) have presented great promise in replacing native ISGs from *T. congolense*, *T. evansi* and *T. brucei gambiense* in diagnostic tests (Tran *et al*, 2008; Tran *et al*, 2009; Fleming *et al*, 2014; Rudramurthy *et al*, 2015). Other recombinant trypanosomal antigens investigated for serodiagnostic testing include: variable surface glycoprotein (VSG) RoTat 1.2 from *T. brucei gambiense* (Lejon *et al*, 2005; Rogé *et al*, 2013; Rogé *et al*, 2014b; Sengupta *et al*, 2014), VSG LiTat 1.5 from *T. brucei gambiense* (Van Nieuwenhove *et al*, 2013) and GM6, flagellar associated-protein from *T. vivax (*Pillay *et al*, 2013).

Lateral flow tests have become increasingly popular and offer rapid testing in the field without requiring expensive equipment or trained personnel. These tests are also easily designed and manufactured to be robust for field use, can often be stored for 12-24 months without cooling conditions and can accommodate small sample volumes with the option of scaling up to cater for larger volumes. Results can be determined visually or through electronic systems (O'Farrell, 2009). Lateral flow tests, HAT Sero-strip and HAT Sero-K-set use native VSG LiTat 1.3 and LiTat 1.5 native proteins from *T. brucei gambiense* (Büscher *et al*, 1991; 2014). The Surra Sero K-SeT lateral flow test uses a recombinant fragment of VSG RoTat 1.2 to detect anti-RoTat 1.2 antibodies from *T. evansi* infected blood, serum and plasma (Birhanua *et al*, 2015). Sullivan and co-workers have also developed a *T. brucei gambiense* recombinant ISG65 based lateral flow device (Sullivan *et al*, 2013). Until recently, lateral flow tests for AAT did not exist, however, Fleming and colleagues have produced a prototype lateral flow test using recombinantly produced ISG from *T. vivax* as the test antigen (Fleming *et al*, 2016). Lateral flow systems are yet to be developed for the diagnosis of *T. congolense* infections.

Egg yolk Immunoglobulin Y (IgY) technology is used to generate antibodies in chickens. Chicken antibodies are isolated from the egg yolks using precipitants such as polyethylene glycol (PEG) (Polson *et al*, 1980), dextran sulfate, ammonium sulfate, sodium sulfate, sodium citrate and caprylic acid (Meenatchisundaram and Michael, 2010). Isolation can also be achieved using chloroform and affinity chromatography. Polyethylene glycol precipitation is effective and yields increased amounts of highly pure antibodies (Meenatchisundaram and Michael, 2010). Generating polyclonal antibodies in chickens does not require bleeding of animals thus preventing stress on the animal; isolation is simple and rapid with various isolation techniques available and increased quantities of highly specific antibodies are obtained in comparison to generating immunoglobulin G (IgG) in rabbits (Pauly *et al*, 2011).

Epitope mapping is a technique used to identify specific antibody binding sites or epitopes in a protein. The epitopes are exposed to the immune system and can take the form of continuous epitopes which are linear peptides capable of binding antibodies raised against the complete protein or discontinuous epitopes comprising of amino acids located further apart from each other but brought together through protein folding (Barlow *et al*, 1986). Epitope mapping is useful in investigating the structure of proteins, parts of an enzyme that are inhibited by antibodies as well as identifying specific regions on a protein capable of eliciting an immune response for use as diagnostic antigens or vaccine candidates. The peptide corresponding to the epitope is synthesised and conjugated to a carrier protein for anti-peptide production. The

chances of detecting the native protein using anti-peptide antibodies is increased if the peptide is accessible to the immune system, has a length of 15-20 amino acids, has structural features that resemble the native protein, is flexible and contains polar and charged amino acids residues. The presence of glycine and proline residues also increases the chance of detecting native proteins by imitating the conformation of the native structure (Trier *et al*, 2012). Anti-peptide antibodies are capable of detecting the denatured protein in a western blot, while detection of the native protein can be done using flow cytometry, immunoprecipitation and immunohistochemistry as well as ELISA.

In this study, antibodies were generated in chickens against recombinantly expressed *Tc*ISG and a *Tc*ISG-N-terminal peptide. The IgY was isolated using PEG precipitation and affinity resins prepared by immobilising *Tc*ISG and *Tc*ISG N-terminal peptide on the affinity matrix. Chicken antibodies were assessed in ELISAs followed by the detection of native *Tc*ISG using western blots. The ability of recombinant *Tc*ISG to differentiate between non-infected and infected cattle sera was evaluated in an indirect ELISA and the detection of *Tc*ISG using anti-*T. b. brucei* ISG and anti-*T. b. gambiense* VSG antibodies was also investigated.

3.2 Materials and Methods

3.2.1 Materials

Preparation of chicken IgY antibodies: Freund's complete and incomplete adjuvants as well as bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO. USA). Polyethylene glycol (PEG) M_r 6 000 was acquired from Merck (Darmstadt, Germany) and the BIOTEK® ELx50™ Microplate washer was purchased from BioTek Instruments Inc. (USA). Nunc-Immuno™ Maxisorp 96-well plates were purchased from Nunc Intermed (Roskilde, Denmark) while the 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was purchased from Roche (Mannheim, Germany). Whatman No. 1 filter paper was purchased from Whatman International Ltd (UK), the Aminolink® and SulfoLink® matrix were obtained from Pierce (Rockford, IL, USA) and Sephadex G-10, G-25 and sodium hydroborocyanide were obtained from Sigma (St. Louis, MO. USA). The *Tc*ISG N-terminal peptide was synthesised by GL Biochem (Shanghai, China).

Antibodies: mouse anti-6xHis antibody HRPO conjugate antibody was obtained from Roche (Mannheim, Germany). Rabbit anti-chicken IgY conjugated to HRPO and rabbit anti-bovine IgG (A8917) conjugate antibody were purchased from Sigma (Munich, Germany). Chicken anti-*T. brucei* ISG65 IgY, chicken anti-*T. brucei* ISG75 IgY, chicken anti-*T. b. gambiense* RoTat 1.2 IgY, week 0 and week 4, chicken anti-*T. b. gambiense* LiTat 1.3 IgY week 0 and week 4, and chicken anti-*T. b. gambiense* LiTat 1.5 IgY week 0 and week 7 were obtained through in-house preparations (Mnkandla, 2013 and Baiyegunhi, 2013).

***T. congolense*-infected sera**: Infected and non-infected cattle sera were obtained from ClinVet International (PTY) LTD, Bloemfontein, South Africa.

Western blot: The Mini Trans-Blot® Cell apparatus was purchased from Biorad (CA, USA). BioTrace™, nitrocellulose membrane was obtained from PALL Corp (Ann Arbor, USA) while 4-choloro-1-naphthol and Kodak BioMax light film were acquired from Sigma (St. Louis, MO, USA). PageRuler™ prestained protein ladder was purchased from Pierce (Rockford, IL, USA).

3.2.2 Selection of *Tc*ISG N-terminal peptide

The complete protein sequence of *Tc*ISG was analysed using the Predict7 (Carmenes *et al*, 1989) epitope prediction software which analyses the surface probability, antigenicity, hydrophilicity and flexibility of the protein sequence. The PEP6 software [developed by Leonard K, BSc (Hons) Computer Science, University of KwaZulu-Natal] takes into consideration the flexibility of the amino acid residues by predicting protein mobility (Karplus and Schulz, 1985), uses the hydrophilicity scale developed by Hopp and Woods (1981) while the surface accessibility plot is in accordance with Emini and co-workers (1985) and was thus used in addition to Predict7. A region corresponding to increased surface probability and hydrophilicity values was selected for antibody production. Since the peptide contained an internal lysine residue, a cysteine residue was added to the N-terminus of *Tc*ISG to facilitate conjugation to rabbit albumin using MBS (maleimidobenzoyl-N-hydroxysuccinimide ester) for antibody production.

3.2.3 Immunisation of chickens with *Tc*ISG N-terminal peptide

3.2.3.1 Coupling of *Tc*ISG N-terminal peptide to rabbit albumin using MBS

Four milligrams of *Tc*ISG N-terminal peptide was dissolved in dimethyl sulfoxide (DMSO) (50 µl) and reducing buffer [100 mM Tris-HCl, 1 mM Na_2EDTA, 0.02% (w/v) NaN_3, pH 8.0 (500 µl)] followed by the addition of 10 mM DTT (500 µl). The mixture was incubated at 37°C for 1.5 h. The reduced peptide was separated from DTT using a Sephadex G-10 column (15 x 110 mm, 0.7 ml/min) previously equilibrated with 60 ml MEC buffer (100 mM NaH_2PO_4, 0.02 % (w/v) NaN_3, pH 7.0). Using MEC buffer, fractions (500 µl) were collected and tested for the presence of reduced peptide by combining each fraction (10 µl) with 10 µl Ellman's reagent [(2 mg 5,5'-Dithio-*bis*-(2-nitrobenzoic acid)] prepared in 500 µl Ellmans reagent buffer (100 mM Tris-HCl, 1 mM Na_2EDTA, 0.1% (w/v) SDS, pH 8.0). Reactions resulting in a yellow color indicated the presence of reduced peptide and were pooled (Kitagawa and Aikawa, 1976).

Rabbit albumin (RA) (3.16 mg) was dissolved in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2 (500 µl)] while MBS (0.58 mg) was dissolved in DMF (dimethylformamide) (200 µl) and PBS (300 µl). Both mixtures were combined in a 1: 40 molar ratio of MBS to rabbit albumin and gently mixed at 37 °C for 30 min. The rabbit albumin-MBS mixture was loaded onto a Sephadex G-25 column (15 x 130 mm) previously equilibrated with MEC buffer. Using MEC buffer, eluted fractions (1 ml) were collected and measured spectrophotometrically at 280 nm for the presence of the activated rabbit albumin carrier. Fractions with absorbance values above 0.3 were pooled and immediately combined with the reduced peptide followed by gentle stirring at RT for 2 h and incubated further at 4°C using end-over-end rotation for 16 h. The solution was distributed into 4 equal aliquots and stored at -20°C until immunisation.

3.2.4 Immunisation of chickens for antibody production

Antibodies against the recombinant *Tc*ISG and *Tc*ISG N-terminal peptide conjugated to rabbit albumin were produced in chickens. Purified *Tc*ISG and *Tc*ISG N-terminal peptide were separately combined with Freund's complete adjuvant (1:1) followed by trituration to form a stable emulsion. Two chickens were immunised intramuscularly on both sides of the breast bone with purified *Tc*ISG (100 µg/ml) and another two chickens were immunised with *Tc*ISG N-terminal peptide conjugated to rabbit albumin (1.25 ml). Using Freund's incomplete adjuvant, booster injections were given to each chicken at weeks 2, 4 and 6. Pre-immune eggs collected

prior to immunisation and all eggs collected daily over a period of 16 weeks were stored at 4°C until required.

3.2.5 Isolation of IgY antibodies from egg yolks

The chicken immunoglobulins (IgY) were isolated from egg yolks using polyethylene glycol precipitation described by Goldring and Coetzer (2003). Briefly, egg yolks were separated from albumin under running water. The egg yolk volume was determined and twice the volume of IgY buffer (100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH 7.6) and 3.5% (w/v) PEG 6000 was added and mixed using magnetic stirring. The mixture was centrifuged (4 420 x *g,* 30 min, 4°C) to remove the lipid fraction and the supernatant filtered through cotton wool until a clear filtrate was obtained. To the filtrate, 8.5% (w/v) PEG 6000 was added and mixed thoroughly followed by centrifugation (12 000 x *g*, 10 min, 4°C). The pellet was subsequently dissolved in IgY buffer equivalent to the original egg yolk volume and 12% (w/v) PEG 6000 was added and mixed. The mixture was centrifuged (12 000 x *g*, 10 min, 4°C). The resulting pellet was dissolved in final IgY buffer (100 mM NaH_2PO_4, 0.1% (w/v) NaN_3, pH 7.6) equivalent to one sixth of the original egg yolk volume and stored at 4°C. Using spectrophotometry, the concentration of the IgY was determined at 280 nm using an extinction coefficient of $E^{1\ mg/ml}_{280\ nm}$ = 1.25 (Goldring and Coetzer, 2003).

3.2.6 Enzyme-linked immunosorbent assay

Production of anti-*Tc*ISG IgY and anti-*Tc*ISG N-terminal peptide IgY antibodies were monitored and evaluated using the ELISA. The 96 well Nunc-Immuno™ Maxisorp ELISA plates were coated with recombinant *Tc*ISG (1 µg/ml, 100 µl per well) or *Tc*ISG N-terminal peptide (5 µg/ml) prepared in PBS and incubated at 4°C for 16 h. The solution was discarded and the wells blocked with 0.5% (w/v) BSA in PBS-0.1% (v/v) Tween-20 blocking buffer (200 µl per well) at 37°C for 1 h. Wells were subsequently washed thrice with PBS containing 0.1% (v/v) Tween-20 using the BIOTEK® ELx50™ Microplate washer. Wells were incubated with anti-*Tc*ISG or anti-*Tc*ISG peptide IgY primary antibody prepared in blocking buffer (100 µg/ml, 100 µl per well) at 37°C for 2 h. Wells were washed as described previously followed by incubation with rabbit anti-chicken IgY-HRPO secondary antibody conjugate prepared in blocking buffer (1:15000, 100 µl per well) at 37°C for 1 h. Wells were washed thrice with PBS containing 0.1% (v/v) Tween-20 and incubated with chromogen-substrate solution [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in 0.15 M citrate-phosphate buffer, pH 5.0 (145 µl per well)]. The reaction was allowed to develop

in the dark before absorbance analysis at 405 nm using the FLUOStar Optima microplate reader (BMG Labtech, Offenburg, Germany).

3.2.7 Affinity purification of anti-*Tc*ISG IgY and anti-*Tc*ISG N-terminal peptide IgY

3.2.7.1 Coupling of *Tc*ISG to Aminolink® affinity column

AminoLink® coupling resin (2 ml) was added to a 10 ml chromatography column and allowed to settle. The storage solution was allowed to drain and the resin equilibrated with coupling buffer [150 mM $Na_3C_6H_5O_7.2H_2O$, 50 mM Na_2CO_3, pH 10 (6 ml)]. Purified *Tc*ISG (8 mg/ ml) was diluted in coupling buffer (4 ml) and 100 µl of this dilution was kept to determine the coupling efficiency. The resin was mixed with *Tc*ISG at RT for 4 h using end-over-end rotation. The column was allowed to remain stationary at RT for 16 h. The unbound fraction was collected and a coupling efficiency of 50% was obtained therefore 4 mg/ml of *Tc*ISG coupled to the resin. The resin was washed with AminoLink® coupling buffer [100 mM NaH_2PO_4, 150 mM NaCl, pH 7.2 (6 ml)] to obtain a neutral pH. In the fume hood, AminoLink® buffer [100 mM NaH_2PO_4, 150 mM NaCl, pH 7.2 (2 ml)] and cyanoborohydride [5 M $NaCNBH_3$ in 1 M NaOH (40 µl)] was gently mixed with the resin and incubated at 4°C for 16 h using end-over-end rotation. The resin was washed with quenching buffer [1 M Tris-HCl, pH 7.4 (4 ml)]. In the fume hood, 2 ml quenching buffer and cyanoborohydride [5 M $NaCNBH_3$ in 1 M NaOH (40 µl)] were gently mixed with the resin and incubated at RT for 30 min using end-over-end rotation. The resin was washed with PBS until an absorbance of zero at 280 nm was obtained. The resin was washed with 6 ml of IgY buffer (100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH7.6) and stored in 2 ml of the IgY buffer at 4°C until required.

3.2.7.2 Coupling of *Tc*ISG N-terminal peptide to Sulfolink® affinity column

The *Tc*ISG N-terminal peptide (5 mg) was reduced as described in Section 3.2.3.1. The SulfoLink® coupling resin (1 ml) was placed into a 10 ml affinity column and washed with 6 ml coupling buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.5). The resin was mixed with the reduced peptide at RT for 15 min using end-over-end rotation and subsequently allowed to remain stationary for 30 min at RT and washed with 3 ml coupling buffer. To the resin, 50 mM L-Cysteine made in Sulfolink® coupling buffer (1 ml) was added and mixed at RT for 15 min using end-over-end rotation and allowed to remain stationary at RT for 30 min. The resin was thereafter washed with 1 M NaCl (16 ml) followed by IgY buffer [100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH 7.6 (2 ml)] and stored in 2 ml IgY buffer at 4°C until required.

3.2.7.3 Purification of anti-*Tc*ISG IgY and anti-*Tc*ISG N-terminal IgY antibodies

The Aminolink® and SulfoLink® resins were equilibrated with 6 ml PBS and antibodies with high antibody titres determined by ELISA (Section 3.2.6) were pooled and filtered through Whatman No. I filter paper. The pooled antibodies were applied to the column and cycled at RT for 16 h (0.5 ml/min). The unbound antibodies were collected and the resin was washed with PBS until the absorbance at 280 nm reached zero. Bound antibodies (900 µl fractions) were eluted with elution buffer (0.1 M glycine-HCl, 0.02% (w/v) NaN_3, pH 2.8) into microcentrifuge tubes containing neutralisation buffer [1 M NaH_2PO_4, 0.02% (w/v) NaN_3, pH 8.5 (100 µl)] and gently mixed by inversion. The absorbance of each fraction was measured at 280 nm and fractions containing purified IgY antibodies were pooled and stored in the presence of 0.1% (w/v) sodium azide at 4°C until required. The resin was subsequently washed with 20 ml PBS, 10 ml IgY buffer (100 mM NaH_2PO_4, 0.02% (w/v) NaN_3, pH 7.6) and stored in 2 ml of IgY buffer at 4°C until required.

3.2.8 Silver staining

The detection of small quantities of protein (300-500 ng) was achieved using the silver staining technique according to Blum and co-workers (1987). All steps were carried out at RT. Following SDS-PAGE, the electrophoresis gel was soaked in fixing solution (50% (v/v) methanol, 12% (v/v) acetic acid, 0.5% (v/v) formaldehyde) for 16 h on an orbital shaker. The gel was subsequently incubated (3 x 20 min) in wash solution (50% (v/v) ethanol) followed by soaking in pre-treatment solution (0.4% (w/v) $Na_2S_2O_3.5H_2O$) for 1 min. The gel was rinsed in distilled water (3 x 20 s) and soaked in impregnation solution (0.2% (w/v) $AgNO_3$, 0.75% (v/v) 37% formaldehyde) for 20 min. Following rinsing with distilled water (3 x 20 s), the gel was incubated in developing solution (6% (w/v) Na_2CO_3, 0.004% (w/v) $Na_2S_2O_3.5H_2O$, 0.5% (v/v) 37% formaldehyde) until the first bands became visible. The developing solution was then replaced with distilled water until the colour of the bands had developed accordingly. The development of the bands was terminated using stopping solution (50% (v/v) methanol, 12% (v/v) acetic acid) for 10 min and the gel stored in 50% (v/v) methanol. Gels were photographed using the Sys Gene® imaging system (Syngene®, USA).

3.2.9 Western Blot analysis of *Tc*ISG fusion protein

*Tc*ISG fusion protein was electrophoresed on a 10% reducing SDS-PAGE gel (Section 2.2.10) and transferred onto a nitrocellulose membrane using the Mini Trans-Blot® Cell apparatus (Biorad, CA, USA). Subsequent steps were performed as described in Section 2.2.11. The blot was incubated with antibodies depicted in Table 1.3, detected using chromogen-substrate solution (0.06% (w/v) 4-chloro-1-naphthol, 0.1% (v/v), H_2O_2 in TBS) at RT in the dark and photographed using the Sys Gene® imaging system (Syngene®, USA).

Table 3.1: Antibodies used for the detection of *Tc*ISG in a western blot.

Primary antibody	Secondary antibody
mouse anti-6xHis-HRPO [1:8000]	none
chicken anti-*Tc*ISG IgY	rabbit anti-chicken IgY- HRPO
chicken anti-*Tc*ISG N-terminal peptide IgY	rabbit anti-chicken IgY- HRPO
T. congolense infected cattle sera [1:100]	rabbit anti-bovine IgG-HRPO

3.2.10 Western blot analysis of *T. congolense* lysate

Trypanosoma congolense (IL3000) BSFs were obtained by centrifugation of the culture medium (2000 x *g*, 15 min, RT), resuspended in PBS and centrifuged (2000 x *g*, 5 min, RT). The resuspension and centrifugation steps were repeated. Parasites were resuspended in lysis buffer (20 mM Tris-HCl buffer pH 7.2, 10 mM Na_2EDTA, 1% (v/v), Triton X-100) containing L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane E64 (10 µM) and an equivalent volume of reducing treatment buffer was added and boiled at 100°C for 10 min. Parasite lysate proteins were separated on a 10% reducing SDS-PAGE gel and transferred onto nitrocellulose. The subsequent western blot steps were conducted as described in Section 2.2.11. The blot was incubated with primary antibodies: affinity purified chicken anti-*Tc*ISG IgY and chicken anti-*Tc*ISG N-terminal peptide IgY (10 µg/ml) antibodies. Rabbit anti-chicken IgY [1:5000] secondary antibody and chromogen-substrate solution (0.06% (w/v) 4-chloro-1-naphthol, 0.1% (v/v), (v/v) H_2O_2 in TBS) was used as a detection system. Blots were photographed using the Sys Gene® imaging system (Syngene®, USA).

3.2.11 Detection of anti-ISG antibodies in infected sera using indirect ELISA

The detection of anti-ISG antibodies in *T. congolense* infected cattle sera was conducted by coating 96 well Nunc-Immuno™ Maxisorp ELISA plates with recombinant *Tc*ISG (5 µg/ml, 100 µl per well) prepared in PBS. Samples were tested in quadruplicate and negative controls included wells coated with PBS with no antigen present. Subsequent steps were performed as described in Section 3.2.6. Wells were incubated with sera from infected, non-infected and drug treated cattle (1:50 and 1:100) prepared in blocking buffer (100 µl per well) at 37°C for 2 h. A no serum control was also included. Blocking buffer was added to these wells. Wells were then washed as described previously followed by incubation with rabbit anti-bovine IgG antibody conjugated to HRPO prepared in blocking buffer (1:5000, 100 µl per well) at 37°C for 1 h. Subsequent steps were performed as described in Section 3.2.6.

3.2.12 Cross reactivity of *Tc*ISG with trypanosomal ISG and VSG antibodies

3.2.12.1 Enzyme-linked immunosorbent assay

The ability of *Tc*ISG to be detected by antibodies produced against ISG and VSG molecules from *T. brucei* and *T. brucei gambiense* was investigated. Nunc-Immuno™ Maxisorp ELISA plates were coated with *Tc*ISG (1 µg/ml; 100 µl per well) prepared in PBS and incubated at 4°C for 16 h. Subsequent steps were performed as described in Section 3.2.6 and wells were probed with chicken anti-*Tc*ISG IgY collected weeks 3-8 post first immunisation from chicken 2, anti-*Tc*ISG IgY pre-immune, chicken anti-*Tc*ISG N-terminal peptide IgY collected weeks 10-16 post first immunisation from chicken 2, chicken anti-*Tc*ISG N-terminal peptide IgY pre-immune, chicken anti-*T. brucei* ISG65 IgY, chicken anti-*T. brucei* ISG75, chicken anti-*T. b. gambiense* VSG RoTat 1.2, week 0 and week 4, chicken anti-*T. b. gambiense* VSG LiTat 1.3 IgY week 0 and week 4 and chicken anti-*T. b. gambiense* VSG LiTat 1.5 IgY week 0 and week 7 [0.316-100 µg/ml prepared in blocking buffer]. Wells were washed as described previously followed by incubation with rabbit anti-chicken IgY secondary antibody conjugated to HRPO prepared in blocking buffer [1:15000, 100 µl per well] at 37°C for 1 h. Subsequent steps were performed as described in Section 3.2.6.

3.2.12.2 Dot blot analysis of antibody cross reactivity

Nitrocellulose strips were spotted with *Tc*ISG (1, 5, 10, 50 and 100 µg/ml) and air dried. Subsequent steps were performed as described in Section 2.2.11. Nitrocellulose strips were

probed with chicken anti-*Tc*ISG IgY, chicken anti-*Tc*ISG IgY pre-immune, chicken anti-*Tc*ISG N-terminal peptide IgY, chicken anti-*Tc*ISG N-terminal peptide IgY pre-immune, chicken anti-*T. brucei* ISG65 IgY, chicken anti-*T. brucei* ISG75, chicken anti-*T. b. gambiense* VSG RoTat 1.2, week 0 and week 4, chicken anti-*T. b. gambiense* VSG LiTat 1.3 IgY week 0 and week 4, and chicken anti-*T. b. gambiense* VSG LiTat 1.5 IgY week 0 and week 7 [0.316 µg/ml and 100 µg/ml]. Wells were washed as described previously followed by incubation with rabbit anti-IgY-HRPO [1:5000] and 4-chloro-1-naphthol·H_2O_2 chromogen-substrate solution. Blots were photographed using the Sys Gene® imaging system (Syngene®, USA).

3.3 Results

3.3.1 Production of chicken anti-*Tc*ISG IgY antibodies

Chicken anti-*Tc*ISG IgY antibodies were isolated from a single egg collected at the start of each week post first immunisation and used in an ELISA to monitor antibody production over the period of 16 weeks. Following the first booster injection at week 2, both chickens began producing antibodies as seen by the rapid increase in absorbance values (Figure 3.1). Antibody production by chicken 1 increased from week 2 to 6 and thereafter remained consistent with a slight decrease during week 10. Peak antibody production was detected during weeks 5, 7 and 9 from eggs from chicken 2 with a minor decrease during weeks 6, 8 and 10 and production remained constant thereafter.

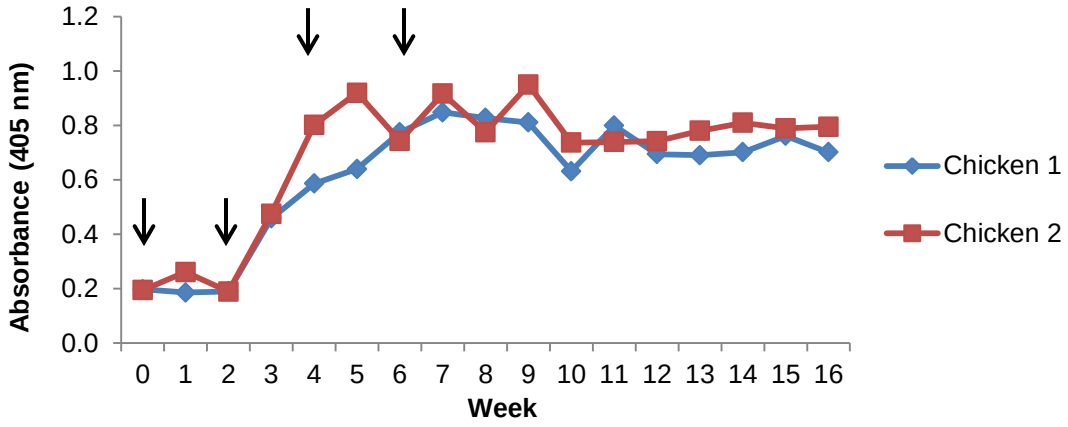

Figure 3.1: Production of anti-*Tc*ISG IgY antibodies over a period of 16 weeks. Antibodies were isolated from a single egg at the start of each week**.** ELISA plates were coated with *Tc*ISG (1 µg/ml) and probed with anti-*Tc*ISG IgY (100 µg/ml) from chickens 1 and 2. Rabbit anti-chicken IgY-HRPO conjugate [1:15000] and chromogen-substrate solution (0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer, pH 5.0) was used to develop the reaction for 30 min in the dark. The absorbance readings at 405 nm represent the average of duplicate experiments. Arrows represent immunisation at week 0 followed by booster injections at weeks 2, 4 and 6.

3.3.2 Purification of chicken anti-*Tc*ISG antibodies

To obtain an anti-*Tc*ISG IgY antibody preparation with improved specificity, eggs collected during weeks corresponding to increased antibody production were pooled and purified using the AminoLink® resin coupled with purified *Tc*ISG. The antibody pools included: weeks 3-7, weeks 8-12 and weeks 9-16 from chicken 1 (Figure 3.2, panel A) and weeks 3-8 and weeks 9-16 from chicken 2 (Figure 3.2, panel B). The respective IgY pools were cycled through the AminoLink®-*Tc*ISG resin and specific antibodies were eluted using an acidic pH and elution monitored spectrophotometrically at 280 nm. High absorbance indicating increased antibody concentration was detected from fractions 3-5 (Figure 3.2, panel A and panel B). All pools contained a significant amount of antibodies as seen by the increased absorbance values for eluates from all pools from both chickens.

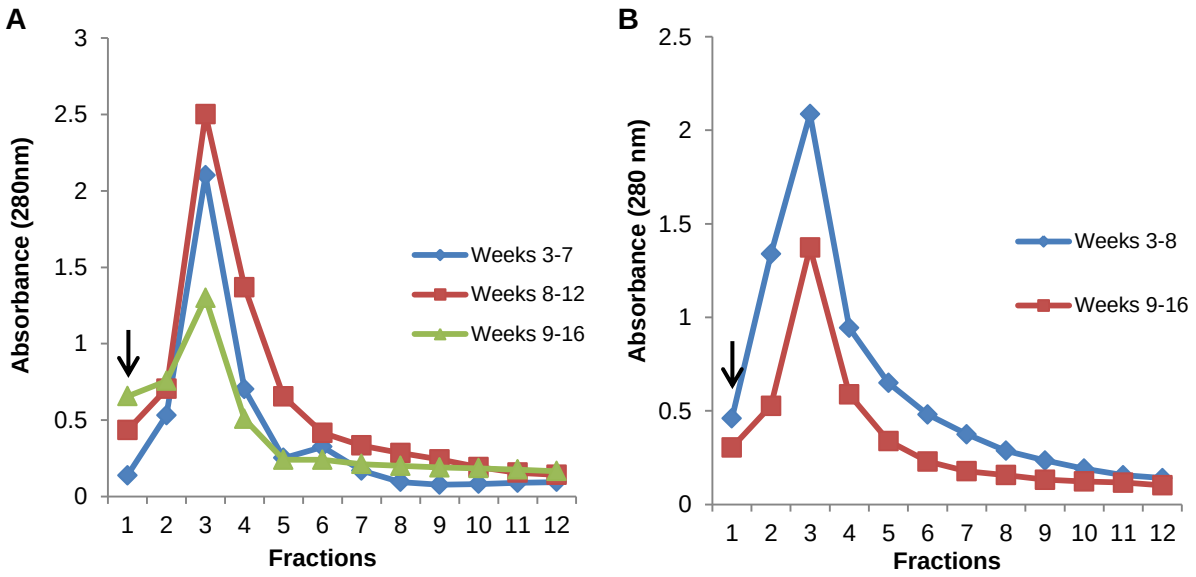

Figure 3.2: Elution profiles for affinity purification of chicken anti-*Tc*ISG IgY antibodies. Antibodies from **(A)** chicken 1 and **(B)** chicken 2 were purified using the AminoLink® resin coupled to *Tc*ISG. The resin was washed with PBS and bound antibodies were eluted using elution buffer (0.1 M glycine, 0.02% NaN_3, pH 2.8) into microcentrifuge tubes containing 100 µl neutralisation buffer (1 M NaH_2PO_4, 0.02% NaN_3, pH 8.5). Absorbance was monitored at 280 nm. Arrows indicate the start of low pH elution.

Affinity purification of anti-*Tc*ISG IgY antibodies and their ability to recognise recombinant *Tc*ISG antigen was investigated in a ELISA. All antibody pools from chicken 1 and 2 displayed increased antibody titres compared to the unbound and pre-immune IgY fractions (Figure 3.3). Highest absorbance values were observed from affinity purified antibody from chicken 1 weeks 3-7 (Figure 3.3, panel A) and weeks 8-12 (Figure 3.3, panel B) as well as from chicken 2 weeks 3-9 (Figure 3.3, panel D). Antibody titres from chicken 1 weeks 9-16 (Figure 3.3, panel C) and chicken 2 weeks 10-16 (Figure 3.3, panel E) were marginally lower than the other pools.

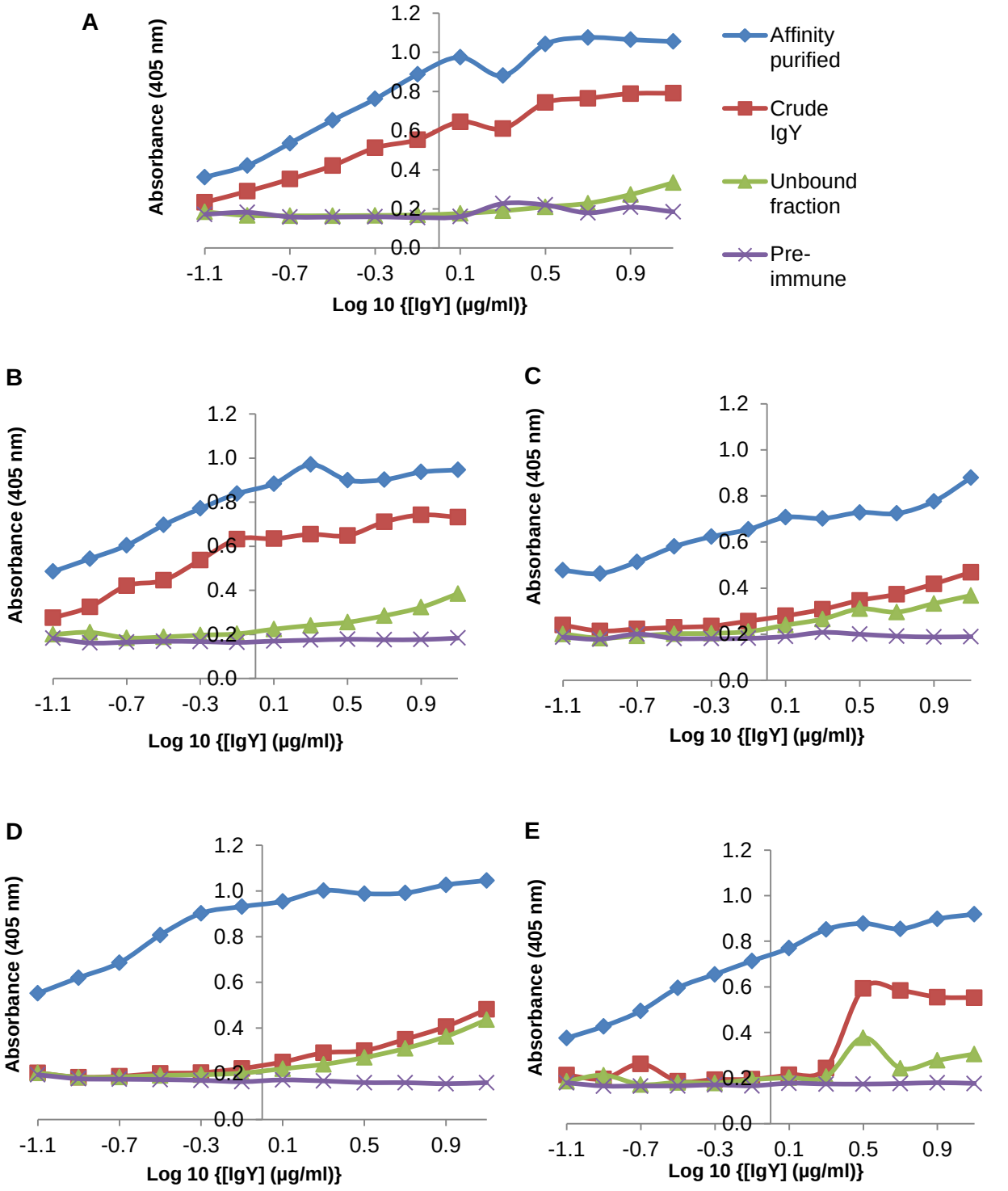

Figure 3.3: ELISA analysis of affinity purified anti-*Tc*ISG IgY antibodies. Wells were coated with *Tc*ISG (1 µg/ml in PBS) and probed with affinity purified chicken anti-*Tc*ISG IgY, crude IgY, unbound fraction and pre-immune diluted from 0.08-12.59 µg/ml. **(A)** Weeks 3-7 chicken 1. **(B)** Weeks 8-12 chicken 1. **(C)** Weeks 9-16 chicken 1. **(D)** Weeks 3-9 chicken 2. **(E)** Weeks 10-16 chicken 2. Rabbit anti-chicken IgY-HRPO conjugate [1:15000] and chromogen-substrate solution (0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer, pH 5.0) was used to develop the reaction for 45 min in the dark. The absorbance readings at 405 nm represent the average of duplicate experiments.

3.3.3 Production of chicken anti-*Tc*ISG N-terminal peptide IgY

The peptide selected for antibody production corresponds to amino acid residues 320-338 which is located within the extracellular domain of *Tc*ISG for maximum exposure to the chicken immune system (Figure 3.4). The peptide possessed highest hydrophilicity and surface probability at the C-terminus and contained a lysine residue within the sequence therefore the peptide was coupled at the N-terminus by replacing the asparagine residue (indicated by the arrow) with a cysteine residue to assist coupling of the peptide to rabbit albumin using MBS for antibody production in chickens.

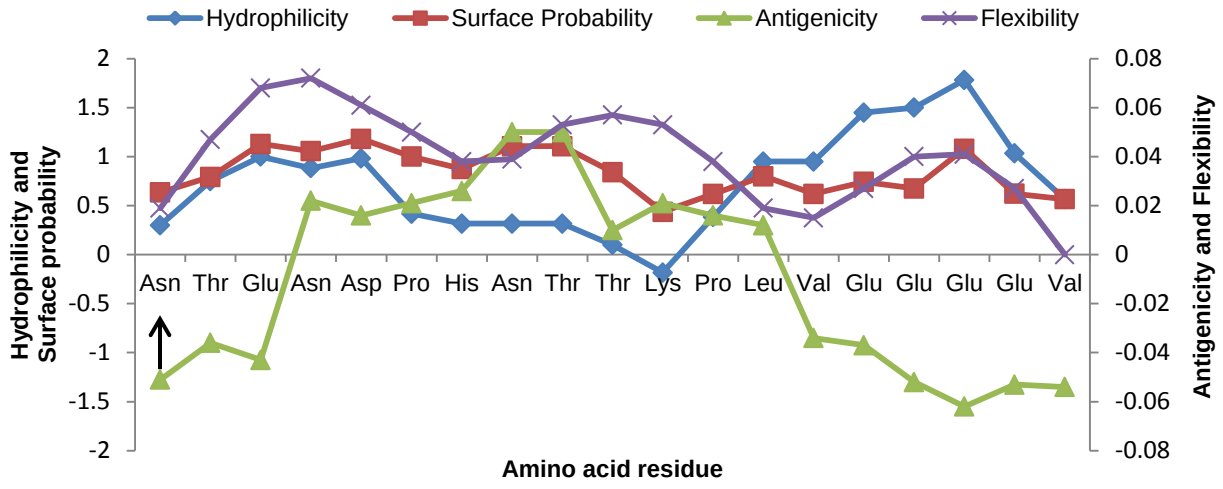

Figure 3.4: Analysis of N-terminal *Tc*ISG N-terminal peptide using Predict7 program. The surface probability and hydrophilicity is plotted on the primary axis while antigenicity and flexibility is displayed on the secondary axis. The three letter amino acid residues are indicated on the horizontal axis.

Antibodies made against *Tc*ISG N-terminal peptide was isolated from a single egg collected at the start of each week over the course of 16 weeks and examined in an ELISA. Antibody production began two weeks post immunisation for both chickens as observed by the increased absorbance values (Figure 3.5). No eggs were laid by chicken 1 during week 3 however; antibody production remained relatively constant with minor decreases during weeks 6 and 11. After week 2, consistent antibody production was detected from chicken 2 with a dramatic decrease during week 9. Antibody production then increased during week 10 and displayed a uniform trend until week 16 where a small increase was observed.

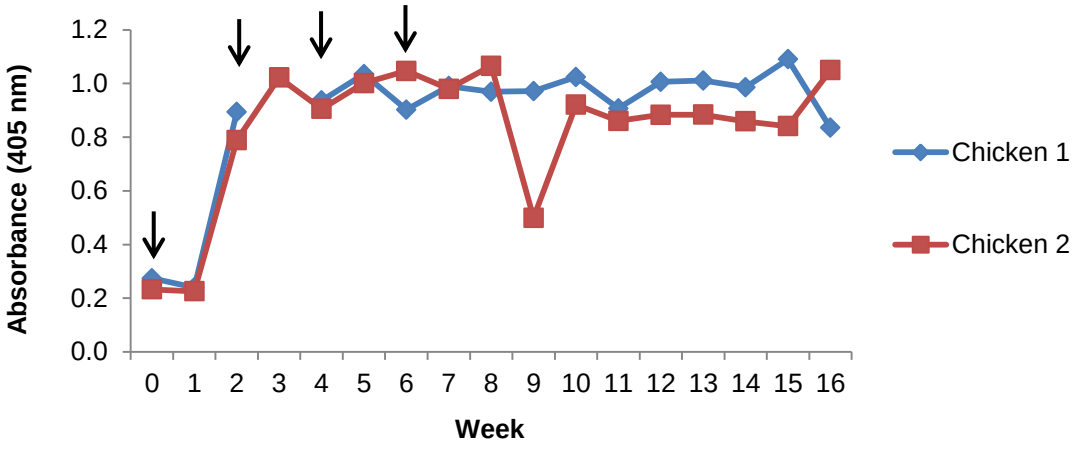

Figure 3.5: Production of chicken anti-*Tc*ISG N-terminal peptide IgY antibodies over a period of 16 weeks. Antibodies were isolated from a single egg collected at the start of each week**.** ELISA plates were coated with *Tc*ISG N-terminal peptide (5 µg/ml in PBS) and probed with anti-*Tc*ISG N-terminal peptide IgY (100 µg/ml) from chickens 1 and 2. Rabbit anti-chicken IgY-HRPO conjugate antibody [1:15000] and chromogen-substrate solution was used to develop the reaction for 30 min in the dark. The absorbance readings at 405 nm represent the average of duplicate experiments. Arrows represent immunisation at week 0 followed by booster injections at week 2, 4 and 6.

3.3.4 Purification of chicken anti-*Tc*ISG N-terminal peptide IgY

Antibodies made against *Tc*ISG N-terminal peptide were purified on a SulfoLink® resin immobilised with *Tc*ISG N-terminal peptide. The antibody pools comprised of weeks 4-9 and weeks 10-16 from chicken 1 (Figure 3.6, panel A) and weeks 3-9 and 10-16 from chicken 2. (Figure 3.6, panel B). Increased absorbance relating to high antibody concentration was detected from fractions 2-4 from chicken 1 antibody pools (Figure 3.6, panel A) and fractions 2-3 from chicken 2 (Figure 3.6, panel B). The highest absorbance readings were detected from chicken 1, weeks 10-16 and chicken 2, weeks 3-9. Nonetheless, all pools contained a considerable amount of antibodies as seen by the increased absorbance values from all antibody pools from both chickens.

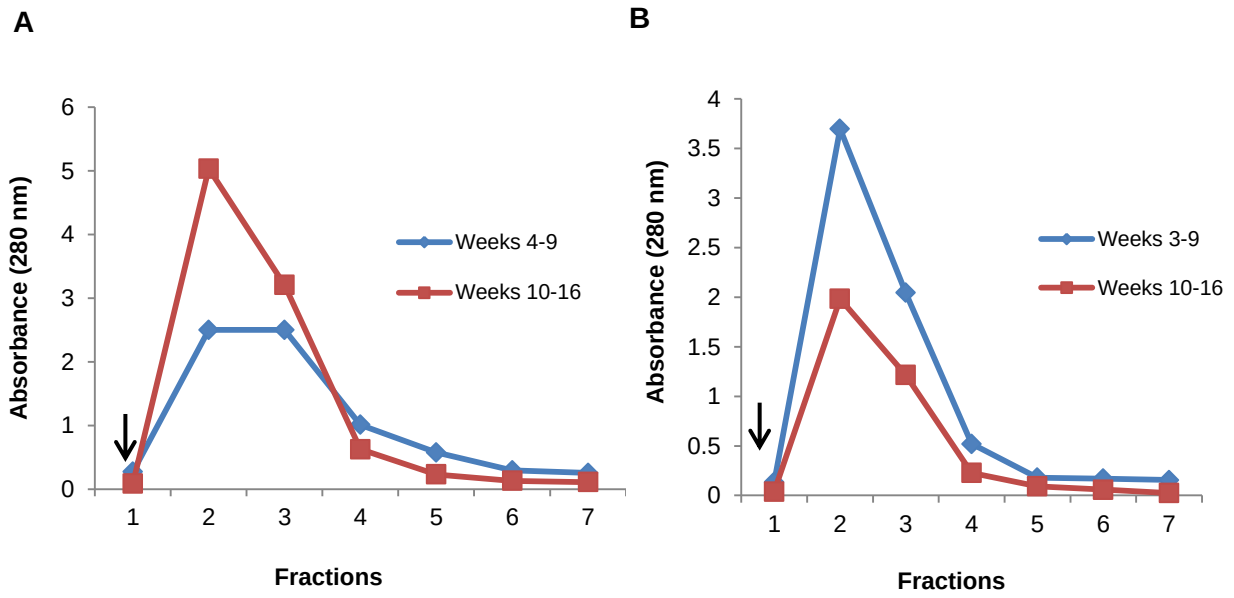

Figure 3.6: Elution profiles for affinity purification of chicken anti-*Tc*ISG N-terminal peptide IgY antibodies. Antibodies from **(A)** chicken 1 and **(B)** chicken 2 were purified using the SulfoLink®-*Tc*ISG N-terminal peptide immobilised resin. The resin was washed with PBS and bound antibodies were eluted using elution buffer (0.1 M glycine, 0.02% NaN_3, pH 2.8) into microcentrifuge tubes containing 100 µl neutralisation buffer (1 M NaH_2PO_4, 0.02% NaN_3, pH 8.5). Absorbance was monitored at 280 nm. Arrows indicate the start of low pH elution**.**

Affinity purification of anti-*Tc*ISG N-terminal peptide IgY antibodies and their specificity was evaluated in a ELISA with different concentrations of *Tc*ISG antigen and the respective antibodies. Antibody pools from chicken 1 and 2 exhibited high antibody titres compared to the crude IgY fraction, unbound and pre-immune fractions (Figure 3.7). However, the absorbance decreased greatly from a log concentration of 0.5 for chicken 1 weeks 3-7 (Figure 3.7, panel A), 0.3 for chicken 1 weeks 8-12 (Figure 3.7, panel B), 0.3 for chicken 2 weeks 3-9 chicken (Figure 3.7, panel C) and weeks 10-16 (Figure 3.7, panel D).

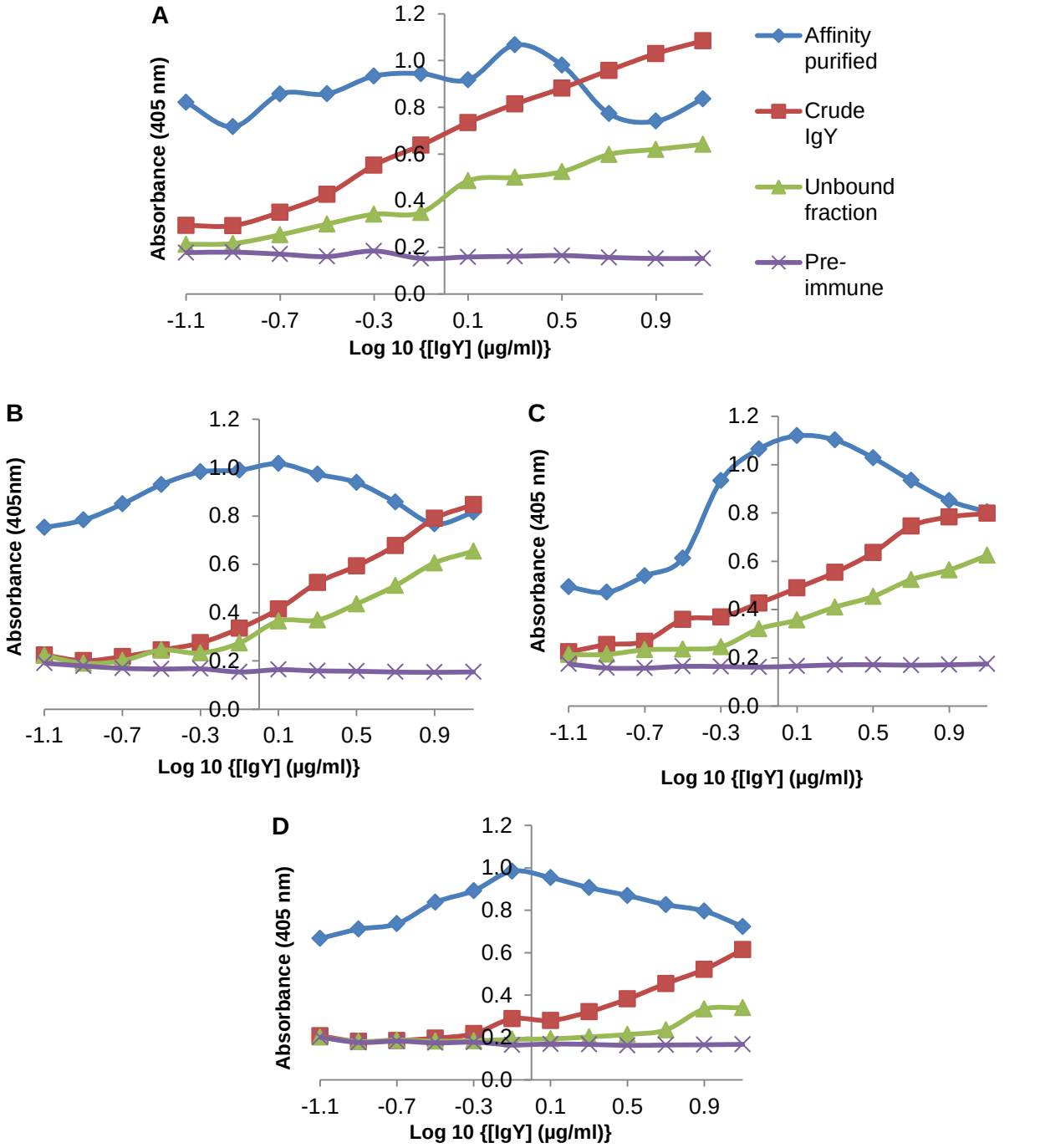

Figure 3.7: ELISA analysis of affinity purified chicken anti-*Tc*ISG N-terminal peptide IgY antibodies. ELISA plates were coated with *Tc*ISG N-terminal peptide (1 µg/ml in PBS) and probed with affinity purified anti-*Tc*ISG N-terminal peptide IgY, crude IgY, unbound fraction and pre-immune diluted from 0.08-12.59 µg/ml. **(A)** Weeks 3-7 chicken 1. **(B)** Weeks 8-12 chicken 1. **(C)** Weeks 9-16 chicken 1. **(D)** Weeks 3-9 chicken 2. **(E)** Weeks 10-16 chicken 2. Rabbit anti-chicken IgY-HRPO conjugate [1:15000] and chromogen-substrate solution (0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer, pH 5.0) was used to develop the reaction for 45 min in the dark. The absorbance readings at 405 nm represent the average of duplicate experiments.

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3.3.5 Evaluation of chicken antibodies by western blot

The concentrations of *Tc*ISG antigen and chicken anti-*Tc*ISG IgY were optimised in a dot blot which showed detection using as little as 1 µg/ml *Tc*ISG and 0.5 µg/ml chicken anti-*Tc*ISG IgY (Appendix I). The chicken antibodies were subsequently evaluated in a western blot against recombinant *Tc*ISG. The *Tc*ISG antigen was electrophoresed on a reducing SDS-PAGE gel and silver stained which presented a single band corresponding to *Tc*ISG protein (Figure 3.8, panel A). No lower molecular weight proteins existed after dialysis and storage in glycerol (Section 2.2.12). Detection of recombinant *Tc*ISG was evident in the western blot probed with affinity purified chicken anti-*Tc*ISG IgY and chicken anti-*Tc*ISG N-terminal peptide IgY antibodies (Figure 3.8, panel B). The mouse anti-6xHis-HRPO conjugate antibody was used as a positive control; while the pre-immune antibody, no primary antibody and no secondary antibody were used a negative controls and showed no non-specific reactions (results not shown).

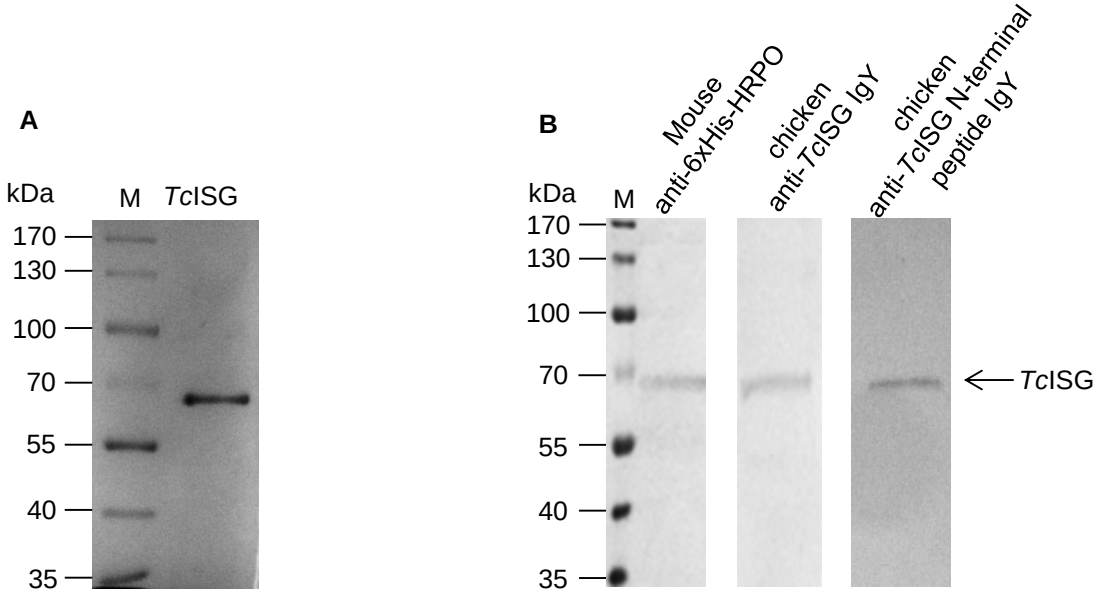


Figure 3.8: Western blot analysis of *Tc*ISG using chicken anti-*Tc*ISG IgY and chicken anti-*Tc*ISG N-terminal peptide IgY antibodies. *Tc*ISG (5 µg/ml) was analysed on a **(A)** 10% reducing SDS-PAGE gel and visualised with silver staining and **(B)** by western blot probed with mouse anti-6xHis-HRPO [1:8000], affinity purified chicken anti-*Tc*ISG (weeks 3-9 chicken 2) [0.3 µg/ml] and chicken anti-*Tc*ISG N-terminal peptide IgY (weeks 3-7 chicken 1) [0.5 µg/ml]. Blots were developed rabbit anti-chicken IgY-HRPO conjugate [1:5000] conjugate and 4-chloro-1-naphthol·H_2O_2 chromogen-substrate solution. M: PageRuler™ prestained protein marker.

3.3.6 Western blot analysis of *T. congolense* lysate

Affinity purified chicken anti-*Tc*ISG IgY and chicken anti-*Tc*ISG N-terminal peptide IgY were tested for their capacity to detect native ISG from *T. congolense* (IL 3000) bloodstream form parasites. The parasite lysate was analysed on a reducing SDS-PAGE gel resulting in the presence of several proteins following Coomassie Blue staining (Figure 3.9, panel A). Western blot analysis showed detection of a single protein approximately 67 kDa in size with both affinity purified chicken anti-*Tc*ISG IgY (Figure 3.9, panel C) and chicken anti-*Tc*ISG N-terminal peptide IgY (Figure 3.9, panel D). The size was much larger than the predicted 46 kDa. An unrelated His-tagged fusion protein was used as a negative control in this experiment and was detected only with mouse anti-6xHis-HRPO conjugate antibody (Figure 3.8, panel B).

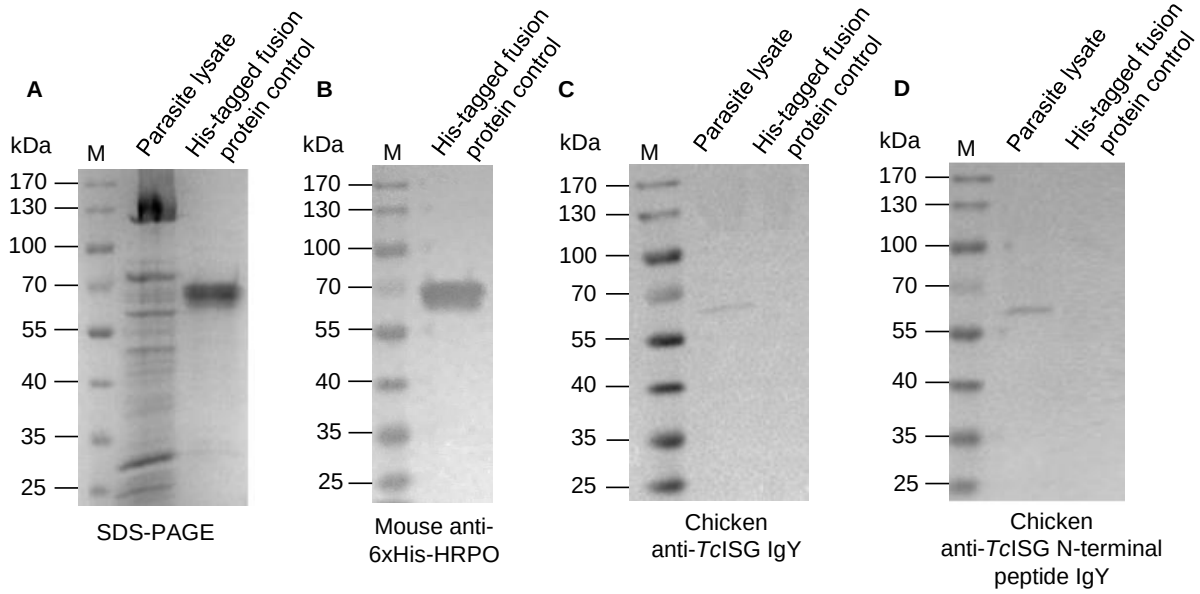


Figure 3.9: Detection of native ISG in *T. congolense* (IL 3000) bloodstream form lysate. **(A)** Parasite proteins and an unrelated His-tagged protein were separated on a reducing 10% SDS-PAGE gel, visualised with silver staining and analysed by western blot probed with **(B)** mouse anti-6xHis-HRPO conjugate antibody [1:8000], **(C)** affinity purified chicken anti-*Tc*ISG (weeks 3-9 chicken 2) [10 µg/ml] and **(D)** chicken anti-*Tc*ISG N-terminal peptide IgY (weeks 3-7 chicken 1) [10 µg/ml]. Blots were developed using rabbit anti-chicken IgY-HRPO conjugate antibody [1:5000] and 4-chloro-1-naphthol·H_2O_2 chromogen-substrate solution. M: PageRuler™ prestained protein marker.

3.3.7 Antibody detection by indirect ELISA

An indirect ELISA was subsequently employed to determine if recombinant *Tc*ISG could discriminate between *T. congolense* infected and non-infected cattle sera. An ELISA plate was coated with *Tc*ISG and tested against sera from non-infected cattle, *T. congolense* infected and cattle treated with trypanocidal drugs. The absorbance from the control wells that were not

coated with *Tc*ISG were subtracted from the experimental absorbance and plotted. The results showed increased absorbance of 0.29 from the infected serum (1:50) indicating detection of anti-ISG antibodies (Figure 3.10). A lower absorbance of 0.045 was obtained with the serum from drug treated cattle (1:50) while absorbance from non-infected cattle was less than 0.03. These results were further validated in a western blot. A single protein band of *Tc*ISG was observed in a silver stained SDS-PAGE gel (Figure 3.11, panel A) and a duplicate gel containing *Tc*ISG transferred onto a nitrocellulose membrane and probed with the sera. Detection of *Tc*ISG was evident using infected serum while no detection was observed with non-infected serum and serum from drug treat cattle (Figure 3.11, panel B).

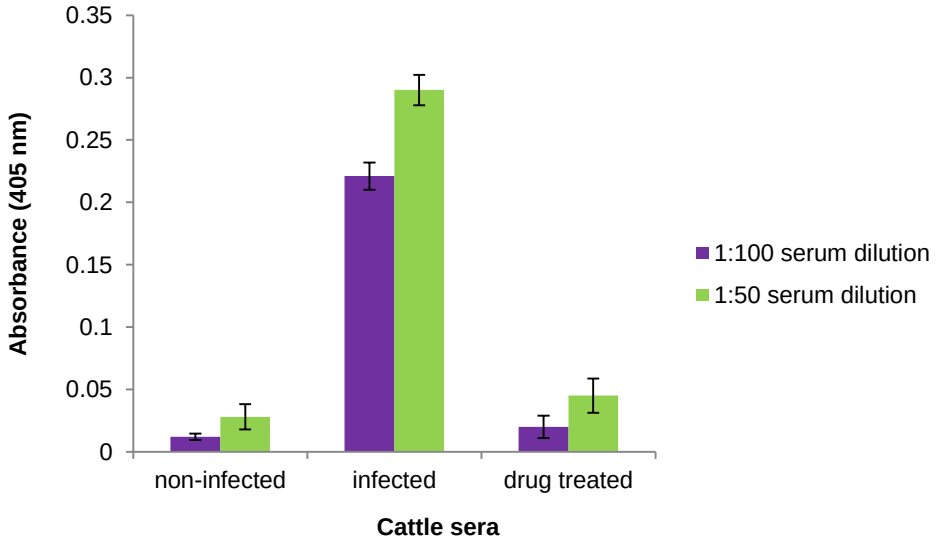


Figure 3.10: Detection of anti-ISG IgG antibodies from *T. congolense* infected sera in an indirect ELISA. A microtitre plate was coated with *Tc*ISG (5 µg/ml) and probed with sera from non-infected cattle, *T. congolense* infected and cattle treated with trypanocidal drugs [1:50 and 1:100]. Anti-bovine IgG-HRPO conjugate antibody [1:5000] and chromogen-substrate solution (0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer, pH 5.0) was used to develop the reaction for 45 min in the dark. The absorbance readings at 405 nm represent the average of quadruplicate experiments. A no coat antigen control and no serum control were included in the experiment.

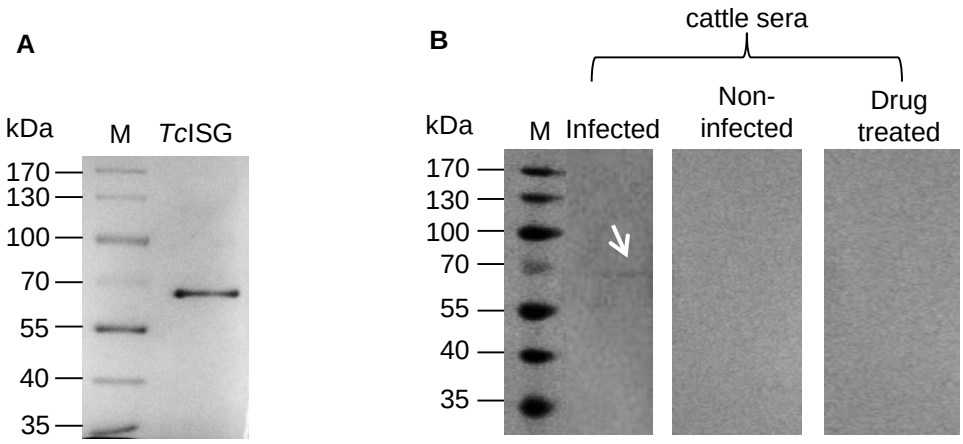
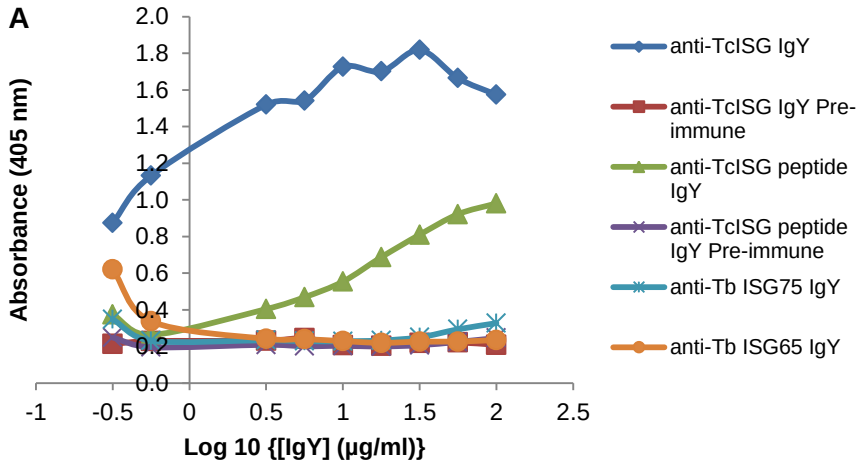


Figure 3.11: Detection of anti-ISG IgG antibodies from *T. congolense* infected sera in a western blot. **(A)** *Tc*ISG (5 µg/ml) was analysed on a 10% reducing SDS-PAGE gel and silver stained and a duplicate gel was transferred onto **(B)** nitrocellulose and probed with sera from non-infected cattle sera, *T. congolense* infected sera and sera from cattle treated with a trypanocidal drug [1:100]. Anti-bovine IgG-HRPO conjugate antibody [1:5000] and 4-chloro-1-naphthol·H_2O_2 chromogen-substrate solution was used to develop the blot. M: PageRuler™ prestained protein marker.

3.3.8 Cross reactivity of anti-trypanosomal ISG and VSG antibodies with *Tc*ISG

*Tc*ISG was detected with highest specificity using chicken anti-*Tc*ISG IgY followed by chicken anti-*Tc*ISG N-terminal peptide IgY (Figure 3.12, panel A). No cross reactivity was observed using chicken antibodies against: *T. brucei* ISG65 and ISG75 (Figure 3.12, panel A), *T. b. gambiense* VSG RoTat 1.2 and *T. b. gambiense* VSG LiTat 1.3 and LiTat 1.5 (Figure 3.12, panel B). These antibodies were further evaluated in a dot blot were only chicken anti-*Tc*ISG IgY and chicken anti-*Tc*ISG N-terminal peptide IgY detected the *Tc*ISG antigen (Figure 3.13).



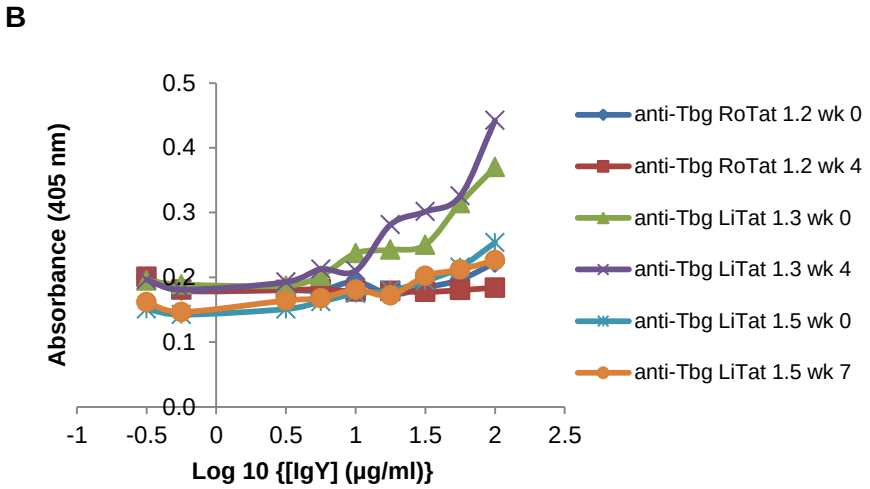


Figure 3.12: Cross reactivity of anti-trypanosomal ISG and VSG antibodies. ELISA plates were coated with *Tc*ISG (1 µg/ml) and probed with **(A)** ISG antibodies: anti-*Tc*ISG IgY weeks 3-8 from chicken 2, anti-*Tc*ISG IgY pre-immune, anti-*Tc*ISG N-terminal peptide IgY weeks 10-16 from chicken 2, anti-*Tc*ISG N-terminal peptide IgY pre-immune, anti-*Tb* ISG65 IgY, anti-*Tb* ISG75, **(B)** VSG antibodies: *Tbg* RoTat 1.2 week 0 and week 4, *Tbg* LiTat 1.3 week 0 and week 4, *Tbg* LiTat 1.5 week 0 and week 7. All antibodies were made in chickens and were serially diluted from 0.316-100 µg/ml in blocking buffer. Rabbit anti-IgY-HRPO conjugate [1:15000] and chromogen-substrate solution (0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in citrate-phosphate buffer, pH 5.0) was used to develop the reaction for 45 min in the dark. The absorbance readings at 405 nm represent the average of duplicate experiments. A no coat antigen control and no primary antibody and secondary antibody control were included in the experiment. Wk: week.

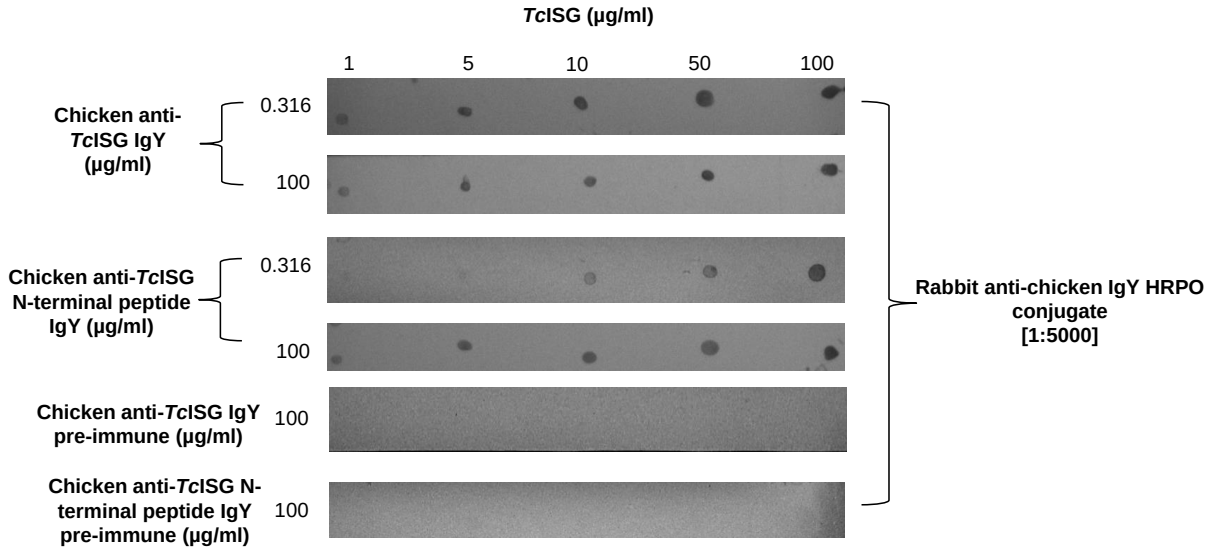


Figure 3.13: Dot blot analysis of cross reactivity of anti-ISG antibodies and anti-VSG antibodies with *Tc*ISG antigen. Nitrocellulose strips were spotted with various concentrations of *Tc*ISG probed with anti-*Tc*ISG IgY weeks 3-8 from chicken 2, anti-*Tc*ISG IgY pre-immune, anti-*Tc*ISG N-terminal peptide IgY 10-16 from chicken 2, anti-*Tc*ISG N-terminal peptide IgY pre-immune, anti-*Tb* ISG65 IgY, anti-*Tb* ISG75, VSG antibodies: *Tbg* RoTat 1.2 week 0 and week 4, *Tbg* LiTat 1.3 week 0 and week 4 and *Tbg* LiTat 1.5 week 0 and week 7 (0.316 µg/ml and 100 µg/ml). All antibodies were made in chickens and blots were developed rabbit anti-IgY-HRPO conjugate antibody [1:5000] and 4-chloro-1-naphthol·H_2O_2 chromogen-substrate solution.

3.4 Discussion

Recombinant *Tc*ISG was expressed and purified as described in Chapter 2, Section 2.2.8 and used to immunise chickens for antibody production. Freund's adjuvant was used to stimulate the immune system by increasing the immunogenicity of the antigen (Freund, 1956; Singh and O'Hagan, 2003). Chicken antibodies are considered favorable since it does not activate complement proteins present in mammalian sera thereby reducing potential interferences and preventing false positive results. Additionally, IgY does not cross react with mammalian IgM or IgG, human anti-mouse IgG antibodies, rheumatoid factors and anti-nuclear antibodies (Larsson *et al*, 1993; Schade *et al*, 2005).

High anti-*Tc*ISG IgY antibody titres were obtained after two injections with *Tc*ISG antigen at the start of week 3 and was maintained until week 16 with a further two booster injections at weeks 4 and 6. The concept that the greater the phylogenetic distance between the immune system and the antigen source, the larger the immune response elicited (Horton *et al*, 1985; Olovsson and Larsson, 1993) may explain the large quantity of antibodies produced by avian chicken against protozoan *Tc*ISG. Antibody yield is also superior in chickens since 20 eggs are laid monthly on average with each egg containing approximately 100 mg of antibodies (Akita and Nakai, 1992) and possesses greater affinity for its respective antigen compared to antibodies made in rabbits (Schade *et al*, 1996). Chicken anti-*Tc*ISG IgY antibodies were affinity purified using an affinity resin on which *Tc*ISG was immobilised to obtain *Tc*ISG specific antibodies. Initial preparation of the AminoLink®-*Tc*ISG resin with a coupling protocol using pH 7.2 led to very low coupling efficiency. A neutral pH was preferred to ensure stability of the *Tc*ISG protein and it is also the pH routinely used to couple IgY to the AminoLink® resin. For this reason, coupling protocol using pH 10 was explored as it offers improved protein immobilisation to the resin by establishing a Schiff base bond between the functional aldehyde groups on the agarose beads and primary amines present on *Tc*ISG in accordance with the AminoLink® Plus Coupling Resin manual (Thermo Scientific, USA). This approach was successful. Increased amounts of *Tc*ISG IgY were purified and displayed improved specificity for *Tc*ISG antigen compared to the crude IgY samples and unbound fractions in an ELISA.

Peptide antibodies offer specific recognition of amino acid sequences present on a protein which is beneficial for the detection of specific regions on the protein (Hancock and O'Reilly, 2005). In this study, *Tc*ISG N-terminal peptide located within the extracellular domain of *Tc*ISG was selected based on the Predict7 epitope prediction program (Carmenes *et al*, 1989) which evaluates the surface probability, hydrophilicity, flexibility and antigenicity of the given protein

sequence. An extracellular region located on *Tc*ISG pertaining to increased hydrophilicity and surface probability values with the possibility of residing on the surface of the protein was chosen for synthesis (Appendix G and H). These two features were vital for maximum exposure to the chicken immune system for antibody production. Peptides are not large enough to ellicit an immune response therefore the *Tc*ISG peptide was coupled to rabbit albumin using MBS before immunisation of chickens for antibody production (Lateef *et al*, 2007). Increased chicken anti-*Tc*ISG N-terminal peptide antibody titres were detected from both chickens over the course of 16 weeks post immunisation. No eggs where laid by chicken 2 during week 3. Studies have shown that Freund's complete adjuvant and Freund's incomplete adjuvant can reduce or completely prevent chickens from laying eggs for several weeks (Bollen and Hau, 1999).

Large amounts of chicken anti-*Tc*ISG N-terminal peptide IgY were affinity purified and demonstrated increased specificity in an ELISA in comparison to the crude IgY samples. Lower absorbance readings were detected when higher concentrations of chicken anti-*Tc*ISG N-terminal peptide IgY were utilised in an ELISA plate coated with *Tc*ISG. This is attributed to the prozone or "hook" effect which is a phenomena caused by high antibody concentrations in the presence of low antigen concentrations resulting in excessive antibody binding sites. This leads to univalent antibody binding to the antigen and thus low signal instead of the typical multivalent binding that cross links one antigen to the next leading to higher absorbance readings (Sateesh, 2003).

Affinity purified chicken anti-*Tc*ISG IgY and chicken anti-*Tc*ISG N-terminal peptide IgY antibodies were able to detect recombinant *Tc*ISG in a western blot. Next, both antibodies were tested against *T. congolense* bloodstream form lysate for detection of native ISG. Histidine affinity tags are small in size and seldom affect the downstream applications of recombinant protein use (Graslund *et al*, 2008). However, an unrelated histidine-tagged fusion protein was used as a control to demonstrate the specificity of anti-*Tc*ISG IgY. Chicken anti-*Tc*ISG IgY did not recognise the unrelated histidine-tagged fusion protein thus the antibody was specific for *Tc*ISG epitopes. Chicken anti-*Tc*ISG IgY and chicken anti-*Tc*ISG N-terminal peptide IgY detected a single band from the parasite lysate approximately 67 kDa in size which was larger than the predicted size of 46 kDa. Potential glycosylation of native *Tc*ISG i.e *Tc*ISG expressed by trypanosomes can be speculated since trypanosomes can post translationally glycosylate its proteins whereas prokaryotes such as the one used in this study cannot.

Furthermore, *Tc*ISG possess conserved domains from the ISG65 and ISG75 superfamily and Ziegelbauer and co-workers have confirmed N-linked glycosylation of ISG65 and ISG75 from *T. brucei* at Asn^{155} and Asn^{334} for ISG65 and Asn^{134} for ISG75 (Ziegelbauer *et al*, 1992). N-linked glycosylation is a process where glycans are attached to the Asn residue in consensus sequences: Asn-X-Ser / Asn-X-Thr where X can be any amino acid except proline (Imperiali and O'Conner, 1999). Sequence analysis of *Tc*ISG revealed 5 potential glycosylation sites possessing the consensus sequence with positions of: Asn^{55}, Asn^{123}, Asn^{133}, Asn^{141} and Asn^{327} within the extracellular domain of *Tc*ISG. Potential glycosylation sites are typically located within the extracytosolic domain and more than ten residues from the transmembrane domain (Landolt-Marticorena and Reithmeier, 1994) which favors the perception that native *Tc*ISG may undergo N-linked glycosylation. Proteins can possess more than one consensus sequence; however, not all sites are glycosylated as some sites may be unsuitable since asparagine must be located near the luminal end of the endoplasmic reticulum (Landolt-Marticorena and Reithmeier, 1994). Glycosylation of ISGs have also been reported in *T. b. gambiense* ISG75 (Rogé *et al*, 2013), *T. b. gambiense* ISG65 (Baiyegunhi, 2013), *T. brucei* ISG100 (Nolan *et al*, 1997), *T. brucei* ISG64 and ISG70 (Jackson *et al*, 1993) while five potential glycosylation sites were identified from *T. congolense* Tc38630 ISG by Mochabo and colleagues (2013).

Preliminary tests were conducted to determine the diagnostic potential of *Tc*ISG in an indirect antibody detection ELISA. Antibodies from infected *T. congolense* cattle serum as well as serum from drug treated cattle identified recombinant *Tc*ISG in comparison to the non-infected cattle serum. This result demonstrated the capability of recombinant *Tc*ISG to distinguish between infected and non-infected cattle sera. The results were confirmed in a western blot where *Tc*ISG was recognised using infected cattle serum while no detection was observed using non-infected serum and serum from drug treated cattle. Even though a signal was detected using serum from drug treated cattle in the ELISA, the concentrations of *Tc*ISG and serum used in a western blot were possibly too low for recognition. Low ELISA signal can be attributed to the use of low concentrations of *Tc*ISG. Additionally, ISGs are shielded by large VSG molecules therefore more antibodies are made against the VSGs compared to ISGs (Ziegelbauer and Overath, 1993; Schwede *et al*, 2015). Further testing of *Tc*ISG against a blinded panel of infected cattle sera will need to be conducted to determine the sensitivity and specificity of using *Tc*ISG as a diagnostic antigen.

The recognition of *Tc*ISG using antibodies produced against ISG and VSG from various *Trypanosoma* species was examined in an ELISA. *Tc*ISG was detected with increased

specificity using chicken anti-*Tc*ISG IgY and chicken and *Tc*ISG N-terminal peptide IgY antibodies. No further cross reactions was detected using chicken anti-*T. brucei* ISG65 and ISG75 as well as *T. b. gambiense* VSG RoTat 1.2, VSG LiTat 1.3 and VSG LiTat 1.5. Antibodies used in the cross reactivity ELISA such as *T. brucei* ISG65 and ISG75 were made specifically against the ISG extracellular domain therefore recognition of epitopes present on other domains of *Tc*ISG would not have occurred thus contributing to the lack of cross reactivity. Additionally, *Tc*ISG does not possess significant sequence similarity to *T. brucei* or *T. b. gambiense* ISGs as evident from the NCBI-BLAST search analysis which only returned hits from *T. congolense* unnamed proteins and with less than 26% identity from *T. brucei* and *T. b. gambiense* ISGs (Appendix J). Multiple sequence alignment of *T. brucei* and *T. b. gambiense* ISG sequences displaying highest query cover showed very little sequence identity and similarity (Appendix K) thus correlates with the lack of cross reactivity between *Tc*ISG antigen and *T. brucei* and *T. b. gambiense* ISG antibodies. Since *Tc*ISG was not recognised by antibodies from other *Trypanosoma* species, it suggests that *Tc*ISG could be used for species identification in diagnostic testing of *Trypanosoma* mixed infections. Although more experiments are required to validate the absence of cross reactivity between *Trypanosoma* species, the lack of ISG sequence identity and similarity among species is less than 10% and 20% respectively (Jackson *et al*, 2013).

In conclusion, antibodies were generated in chickens against *Tc*ISG and *Tc*ISG N-terminal peptide and analysed using a dot blot, ELISAs and western blots. Both antibodies were produced in large quantities and affinity purified preparations displayed increased specificity for their respective antigens. Native *T. congolense* ISG (TcIL3000.0.29290) was detected using both chicken anti-*Tc*ISG IgY and chicken anti-*Tc*ISG N-terminal peptide IgY. Additionally, anti-ISG IgG antibodies from *T. congolense* infected cattle serum detected *Tc*ISG in an antibody detection indirect ELISA and no detection was observed using non-infected cattle serum. These results were further confirmed in a western blot. No cross reactivity was detected between *Tc*ISG antigen and antibodies against *T. brucei* ISG65 and ISG75 and *T. b. gambiense* VSGs: RoTat 1.2, LiTat 1.3 and LiTat 1.5. However the experiment could be improved by coating the ELISA plate with *T. brucei* ISG65 and ISG75, *T. b. gambiense* RoTat 1.2 antigen and *T. b. gambiense* LiTat 1.3 and LiTat 1.5 antigens to show how each antigen is recognised by its specific antibody.

CHAPTER 4

GENERAL DISCUSSION

Animal African trypanosomiasis (AAT) also known as 'Nagana' is a neglected disease prevalent in poor remote countries in Sub-Saharan Africa. Nagana is mainly caused by *T. congolense* and *T. vivax* parasites which affects livestock while *T. brucei gambiense* and *T. brucei rhodesiense* are human pathogens causing human African trypanosomiasis (Schofield and Kabayo, 2008). Animal African trypanosomiasis accounts for major financial losses in animal and crop farming and is transmitted by hematophagous tsetse flies located within the tsetse fly belt with approximately 37 countries considered unsuitable for cattle farming (Kuzoe, 1993; Baral, 2010).

Trypanosoma congolense alternates its life cycle stages between the host bloodstream and the tsetse fly midgut. Each life cycle stage is characterised by the expression of a different surface coat known as a variable antigen type coding for a variable surface glycoprotein (VSG) in a process called antigenic variation (Gruszynski *et al*, 2006). Antigenic variation and the lack of B cell memory which typically leads to a long term IgM response makes vaccine development unlikely, therefore effective control of the disease is required (Magez and Radwanska, 2014). Trypanocidal drugs such as diminazene, isometamidium and homidium have been used since the 1950s however; drug resistance is rapidly increasing (Kuriakose and Uzonna, 2014). Insecticide spraying and sterile insect technique are typically employed to control tsetse fly populations but have met with variable degrees of success (Dyck *et al*, 2005; Torr *et al*, 2007; Vreysen *et al*, 2013).

In association with effective disease control, accurate diagnosis is essential. Direct and indirect detection of trypanosomes in infected blood samples are not species specific or sensitive diagnostic tests. Molecular techniques such as polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) and fluorescence in situ hybridisation testing using nucleic acid probes offer increased specificity since specific genes are targeted for diagnosis (Notomi *et al*, 2000; Desquesnes and Dávila, 2002; Radwanska *et al*, 2002). However, these tests are not suitable for field use, are expensive and trained personnel are required to perform the test and interpret the results (Deborggraeve and Büscher, 2010). Serological tests provide increased sensitivity and specificity in the detection of trypanosome-induced antibodies in infected sera. Antibody detection tests such as enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and card agglutination test (CATT) are commonly used to

diagnose HAT infections due to their affordability, simplicity and suitability for field use (Voller *et al*, 1975; Songa and Hamers, 1988; Nantulya and Lindwvist, 1989). Such tests use native trypanosome proteins thus requiring either *in vivo* or *in vitro* cultivation of parasites which results in a lack of standardisation testing, raises ethical concerns for propagation of parasites in laboratory animals and poses as a health risk to personnel (Greiner *et al*, 1997; Hopkins *et al*, 1998; Desquesnes *et al*, 2009).

In response to these challenges, trypanosome virulence associated proteins have been identified with the potential to replace native antigens. Variable surface glycoproteins are abundantly expressed on trypanosome bloodstream forms and are capable of stimulating an antibody response (Cornellissen *et al*, 1985; La Greca and Magez, 2011) thus possessing key features of an ideal diagnostic antigen (Hutchinson *et al*, 2004). Variable surface glycoprotein based tests such as ELISA/*T. evansi* (Büscher *et al*, 1995) and LATEX/*T. b. gambiense* (Verloo *et al*, 2001) are predominantly used for the detection of anti-VSG RoTat 1.2 antibodies in HAT patient sera. However, both tests may lead to misdiagnosis since the parasite is capable of switching its surface coat to express a different VSG molecule than the VSG used in the test (Tran *et al*, 2008) therefore a surface protein that does not undergo antigenic variation would serve as a better alternative (Hutchinson *et al*, 2004).

Invariant surface glycoproteins (ISGs) are transmembrane proteins expressed on trypanosome bloodstream forms with an estimated 70 000 copies of ISG65 and 50 000 copies of ISG75 per cell and these proteins do not exhibit antigenic variation (Ziegelbauer and Overath, 1992; Tran *et al*, 2008). Invariant surface glycoproteins are also immunogenic since they are capable of eliciting an antibody response (Ziegelbauer and Overath, 1993) and have been proposed as a possible DNA vaccine candidate by activating a humoral response, conferring partial protection to immunised mice and stimulating a Th1 antibody profile (Lança *et al*, 2011). Due to these features, ISGs have been proposed as an alternate immunodiagnostic antigen to VSGs. In the present study, *Trypanosoma congolense* ISG (*Tc*ISG) was cloned and recombinantly expressed with the aim of testing its diagnostic potential for animal African trypanosomiasis.

The DNA construct prepared for this purpose included all four domains of *Tc*ISG (Figure 2.1) and was recombinantly expressed as a histidine-tagged fusion protein within inclusion bodies in *E. coli* BL21 (DE3) cells using IPTG induction. Other ISGs that have been recombinantly expressed are displayed in Table 4.1.

Table 4.1: Summary of *Trypanosoma* ISG expression and purification details and their applications.

***Trypanosoma* species**	**ISG name**	**Expression system**	**Purification method**	**Protein size (kDa)**	**Application**	**Reference**
T. brucei gambiense	ISG75	pET-1b in *E. coli* Origami B (DE3) cells	Nickel affinity matrix	52 105	Diagnostics	Tran *et al*, 2008
T. b. brucei GVR 35/1.5	ISG	pET-28a in *E. coli* BL21 (DE3) cells	None	60	DNA construct coding for ISG was tested as a vaccine candidate	Lança *et al*, 2011
T. brucei gambiense	ISG75	*P. pastoris* M5 yeast strain	Anti-E tag affinity matrix	40-45 & 60-65	Diagnosis of *T. evansi* infections	Rogé *et al*, 2013
T. brucei (EATRO1125 Lister 427)	ISG65-1 ISG65-2 ISG64-1 ISG64-2 ISG64-3 ISG75-1	pET vectors in *E.coli* BL21(DE3) RIPL cells	Nickel affinity matrix	42 43 40 & 30 40 35 48	Development of lateral flow device using ISG65-1 ISG	Sullivan *et al*, 2013
T. congolense (IL3000)	Tc38630	pGEX-4T-1 in *E. coli* DH5α	Glutathione affinity matrix	70	Diagnostics	Mochabo *et al*, 2013
T. congolense (IL3000)	TcIL3000.29290 TcIL3000.51750 Tc38630	pET15b in *E. coli* BL21-CodonPlus (DE3) RIPL cells	Nickel affinity matrix Molecular exclusion chromatography	Not reported	Identification of immunodiagnostic antigens	Fleming *et al*, 2014
T. evansi	ISG75	pET-28a in *E. coli* BL21 Star (DE3) pLysS cells	Nickel affinity matrix	55	Diagnostics	Rudramurthy *et al*, 2015
T. brucei	ISG65	*Leishmania tarentolae* LEXSYS pLEX hyg2 vector in LEXSYS host strain p10	Nickel affinity matrix	50–60	Expression of proteins from kinetoplastid organisms	Rooney et al, 2015
*T. vivax (*ILRAD V34)	TvY486_0019690 TvY486_0045500	pET-15b in *E. coli* BL21-CodonPlus (DE3) RIPL cells	Nickel affinity matrix	Not reported	Development of lateral flow device using TvY486_0045500	Fleming *et al*, 2016

Inclusion body formation was attributed to the hydrophobic nature (Ziegelbauer *et al*, 1995) of *Tc*ISG (Ziegelbauer *et al*, 1995; de Groot *et al*, 2008). The expression of recombinant proteins enters several pathways that are regulated at a transcriptional and translational level and a high copy number of the gene of interest in association with a strong promoter and increased concentrations of the inducer such as IPTG can also lead to protein aggregation since the bacterial cells experience increased levels of metabolic burden (Allen *et al*, 1992; Bakau, 1993). Typically, inclusion bodies also form when expression of the target protein exceeds 2% of the total cellular proteins (Mitraki *et al*, 1991).

Purification of proteins expressed within inclusion bodies undergoes four steps: isolation and solubilisation of inclusion bodies followed by refolding of the solubilised protein and purification. Inclusion bodies were isolated using centrifugation and sonication while solubilisation of *Tc*ISG was accomplished by use of SDS and sarkosyl detergents according to Schalger and co-workers (2012). *Tc*ISG could have been solubilised using urea and guanidine hydrochloride which are commonly used chaotropic agents, however, both detergents are considered strong protein denaturants and possible protein aggregation may occur during the refolding process which is undesirable. Solubilisation with urea also requires several dialysis steps thus large amounts of buffers are needed and protein loss can occur which is not feasible for large scale protein solubilisation (Singh *et al*, 2012). Sarkosyl is a mild solubilisation agent and is preferred over urea and guanidine hydrochloride because it does not completely denature the inclusion bodies that possess native-like secondary structures significant for protein functionality. Additionally, protein aggregation is restricted during the refolding process and yields increased quantities of bioactive proteins (Singh *et al*, 2012).

Following solubilisation, *Tc*ISG was subjected to on-column refolding and purification using a nickel chelate resin under denaturing conditions. On-column refolding does not require large volumes of refolding buffer; high protein concentrations can be applied to the resin and removal of the denaturant is achieved simultaneous to protein purification (Jungbauer *et al*, 2004; Li *et al*, 2004). Protein aggregation usually occurs during refolding steps therefore certain additives are used to prevent this. Common additives include guanidine hydrochloride (Tsumoto *et al*, 2003), urea (Singh and Panda, 2005), arginine (Buchner and Rudolph, 1991; Xia *et al*, 2007), glycine (Ou *et al*, 2002), proline (Samuel *et al*, 2000), sucrose (Kim *et al*, 2006), sorbitol (Majumder *et al*, 2001), polyethylene glycol (Cleland *et al*, 1992) and glycerol (Sawano *et al*, 1992). Since *Tc*ISG was refolded using affinity chromatography in the presence of sarkosyl detergent, protein aggregation did not occur therefore additives were not required.

The *Tc*ISG fusion protein migrated at a size of approximately 66 kDa on a reducing SDS-PAGE gel and possible bacterial proteins were detected within the first three fractions eluted from the affinity resin. Additionally, a protein approximately 55 kDa in size was present in the solubilised *Tc*ISG fraction, unbound fraction and eluted fractions 1-2. Since this protein was detected using anti-histidine antibody, it is speculated to be a bacterial protein that interacted with the hydrophobic residues from *Tc*ISG during solubilisation, refolding and purification procedures. Furthermore, *E. coli* cellular proteins rich in histidine possess a high affinity for nickel ions and can therefore bind and co-elute with the target protein (Schmitt *et al*, 1993, Bornhorst and Falke, 2002). Expression and purification of *T. brucei gambiense* ISG75 resulted in degradation products. Edman degradation revealed the presence of cleavage sites (Rogé *et al*, 2013) therefore for future studies it will be worthwhile to determine potential cleavage sites in *Tc*ISG which could facilitate identification of the 55 kDa protein. Due to the inability to identify the 55 kDa protein, only fractions containing the 66 kDa fusion protein was used further in this study.

Interestingly, the size of recombinant *Tc*ISG is approximately 66 kDa and was larger than the anticipated predicted size of 46 kDa calculated from the amino acid residues constituting *Tc*ISG. A larger size of *T. brucei* ISG65 and ISG75 was also observed during SDS-PAGE analysis by Ziegelbauer and co-workers (1992) while native *T. brucei* ISG70 was reported to migrate slower than expected and was depicted as a diffuse band (Jackson *et al*, 1993). This irregularity is commonly referred to as ‘gel shifting’ (Shi *et al*, 2012). Invariant surface glycoproteins 65 and ISG75 possess numerous negatively charged amino acids which may contribute to the reduced binding of SDS to the ISGs resulting in slow migration compared to the standard molecular weight markers thus the ISGs appear larger than expected (Ziegelbauer *et al*, 1992). Gel shifting was also observed with other trypanosomal proteins such as *T. brucei* VSG AnTat 1.1A (Nolan *et al*, 2000). Additionally, Using the ProtParam program on the Expasy server (http://web.expasy.org/protparam/), amino acid sequence analysis of *Tc*ISG demonstrated a total of 53 negatively charged amino acids (aspartate and glutamate) which constitutes 13% of the total protein and thus correlates with the concept of reduced binding *Tc*ISG to SDS.

Analysis of the *Tc*ISG amino acid sequence, led to the identification of *Tc*ISG N-terminal peptide, predicted to be hydrophilic and surface exposed and was coupled to rabbit albumin using MBS for antibody production in chickens. Polyclonal anti-*Tc*ISG and anti-*Tc*ISG N-terminal peptide antibodies were produced in chickens since large quantities of antibodies can be obtained from chicken egg yolks using a variety of simple isolation and purification methods (Pauly *et al*, 2011). Chicken anti-*Tc*ISG IgY and *Tc*ISG N-terminal peptide IgY were purified on affinity resins

immobilised with recombinant *Tc*ISG antigen and *Tc*ISG N-terminal peptide respectively. Large quantities of antibodies were obtained and demonstrated increased specificity in an ELISA compared to the non-affinity purified antibodies. One of the main benefits of using chickens to generate antibodies instead of mammals is the isolation of antibodies from egg yolks which obviates bleeding of the animal and hence this approach to antibody production is recommended by the European Centre for the Validation of Alternative Methods (Schade *et al*, 1996). Additionally, IgY antibodies remain stable for more than ten years in a buffer containing a preservative such a sodium azide without losing its titre (Carlander *et al*, 1999), display increased stability over a variety of salt concentrations and pH and are often used in immunofluorescence and immunohistochemistry studies since they are able to recognise and bind to multiple epitopes on a chemically fixed sample (Lipman *et al*, 2005). Furthermore, polyclonal antibodies are produced against numerous epitopes which increases the possibility of detecting the recombinant and native protein however, cross reactivity may arise (Trier *et al*, 2012). In this case monoclonal antibodies are preferred but these antibodies are typically generated in mice therefore bleeding of the animal is required and the production process is complex. Alternatively, anti-peptide antibodies offer increased specificity since antibodies are raised against a specific region or epitope on a protein (Pauly *et al*, 2011; Trier *et al*, 2012).

Affinity purified anti-*Tc*ISG IgY and anti-*Tc*ISG N-terminal peptide IgY identified native ISG from *T. congolense* bloodstream form lysates in a western blot. The expected size of 46 kDa, as calculated from the *Tc*ISG amino acid sequence, was not detected but instead a larger size of approximately 67 kDa was observed. In this study, a prokaryotic expression system was used to express recombinant *Tc*ISG therefore post translational glycosylation could not occur. However, in trypanosomes, native ISGs can undergo glycosylation therefore the amino acid sequence of *Tc*ISG was examined for potential glycosylation sites. Five possible N-linked glycosylation sites containing the consensus sequences Asn-X-Ser/Asn-X-Thr (Imperiali and O'Conner, 1999) were identified in the *Tc*ISG amino acid sequence. This would explain the larger size of native *Tc*ISG found in trypanosomes.

Other ISGs that undergo glycosylation are *T. brucei* ISG64 and ISG70 (Jackson *et al*, 1993), *T. brucei* ISG100 (Nolan *et al*, 1997), *T. b. gambiense* ISG75 (Rogé *et al*, 2013) and *T. b. gambiense* ISG65 (Baiyegunhi, 2013). Furthermore, Mochabo and colleagues (2013) have determined five possible glycosylation sites in *T. congolense* Tc38630 ISG. Considering the potential of native *Tc*ISG to undergo glycosylation, a eukaryotic expression may be more useful in validating this post translational modification since glycosylation, disulfide bond formation,

proteolytic processing and protein folding can be achieved using a eukaryotic expression system (Macauley-Patrick *et al*, 2005). Eukaryotic expression systems include insect cells such as the baculovirus infected cell line (Contreras-Gomez *et al*, 2014), mammalian cells such the human embryo kidney and Chinese hamster ovary (Khan, 2013) as well as yeast such as *Pichia pastoris* and *Saccharomyces cerevisiae* (Strausberg and Strausberg, 2001; Bornert *et al*, 2012). Insect and mammalian expression systems have complex growth media and cultivation conditions are time consuming therefore yeast cells offer a suitable alternative as a simple and affordable growth medium with increased yield of soluble proteins (Macauley-Patrick *et al*, 2005). Soluble *T. b. gambiense* ISG75 has been expressed in *P. pastoris* with a yield of 10 mg/ L of culture media (Rogé *et al*, 2013). However, the limited license issues with using *P. pastoris* for recombinantly expressing proteins for use in a diagnostic test (Research Corporation Technologies, Tuscon, AZ, USA) necessitates the search for intellectual property free recombinant expression systems.

Seeing that native *Tc*ISG was detected by both chicken anti-*Tc*ISG IgY and chicken anti-*Tc*ISG N-terminal peptide IgY antibodies, immunoprecipitation and immuno-affinity chromatography can be used to isolate native *Tc*ISG from bloodstream form parasite lysates (Lipman *et al*, 2005) and tested for glycosylation using Endoglycosidase H enzyme. Immuno-localisation experiments ought to be conducted in the future for the detection of native *Tc*ISG. Moreover, studies have reported localisation of ISGs on the surface of trypanosomes (Ziegelbauer *et al*, 1992, Baiyegunhi, 2013).

The extracellular domain of *T. congolense* ISG (*Tc*ISG) with the gene name TcIL3000.0.29292, could discriminate between infected and non-infected cattle sera in an ELISA but was however not evaluated further (Fleming *et al*, 2014). In the present study *Tc*ISG expressed with all four domains was able to differentiate between non-infected and *T. congolense* infected cattle sera in an ELISA and western blot.

To further examine the diagnostic potential of *Tc*ISG, an inhibition ELISA can be performed using recombinant *Tc*ISG antigen or chicken anti-*Tc*ISG IgY (Figure 4.1 panel B and C). The ELISA plate will be coated with *Tc*ISG and incubated with infected cattle serum containing anti-*Tc*ISG IgG antibodies that will bind to *Tc*ISG (Figure 4.1, panel A) compared to non-infected cattle serum which will not contain anti-*Tc*ISG IgG antibodies and will therefore be washed away. Chicken anti-*Tc*ISG IgY will subsequently be added and washed away since antibodies from the infected serum (anti-*Tc*ISG IgG) would have already bound to *Tc*ISG. If non-infected cattle serum was used as the test sample, chicken anti-*Tc*ISG IgY will bind to *Tc*ISG since the binding

site will be available (Figure 4.1, panel C). Rabbit anti-chicken IgY-HRPO conjugate antibody is used as a detection antibody. Decreased ELISA signal is obtained when using infected cattle serum since chicken anti-*Tc*ISG IgY-HRPO conjugate antibody is washed away.

The inhibition ELISA test requires antibody production against the antigen in question and anti-trypanosomal antibodies from different species can be tested using a single detection antibody in comparison to the indirect ELISA (Figure 4.1, panel A) which requires a single species enzyme conjugate (Crowther, 1995). The inhibition ELISA test offers increased sensitivity possibly due to the detection of both IgM and IgG compared to an indirect ELISA that only detects IgG antibodies (Bossard *et al*, 2010).

It must be noted that anti-trypanosomal antibodies can persist in the bloodstream of an infected animal after treatment with trypanocidal drugs or self-cure, hence antibody detection tests cannot detect active infections (Boulangé *et al*, 2002). For future work, an antigen detection ELISA can be developed which permits the detection of trypanosomal antigens circulating in the blood of infected animals and hence identify active infections (Nantulya & Lindqvist, 1989). The antigen detection ELISA would entail coating the microtiter plate with chicken anti-*Tc*ISG IgY and probing with infected bovine serum or blood containing circulating trypanosomal antigens (Figure 4.2). Rabbit anti-bovine IgG-HRPO conjugate antibody and chromogen-substrate solution will be used as a detection system.

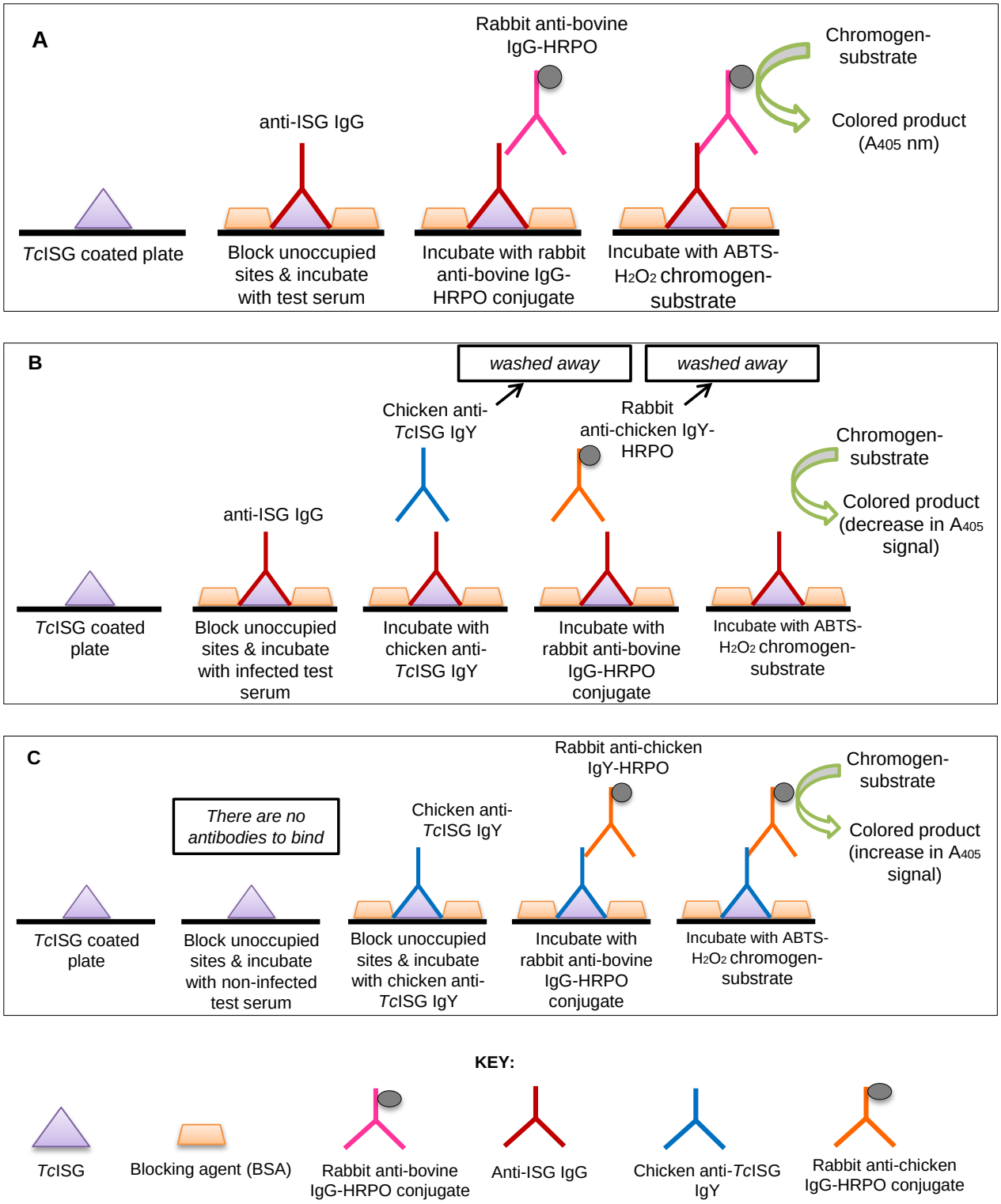


Figure 4.1: Diagrammatic representation of an indirect and inhibition ELISA. **(A)** Indirect ELISA and **(B)** inhibition ELISA using infected serum as the test sample. **(C)** Inhibition ELISA using non-infected serum.

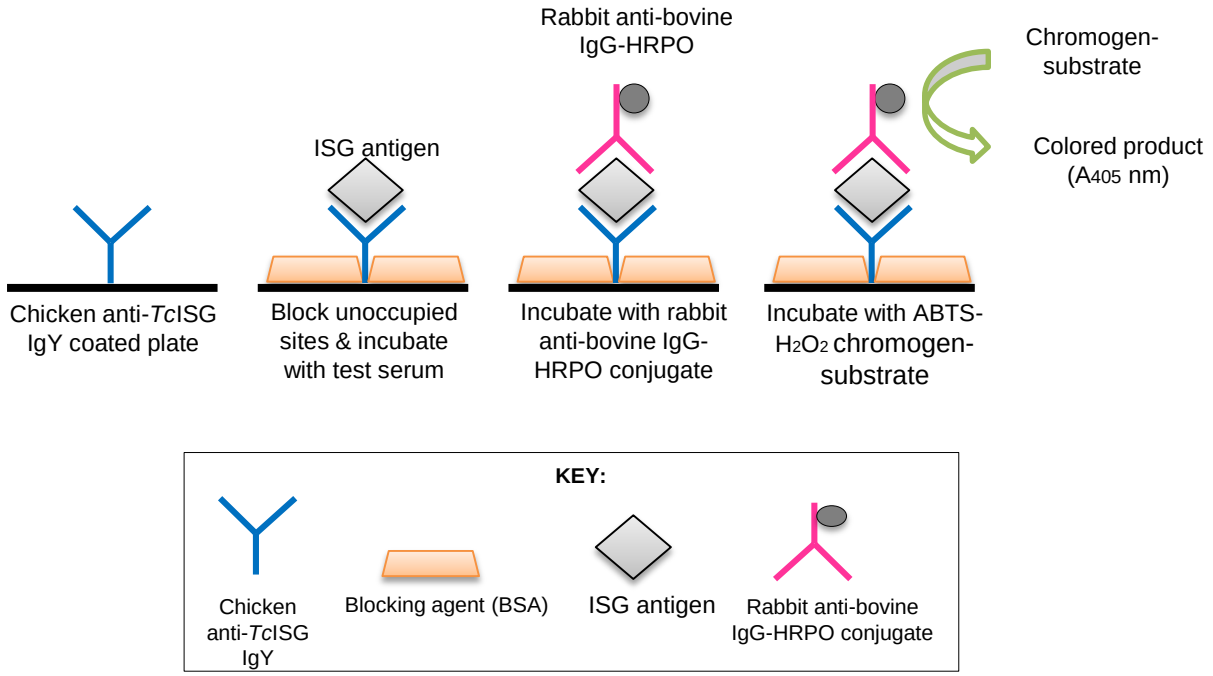


Figure 4.2: Schematic diagram of an antigen detection ELISA test. *T. congolense* ISG in infected serum or a blood sample is captured by chicken anti-*Tc*ISG IgY and detected by rabbit anti-bovine IgG-HRPO conjugate and ABTS-H_2O_2 chromogen-substrate solution.

In the present study, *Tc*ISG antigen was not detected by either anti-*T. brucei* ISG65 and ISG75 or antibodies against *T. b. gambiense* VSGs RoTat 1.2, LiTat 1.3 and LiTat 1.5. The NCBI-BLAST search of *Tc*ISG returned hits to *T. congolense* unnamed proteins, *T. brucei* and *T. b. gambiense* ISGs with less than 26% identity to the *Tc*ISG sequence. Sequence similarity and identity of ISGs among trypanosome species is less than 20% and 10% respectively (Jackson *et al*, 2013). Diagnosis of *T. congolense* and *T. vivax* infections using an indirect antibody detection ELISA plate coated with whole parasite lysate has been developed (Rebeski *et al,* 1999), however, the use of native proteins or parasite lysate is not recommended therefore recombinant proteins such *Tc*ISG can potentially be used as a species specific diagnostic antigen. Recombinant *Tc*ISG can also be tested against a blinded *T. congolense* and *T. vivax* infected sera panel in an indirect and inhibition ELISA format to further verify the diagnostic potential of *Tc*ISG.

Other potential *T. congolense* diagnostic antigens are 69 kDa protein, a member of the 70 kDa heat shock protein (hsp70) family (Boulangé *et al*, 2002), maltose binding protein-HSP70/BiP (Bossard *et al*, 2010), TcP46 invariant antigen (Zhou *et al*, 2014) and Tc38630 ISG (Gene ID: TcIL3000.0.38630) (Mochabo *et al*, 2013; Fleming *et al*, 2014). This study provides a stepping

stone to evaluate *T. congolense* ISGs for use in lateral flow systems similar to the p310 lateral flow test established for diagnosis of *T. vivax* infections (Fleming *et al*, 2016) with the ultimate goal of developing rapid antigen detection tests for AAT diagnosis.

The use of phage display technology has not yet been explored for *T. congolense* ISGs and can be used for the production of anti-*Tc*ISG single-chain variant fragments which will eliminate the need to immunise animals. Phage display antibodies such as the Nkuku® library are produced with high specificity and in large quantities using simple expression methods thus reducing cost and time constraints (Van Wyngaardt *et al,* 2004). The signal peptide of ISGs is understood to undergo cleavage upon maturation of ISG. Since the construct used in the present study included the signal peptide, extracellular domain, transmembrane region and intracellular transmembrane region, it will be worthwhile to generate anti-peptide antibodies against these domains to monitor its processing to determine it significance since characterisation of ISGs are poorly reported. Crystallisation studies can also be conducted for resolution of the *Tc*ISG structure to completely understand the function of ISGs as structural information on this protein is lacking.

In conclusion, encouraging results were obtained in the present study as *Tc*ISG discriminated between *T. congolense* non-infected and infected cattle sera in an antibody detection indirect ELISA and was not recognised by anti-*T. brucei* ISGs and anti-*T. b. gambiense* VSGs antibodies. Additionally, anti-*Tc*ISG IgY and *Tc*ISG N-terminal peptide IgY antibodies were produced in large quantities with high specificity and can be further evaluated in antibody and antigen detection ELISAs to completely validate the diagnostic potential of *Tc*ISG in AAT.

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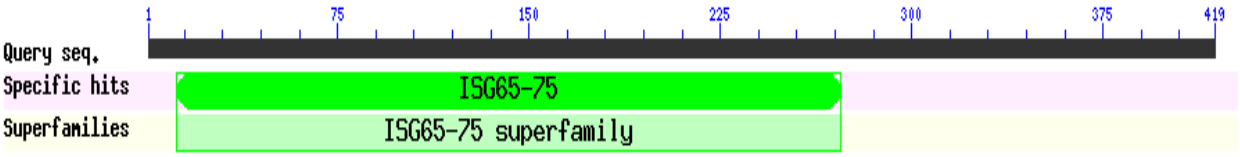
APPENDICES

Appendix A:

Prediction of *T. congolense* ISG conserved domains

Conserved domain predictions from *Trypanosoma congolense* (TcIL3000.0.29290, Accession CCD12004) ISG using the NCBI conserved domain database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb/).



Hits with scores that pass a domain-specific threshold (specific hits) are drawn in bright green colors. Non-specific hits or superfamilies are drawn in light green.

pfam11727: **ISG65-75**

Invariant surface glycoprotein

This family is found in Trypanosome species, and appears to be one of two invariant surface glycoproteins, ISG65 and ISG75 that are found in the mammalian stage of the parasitic protozoan. The sequence suggests the two families are polypeptides with N-terminal signal sequences, hydrophilic extracellular domains, single trans-membrane alpha-helices and short cytoplasmic domains. They are both expressed in the bloodstream form but not in the midgut stage. Both polypeptides are distributed over the entire surface of the parasite.

Appendix B:

Prediction of *T. congolense* ISG transmembrane domains

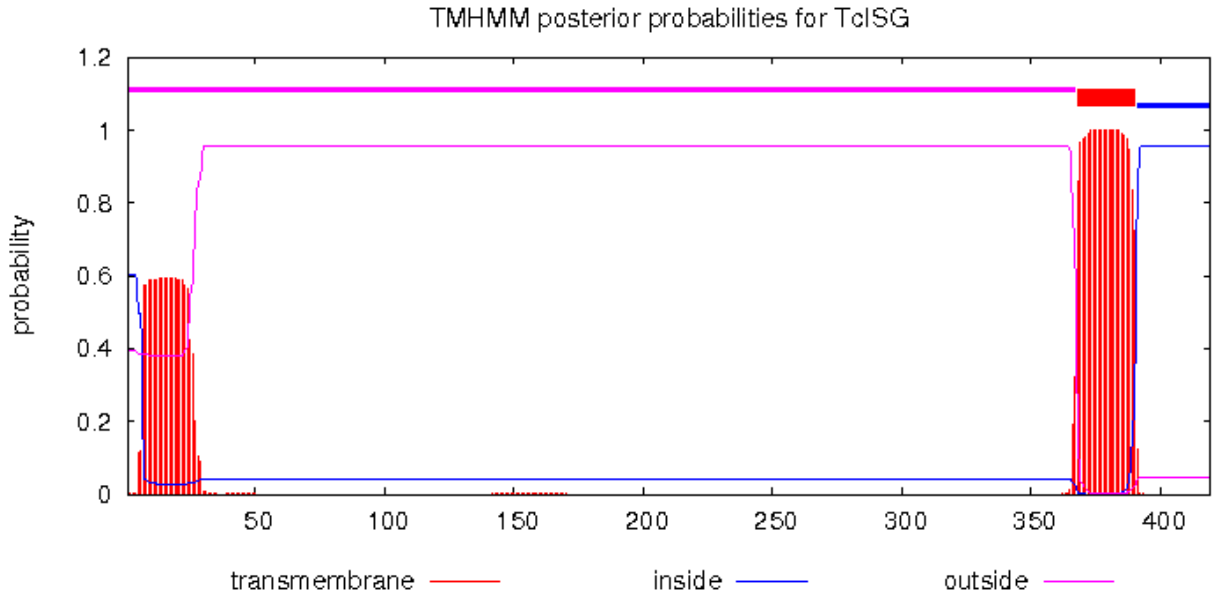
Domain predictions of *Trypanosoma congolense* (TcIL3000.0.29290, Accession CCD12004) using the web-based TMHMM server v. 2.0 (http://www.cbs.dtu.dk/ services/TMHMM-2.0/).

```
# TcISG Length: 419
# TcISG Number of predicted TMHs:  1
# TcISG Exp number of AAs in TMHs: 34.93968
# TcISG Exp number, first 60 AAs:  12.02008
# TcISG Total prob of N-in:        0.60616
# TcISG POSSIBLE N-term signal sequence
TcISG TMHMM2.0    outside          1   367
TcISG TMHMM2.0    TMhelix        368   390
TcISG TMHMM2.0    inside         391   419
```

KEY:

AA-amino acid

TMH-transmembrane helix



Appendix C:

The DNA sequence coding for *T. congolense* ISG

Trypanosoma congolense (IL3000) ISG DNA sequence (TcIL3000.0.29290) was retrieved from GenBank (http://www.ncbi.nlm.nih.gov) with Accession CCD12004. The forward and reverse primers are highlighted in blue and start and stop codons are underlined in bold.

```
   1 ATGGGGATGC AACGTATTGT TTGTGTTGGA TCGCTTTTGG TGGCTGTGTT
  51 GCTTTCCGGA GGGGCCACTG GGAATGCTGG CAATTCTAAT GGTGTCTGTA
 101 GACTGAATGA GAACGCTGCA GGACTCTTGT GTACCATTGC CAAACTTGTG
 151 GAAAAGGCCA AGAATATTAC TGAAAATCAT GACTACAAGG ATATTGACGA
 201 AACATGGGGT TACGTGGCAC TGCATAAGGA AGTGGTTGAT CACCGAGTAA
 251 AAAATTTACC AGATATAATT GAGACAGCTA AAGCAAAAGG AACTTTGACG
 301 GTTAAGGATG CCGAACATCT GACGACGCTT TATCTTGACG CACATAACAA
 351 GAATACTCAG CAACATAATA AGTCCAAAGC AGCCATGGAT GCACACAACA
 401 AAACACATGA GGATGCAAAG AATTCAACAG CGTTGGCGCT GGGTGAAGGC
 451 TACGTCACAG GTAACTGCAA CATGGTCTCA TCGCTCCTGG GTATCCTCCA
 501 GTGCTATGTT AAAGGTGAAC AACCGCACAG TAACCTCAAT GTGGAAACAC
 551 TTTGTAAAGA AAAGAACTAT AATTTGGATG AAAGCCAACA TACACTTCTC
 601 ACAAACTGCA ATAAAATAGG CAATCGCAAA ACGTACTGCA ACGGCACCGG
 651 TGCAGCACTC AAAGTAGCAC TGGACAAGTG GAATGGAATG GACAAGAAAA
 701 AGGCCGCTGA TAATGGAAAC TGTGAGGTCA AAAAGGATTG GGAAGAGCGC
 751 ACCAAGAAGG CCCAAGAACA CATGAGCCGT CTGGACGAGC ACGTACAGAT
 801 CATACACGAC GCCAAACTCT TGACTACCGC CTACTTCTCC ATCGTTGACA
 851 AAATAAAGAC TGGTGTTGAA AACGGCAAAC CCATGAAAGT AATTGTCGCC
 901 AATGCGCGTG AGGCCGGTCA AAAGGGCGCC AAAGTTGTGG TGGAAAAGCT
 951 TAGTGTAAAC ACAGAGAATG ACCCCCATAA CACAACCAAA CCGCTGGTGG
1001 AGGAGGAAGA AGTCAATGTC AACGTCCAAC TCGATGGGCT GAAGTTTGAT
1051 GAGGATGAAA ATGGCCCTGC CCATTCAAAG GAGAGCTTCC CAAAAATTTA
1101 CATTTATCTC TTATCTATTC TTCTCCCGCT CTTCTGCCTG GTCATGGGCA
1151 TTACCCTGTA TTGCCTCATC AGTAAACCCA GGTCCTCACA CCCAGAGAAG
1201 AGCATCCCGG TGGATGGTGC CACTATGAAC AAGGCTGGAG ATACACAGGC
1251 ACACTTTTAA CTTTATCCAA
```

Appendix D:

Nucleotide sequence alignment of pGEM®-T-*Tc*ISG and TcIL3000.0.29290.

EMBOSS Needle Pairwise nucleotide sequence alignment of *T. congolense ISG* Clone (obtained from sequencing of pGEM®-T-*Tc*ISG) [Clone] with TcIL3000.0.29290 gene (Accession CCD12004) [ISG].

```
Aligned_sequences: 2
# 1: ISG
# 2: Clone

# Length: 1260
# Identity:    1247/1260 (99.0%)
# Similarity:  1247/1260 (99.0%)
# Gaps:           0/1260 ( 0.0%)
# Score: 7214.0

ISG                1 ATGGGGATGCAACGTATTGTTTGTGTTGGATCGCTTTTGGTGGCTGTGTT     50
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone              1 ATGGGGATGCAACGTATTGTTTGTGTTGGATCGCTTTTGGTGGCTGTGTT     50

ISG               51 GCTTTCCGGAGGGGCCACTGGGAATGCTGGCAATTCTAATGGTGTCTGTA    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone             51 GCTTTCCGGAGGGGCCACTGGGAATGCTGGCAATTCTAATGGTGTCTGTA    100

ISG              101 GACTGAATGAGAACGCTGCAGGACTCTTGTGTACCATTGCCAAACTTGTG    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            101 GACTGAATGAGAACGCTGCAGGACTCTTGTGTACCATTGCCAAACTTGTG    150

ISG              151 GAAAAGGCCAAGAATATTACTGAAAATCATGACTACAAGGATATTGACGA    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            151 GAAAAGGCCAAGAATATTACTGAAAATCATGACTACAAGGATATTGACGA    200

ISG              201 AACATGGGGTTACGTGGCACTGCATAAGGAAGTGGTTGATCACCGAGTAA    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            201 AACATGGGGTTACGTGGCACTGCATAAGGAAGTGGTTGATCACCGAGTAA    250

ISG              251 AAAATTTACCAGATATAATTGAGACAGCTAAAGCAAAAGGAACTTTGACG    300
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            251 AAAATTTACCAGATATAATTGAGACAGCTAAAGCAAAAGGAACTTTGACG    300

ISG              301 GTTAAGGATGCCGAACATCTGACGACGCTTTATCTTGACGCACATAACAA    350
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            301 GTTAAGGATGCCGAACATCTGACGACGCTTTATCTTGACGCACATAACAA    350

ISG              351 GAATACTCAGCAACATAATAAGTCCAAAGCAGCCATGGATGCACACAACA    400
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            351 GAATACTCAGCAACATAATAAGTCCAAAGCAGCCATGGATGCACACAACA    400

ISG              401 AAACACATGAGGATGCAAAGAATTCAACAGCGTTGGCGCTGGGTGAAGGC    450
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            401 AAACACATGAGGATGCAAAGAATTCAACAGCGTTGGCGCTGGGTGAAGGC    450

ISG              451 TACGTCACAGGTAACTGCAACATGGTCTCATCGCTCCTGGGTATCCTCCA    500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            451 TACGTCACAGGTAACTGCAACATGGTCTCATCGCTCCTGGGTATCCTCCA    500

ISG              501 GTGCTATGTTAAAGGTGAACAACCGCACAGTAACCTCAATGTGGAAACAC    550
```

KEY:

| =identical
. =different

```
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone          501 GTGCTATGTTAAAGGTGAACAACCGCACAGTAACCTCAATGTGGAAACAC    550

ISG            551 TTTGTAAAGAAAAGAACTATAATTTGGATGAAAGCCAACATACACTTCTC    600
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone          551 TTTGTAAAGAAAAGAACTATAATTTGGATGAAAGCCAACATACACTTCTC    600

ISG            601 ACAAACTGCAATAAAATAGGCAATCGCAAAACGTACTGCAACGGCACCGG    650
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone          601 ACAAACTGCAATAAAATAGGCAATCGCAAAACGTACTGCAACGGCACCGG    650

ISG            651 TGCAGCACTCAAAGTAGCACTGGACAAGTGGAATGGAATGGACAAGAAAA    700
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone          651 TGCAGCACTCAAAGTAGCACTGGACAAGTGGAATGGAATGGACAAGAAAA    700

ISG            701 AGGCCGCTGATAATGGAAACTGTGAGGTCAAAAAGGATTGGGAAGAGCGC    750
                    ||||||||||.|||||||||||||.|||||||||||||||||||||||||
Clone          701 AGGCCGCTGAGAATGGAAACTGTGAAGTCAAAAAGGATTGGGAAGAGCGC    750

ISG            751 ACCAAGAAGGCCCAAGAACACATGAGCCGTCTGGACGAGCACGTACAGAT    800
                    ||||||||||||||||||||||||||||||||||||||||||||||||||.
Clone          751 ACCAAGAAGGCCCAAGAACACATGAGCCGTCTGGACGAGCACGTACAGAG    800

ISG            801 CATACACGACGCCAAACTCTTGACTACCGCCTACTTCTCCATCGTTGACA    850
                    |||||||.|||||||||||||||||||||||||||||||||||||||||||
Clone          801 CATACACAACGCCAAACTCTTGACTACCGCCTACTTCTCCATCGTTGACA    850

ISG            851 AAATAAAGACTGGTGTTGAAAACGGCAAACCCATGAAAGTAATTGTCGCC    900
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone          851 AAATAAAGACTGGTGTTGAAAACGGCAAACCCATGAAAGTAATTGTCGCC    900

ISG            901 AATGCGCGTGAGGCCGGTCAAAAGGGCGCCAAAGTTGTGGTGGAAAAGCT    950
                    ||||||||||||||||||||||.||.||||||||||||||||||||||||
Clone          901 AATGCGCGTGAGGCCGGTCAAAAAGGTGCCAAAGTTGTGGTGGAAAAGCT    950

ISG            951 TAGTGTAAACACAGAGAATGACCCCCATAACACAACCAAACCGCTGGTGG   1000
                    ||||.||.|||||||..|||||||||||||||||||||||||||||.||||
Clone          951 TAGTATAGACACAGNNAATGACCCCCATAACACAACCAAACCGCTTGTGG   1000

ISG           1001 AGGAGGAAGAAGTCAATGTCAACGTCCAACTCGATGGGCTGAAGTTTGAT   1050
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone         1001 AGGAGGAAGAAGTCAATGTCAACGTCCAACTCGATGGGCTGAAGTTTGAT   1050

ISG           1051 GAGGATGAAAATGGCCCTGCCCATTCAAAGGAGAGCTTCCCAAAAATTTA   1100
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone         1051 GAGGATGAAAATGGCCCTGCCCATTCAAAGGAGAGCTTCCCAAAAATTTA   1100

ISG           1101 CATTTATCTCTTATCTATTCTTCTCCCGCTCTTCTGCCTGGTCATGGGCA   1150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone         1101 CATTTATCTCTTATCTATTCTTCTCCCGCTCTTCTGCCTGGTCATGGGCA   1150

ISG           1151 TTACCCTGTATTGCCTCATCAGTAAACCCAGGTCCTCACACCCAGAGAAG   1200
                    ||||||||||||||||||||.||||||||||||||||||||||||||||||
Clone         1151 TTACCCTGTATTGCCTCATCNGTAAACCCAGGTCCTCACACCCAGAGAAG   1200

ISG           1201 AGCATCCCGGTGGATGGTGCCACTATGAACAAGGCTGGAGATACACAGGC   1250
                    ||||||||.|||||||||||||||||||||||||||||||||||||||||||
Clone         1201 AGCATCCCNGTGGATGGTGCCACTATGAACAAGGCTGGAGATACACAGGC   1250

ISG           1251 ACACTTTTAA   1260
                    ||||||||||
Clone         1251 ACACTTTTAA   1260
```

Appendix E:

Nucleotide sequence alignment of pET-32a-*Tc*ISG and TcIL3000.0.29290

EMBOSS Needle Pairwise nucleotide sequence alignment of *T. congolense ISG* Clone (obtained from sequencing of pET-32a-*Tc*ISG) [Clone] with TcIL3000.0.29290 gene (Accession CCD12004) [ISG].

KEY:

| =identical
. =different

- Aligned_sequences: 2
- 1: ISG
- 2: Clone

- Length: 1260
- Identity: 1247/1260 (99.0%)
- Similarity: 1247/1260 (99.0%)
- Gaps: 0/1260 (0.0%)
- Score: 7208.0

```
ISG               1 ATGGGGATGCAACGTATTGTTTGTGTTGGATCGCTTTTGGTGGCTGTGTT     50
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone             1 ATGGGGATGCAACGTATTGTTTGTGTTGGATCGCTTTTGGTGGCTGTGTT     50

ISG              51 GCTTTCCGGAGGGGCCACTGGGAATGCTGGCAATTCTAATGGTGTCTGTA    100
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            51 GCTTTCCGGAGGGGCCACTGGGAATGCTGGCAATTCTAATGGTGTCTGTA    100

ISG             101 GACTGAATGAGAACGCTGCAGGACTCTTGTGTACCATTGCCAAACTTGTG    150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           101 GACTGAATGAGAACGCTGCAGGACTCTTGTGTACCATTGCCAAACTTGTG    150

ISG             151 GAAAAGGCCAAGAATATTACTGAAAATCATGACTACAAGGATATTGACGA    200
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           151 GAAAAGGCCAAGAATATTACTGAAAATCATGACTACAAGGATATTGACGA    200

ISG             201 AACATGGGGTTACGTGGCACTGCATAAGGAAGTGGTTGATCACCGAGTAA    250
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           201 AACATGGGGTTACGTGGCACTGCATAAGGAAGTGGTTGATCACCGAGTAA    250

ISG             251 AAAATTTACCAGATATAATTGAGACAGCTAAAGCAAAAGGAACTTTGACG    300
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           251 AAAATTTACCAGATATAATTGAGACAGCTAAAGCAAAAGGAACTTTGACG    300

ISG             301 GTTAAGGATGCCGAACATCTGACGACGCTTTATCTTGACGCACATAACAA    350
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           301 GTTAAGGATGCCGAACATCTGACGACGCTTTATCTTGACGCACATAACAA    350

ISG             351 GAATACTCAGCAACATAATAAGTCCAAAGCAGCCATGGATGCACACAACA    400
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           351 GAATACTCAGCAACATAATAAGTCCAAAGCAGCCATGGATGCACACAACA    400

ISG             401 AAACACATGAGGATGCAAAGAATTCAACAGCGTTGGCGCTGGGTGAAGGC    450
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           401 AAACACATGAGGATGCAAAGAATTCAACAGCGTTGGCGCTGGGTGAAGGC    450

ISG             451 TACGTCACAGGTAACTGCAACATGGTCTCATCGCTCCTGGGTATCCTCCA    500
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           451 TACGTCACAGGTAACTGCAACATGGTCTCATCGCTCCTGGGTATCCTCCA    500
```

```
ISG              501 GTGCTATGTTAAAGGTGAACAACCGCACAGTAACCTCAATGTGGAAACAC    550
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            501 GTGCTATGTTAAAGGTGAACAACCGCACAGTAACCTCAATGTGGAAACAC    550

ISG              551 TTTGTAAAGAAAAGAACTATAATTTGGATGAAAGCCAACATACACTTCTC    600
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            551 TTTGTAAAGAAAAGAACTATAATTTGGATGAAAGCCAACATACACTTCTC    600

ISG              601 ACAAACTGCAATAAAATAGGCAATCGCAAAACGTACTGCAACGGCACCGG    650
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            601 ACAAACTGCAATAAAATAGGCAATCGCAAAACGTACTGCAACGGCACCGG    650

ISG              651 TGCAGCACTCAAAGTAGCACTGGACAAGTGGAATGGAATGGACAAGAAAA    700
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            651 TGCAGCACTCAAAGTAGCACTGGACAAGTGGAATGGAATGGACAAGAAAA    700

ISG              701 AGGCCGCTGATAATGGAAACTGTGAGGTCAAAAAGGATTGGGAAGAGCGC    750
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            701 AGGCCGCTGATAATGGAAACTGTGAGGTCAAAAAGGATTGGGAAGAGCGC    750

ISG              751 ACCAAGAAGGCCCAAGAACACATGAGCCGTCTGGACGAGCACGTACAGAT    800
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||.
Clone            751 ACCAAGAAGGCCCAAGAACACATGAGCCGTCTGGACGAGCACGTACAGAA    800

ISG              801 CATACACGACGCCAAACTCTTGACTACCGCCTACTTCTCCATCGTTGACA    850
                     |||||||.|||||||||||||||||||||||||||||||||||||||||||
Clone            801 CATACACCACGCCAAACTCTTGACTACCGCCTACTTCTCCATCGTTGACA    850

ISG              851 AAATAAAGACTGGTGTTGAAAACGGCAAACCCATGAAAGTAATTGTCGCC    900
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            851 AAATAAAGACTGGTGTTGAAAACGGCAAACCCATGAAAGTAATTGTCGCC    900

ISG              901 AATGCGCGTGAGGCCGGTCAAAAGGGCGCCAAAGTTGTGGTGGAAAAGCT    950
                     |||||||||||||||||||||||.|..|||||||||||||||||||||||||
Clone            901 AATGCGCGTGAGGCCGGTCAAAAAGAAGCCAAAGTTGTGGTGGAAAAGCT    950

ISG              951 TAGTGTAAACACAGAGAATGACCCCCATAACACAACCAAACCGCTGGTGG   1000
                     ||||.||||||||||.||||||||||||||||||||||||||||||||||||
Clone            951 TAGTATAAACACAGAAAATGACCCCCATAACACAACCAAACCGCTGGTGG   1000

ISG             1001 AGGAGGAAGAAGTCAATGTCAACGTCCAACTCGATGGGCTGAAGTTTGAT   1050
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           1001 AGGAGGAAGAAGTCAATGTCAACGTCCAACTCGATGGGCTGAAGTTTGAT   1050

ISG             1051 GAGGATGAAAATGGCCCTGCCCATTCAAAGGAGAGCTTCCCAAAAATTTA   1100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           1051 GAGGATGAAAATGGCCCTGCCCATTCAAAGGAGAGCTTCCCAAAAATTTA   1100

ISG             1101 CATTTATCTCTTATCTATTCTTCTCCCGCTCTTCTGCCTGGTCATGGGCA   1150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone           1101 CATTTATCTCTTATCTATTCTTCTCCCGCTCTTCTGCCTGGTCATGGGCA   1150

ISG             1151 TTACCCTGTATTGCCTCATCAGTAAACCCAGGTCCTCACACCCAGAGAAG   1200
                     ||||||||||||||||||||.||||||||||||||||||||||||||||||||
Clone           1151 TTACCCTGTATTGCCTCATCCGTAAACCCAGGTCCTCACACCCAGAGAAG   1200

ISG             1201 AGCATCCCGGTGGATGGTGCCACTATGAACAAGGCTGGAGATACACAGGC   1250
                     ||||||||.||||||||||||||||.||||||||..||||.|||||||
Clone           1201 AGCATCCCCGTGGATGGTGCCACTATTAACAAGGCTGCGGATAAACAGGC   1250

ISG             1251 ACACTTTTAA   1260
                     ||||||||||
Clone           1251 ACACTTTTAA   1260
```

Appendix F:

Protein sequence alignment of pET-32a-*Tc*ISG and TcIL3000.0.29290

EMBOSS Needle Pairwise protein sequence alignment of *T. congolense* ISG Clone (obtained from sequencing of pET-32a-*Tc*ISG) [Clone] with TcIL3000.0.29290 ISG gene (Accession CCD12004) [ISG].

KEY:

| =identical
. =different

- Aligned_sequences: 2
- 1: ISG
- 2: Clone

- Length: 419
- Identity: 411/419 (98.1%)
- Similarity: 411/419 (98.1%)
- Gaps: 0/419 (0.0%)
- Score: 2153.0

```
ISG                1 MGMQRIVCVGSLLVAVLLSGGATGNAGNSNGVCRLNENAAGLLCTIAKLV     50
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
Clone              1 MGMQRIVCVGSLLVAVLLSGGATGNAGNSNGVCRLNENAAGLLCTIAKLV     50

ISG               51 EKAKNITENHDYKDIDETWGYVALHKEVVDHRVKNLPDIIETAKAKGTLT    100
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone             51 EKAKNITENHDYKDIDETWGYVALHKEVVDHRVKNLPDIIETAKAKGTLT    100

ISG              101 VKDAEHLTTLYLDAHNKNTQQHNKSKAAMDAHNKTHEDAKNSTALALGEG    150
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            101 VKDAEHLTTLYLDAHNKNTQQHNKSKAAMDAHNKTHEDAKNSTALALGEG    150

ISG              151 YVTGNCNMVSSLLGILQCYVKGEQPHSNLNVETLCKEKNYNLDESQHTLL    200
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            151 YVTGNCNMVSSLLGILQCYVKGEQPHSNLNVETLCKEKNYNLDESQHTLL    200

ISG              201 TNCNKIGNRKTYCNGTGAALKVALDKWNGMDKKKAADNGNCEVKKDWEER    250
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
Clone            201 TNCNKIGNRKTYCNGTGAALKVALDKWNGMDKKKAADNGNCEVKKDWEER    250

ISG              251 TKKAQEHMSRLDEHVQIIHDAKLLTTAYFSIVDKIKTGVENGKPMKVIVA    300
                     ||||||||||||||.||.||||||||||||||||||||||||||||||||
Clone            251 TKKAQEHMSRLDEHVQXIHXAKLLTTAYFSIVDKIKTGVENGKPMKVIVA    300

ISG              301 NAREAGQKGAKVVVEKLSVNTENDPHNTTKPLVEEEEVNVNVQLDGLKFD    350
                     ||||||||.|||||||||.||||||||||||||||||||||||||||||||
Clone            301 NAREAGQKXAKVVVEKLSXNTENDPHNTTKPLVEEEEVNVNVQLDGLKFD    350

ISG              351 EDENGPAHSKESFPKIYIYLLSILLPLFCLVMGITLYCLISKPRSSHPEK    400
                     ||||||||||||||||||||||||||||||||||||||||.|||||||||
Clone            351 EDENGPAHSKESFPKIYIYLLSILLPLFCLVMGITLYCLIXKPRSSHPEK    400

ISG              401 SIPVDGATMNKAGDTQAHF    419
                     ||||||||.|||.|.||||
Clone            401 SIPVDGATXNKAXDXQAHF    419
```

Appendix G:

Selection of *Tc*ISG N-terminal peptide using the Predict7 program

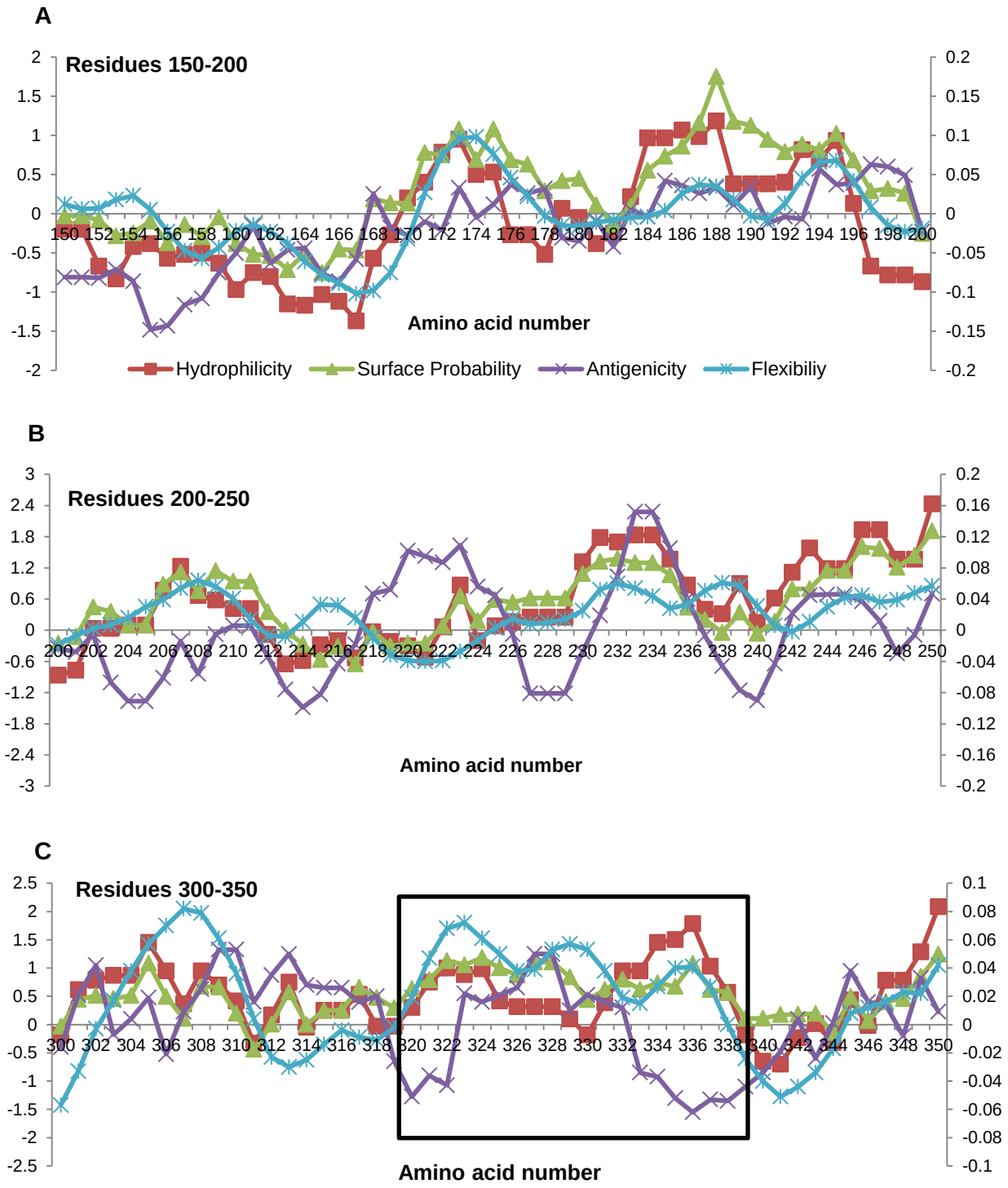


Figure A 1: Analysis of *Tc*ISG extracellular domain using the Predict7 program. **(A)** *Tc*ISG Amino acid residues 150-200 **(B)** 250-300 and **(C)** 300-250. The surface probability and hydrophilicity is plotted on the primary axis while antigenicity and flexibility is displayed on the secondary axis. *Tc*ISG N-terminal peptide selected for this study is indicated within the black square.

Appendix H:

Selection of *Tc*ISG N-terminal peptide using the PEP6 program

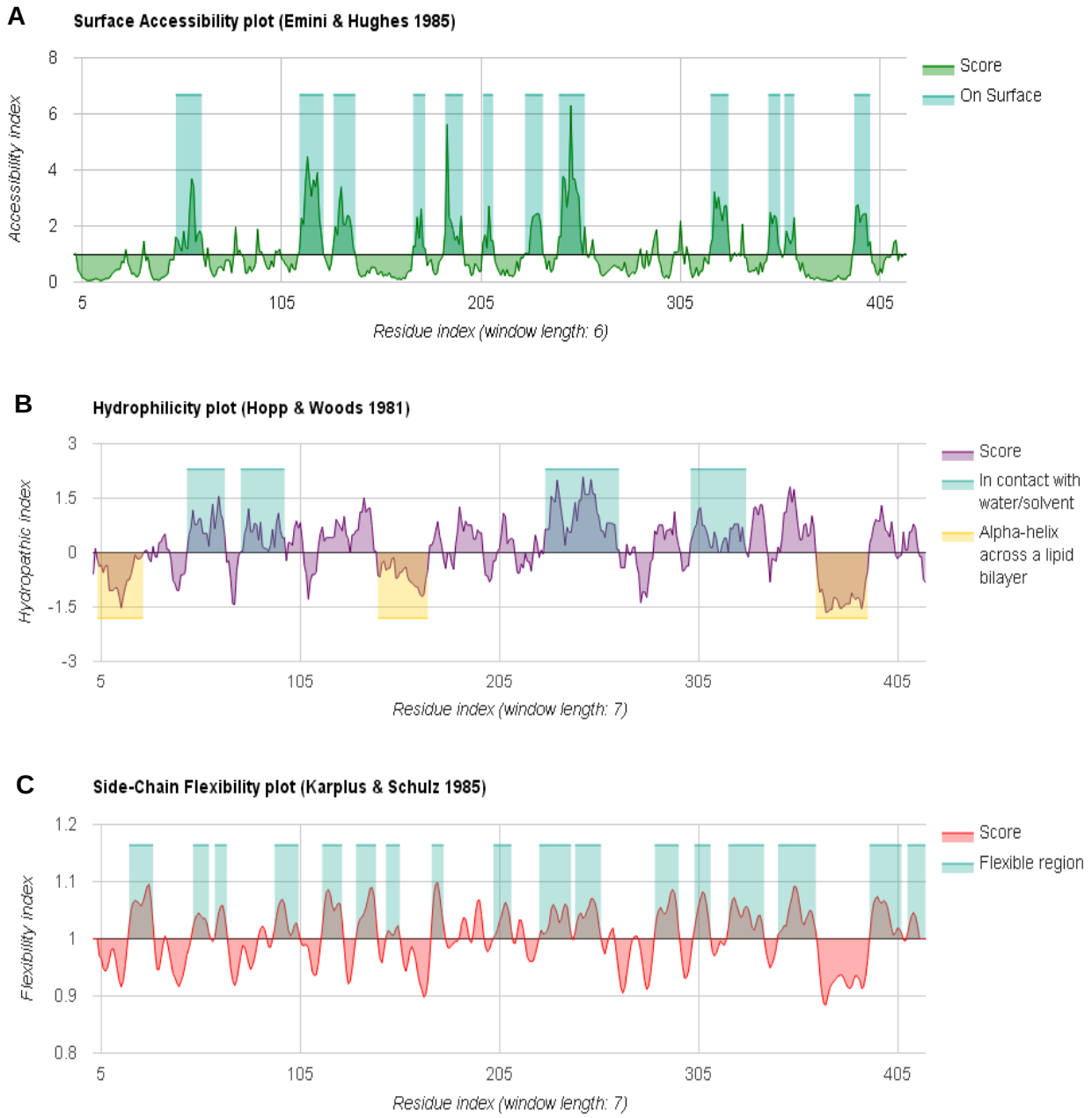


Figure A 2: Analysis of *Tc*ISG N-terminal peptide using PEP6 program. **(A)** The surface accessibility, **(B)** hydrophilicity and **(C)** side chain flexibility is plotted. The amino acid numbers are indicated on the horizontal axis.

Appendix I:

Optimisation of *Tc*ISG antigen and chicken anti-*Tc*ISG IgY in a dot blot.

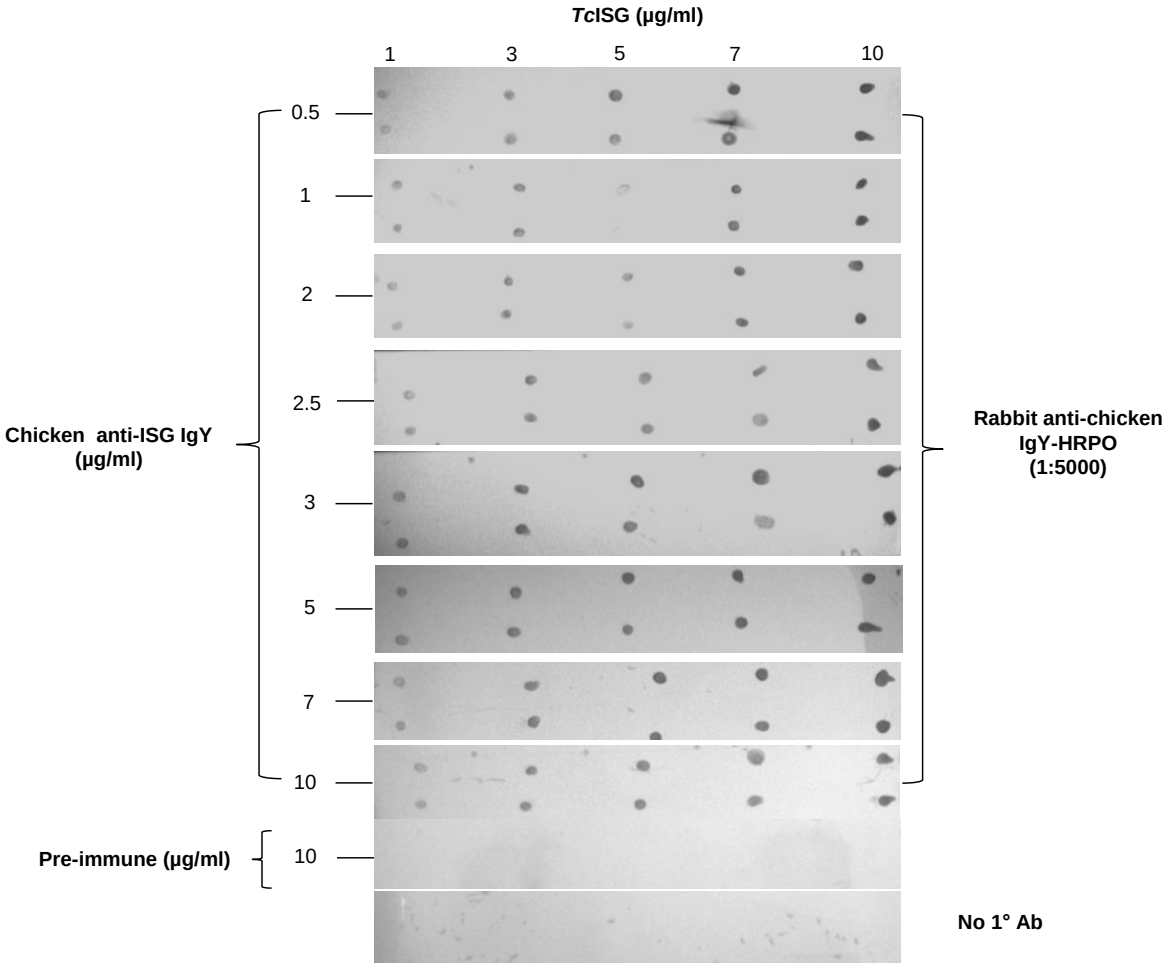


Figure A 3: Dot blot optimisation of *Tc*ISG and chicken anti-*Tc*ISG IgY antibodies. The nitrocellulose membrane was cut into strips and spotted with *Tc*ISG (1-10 µg/ml in duplicate) and probed with chicken anti-*Tc*ISG IgY (0.5-10 µg/ml). Blots were developed rabbit anti-chicken IgY-HRPO conjugate [1:5000] and 4-chloro-1-naphthol·H_2O_2 chromogen-substrate solution. Controls included: pre-immune antibody and the absence of primary antibody.

Appendix J:

NCBI-BLAST search result for *Tc*ISG sequence (TcIL3000.0.29290)

The NCBI-BLAST search was used to identify similar sequences to *Tc*ISG within the *Trypanosoma* species.

Table A 1: NCBI-BLAST search showing sequences similar to *T. congolense. ISG* sequence (Accession CCD12004).

DESCRIPTION	MAX SCORE	TOT SCORE	QUERY COVER	E VALUE	IDENTITY	ACCESSION
unnamed protein product [Trypanosoma congolense IL3000]	862	862	100%	0	100%	CCD12004.1
unnamed protein product [Trypanosoma congolense IL3000]	834	834	100%	0	97%	CCD12655.1
unnamed protein product [Trypanosoma congolense IL3000]	539	539	93%	0	71%	CCD17109.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma congolense IL3000]	530	530	99%	0	63%	CCD16307.1
unnamed protein product [Trypanosoma congolense IL3000]	468	468	99%	1.00E-160	59%	CCD12686.1
unnamed protein product [Trypanosoma congolense IL3000]	393	393	93%	3.00E-131	54%	CCD16692.1
unnamed protein product [Trypanosoma congolense IL3000]	392	392	97%	4.00E-131	50%	CCD13471.1
unnamed protein product [Trypanosoma congolense IL3000]	363	363	43%	4.00E-123	97%	CCD12882.1
unnamed protein product [Trypanosoma congolense IL3000]	354	354	69%	7.00E-118	61%	CCD13936.1
unnamed protein product [Trypanosoma congolense IL3000]	331	331	97%	5.00E-107	44%	CCD13472.1
unnamed protein product [Trypanosoma congolense IL3000]	327	327	97%	2.00E-105	44%	CCD17385.1
unnamed protein product [Trypanosoma congolense IL3000]	325	325	97%	1.00E-104	44%	CCD13473.1
unnamed protein product [Trypanosoma congolense IL3000]	295	295	89%	1.00E-93	45%	CCD16831.1
unnamed protein product [Trypanosoma congolense IL3000]	269	269	45%	4.00E-86	71%	CCD15064.1
unnamed protein product [Trypanosoma congolense IL3000]	228	228	96%	9.00E-66	35%	CCD15069.1
unnamed protein product [Trypanosoma congolense IL3000]	214	214	96%	5.00E-61	34%	CCD14465.1
unnamed protein product [Trypanosoma congolense IL3000]	203	203	47%	5.00E-60	50%	CCD16161.1

unnamed protein product [Trypanosoma congolense IL3000]	179	179	41%	2.00E-50	55%	CCD11875.1
unnamed protein product [Trypanosoma congolense IL3000]	160	160	38%	3.00E-44	57%	CCD11868.1
unnamed protein product [Trypanosoma congolense IL3000]	149	149	37%	5.00E-40	48%	CCD17495.1
hypothetical protein, unlikely [Trypanosoma congolense IL3000]	144	144	27%	2.00E-38	62%	CCD16166.1
unnamed protein product [Trypanosoma congolense IL3000]	141	141	35%	4.00E-37	58%	CCD11876.1
unnamed protein product [Trypanosoma congolense IL3000]	144	144	94%	3.00E-35	27%	CCD12049.1
unnamed protein product [Trypanosoma congolense IL3000]	133	133	33%	7.00E-34	48%	CCD16162.1
unnamed protein product [Trypanosoma congolense IL3000]	132	132	94%	1.00E-30	25%	CCD17383.1
unnamed protein product [Trypanosoma congolense IL3000]	126	126	76%	5.00E-28	27%	CCD13429.1
unnamed protein product [Trypanosoma congolense IL3000]	120	120	68%	2.00E-27	29%	CCD12050.1
unnamed protein product [Trypanosoma congolense IL3000]	116	116	62%	2.00E-26	29%	CCD15617.1
unnamed protein product [Trypanosoma congolense IL3000]	120	120	99%	5.00E-26	24%	CCD13470.1
unnamed protein product [Trypanosoma congolense IL3000]	93.2	93.2	32%	6.00E-19	40%	CCD17055.1
unnamed protein product [Trypanosoma congolense IL3000]	95.1	95.1	74%	2.00E-18	25%	CCD16246.1
unnamed protein product [Trypanosoma congolense IL3000]	87.8	87.8	73%	9.00E-16	25%	CCD12052.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	82.8	82.8	92%	1.00E-13	23%	XP_011771729.1
65 kDa invariant surface glycoprotein [Trypanosoma brucei brucei TREU927]	82.4	82.4	86%	2.00E-13	23%	XP_951583.1
65 kDa invariant surface glycoprotein [Trypanosoma brucei brucei TREU927]	82	82	86%	2.00E-13	23%	XP_951586.1
65 kDa invariant surface glycoprotein [Trypanosoma brucei brucei TREU927]	81.6	81.6	86%	3.00E-13	23%	XP_951585.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	80.9	80.9	94%	5.00E-13	23%	XP_011771734.1
65 kDa invariant surface glycoprotein [Trypanosoma brucei brucei TREU927]	80.5	80.5	86%	7.00E-13	23%	XP_951584.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	78.6	78.6	92%	3.00E-12	23%	XP_011771731.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	77.8	77.8	87%	6.00E-12	23%	XP_011771739.1
unnamed protein product [Trypanosoma congolense	78.2	78.2	79%	6.00E-12	23%	CCD16642.1

IL3000]						
65 kDa invariant surface glycoprotein [Trypanosoma brucei brucei TREU927]	77	77	93%	1.00E-11	22%	XP_951582.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	76.6	76.6	87%	1.00E-11	23%	XP_011771732.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	74.3	74.3	92%	6.00E-11	24%	XP_011771728.1
unnamed protein product [Trypanosoma congolense IL3000]	74.3	74.3	79%	1.00E-10	23%	CCD16641.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	73.6	73.6	87%	1.00E-10	22%	XP_011771745.1
variant surface glycoprotein [Trypanosoma brucei]	72.8	72.8	87%	2.00E-10	25%	AAF14218.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	72.8	72.8	87%	2.00E-10	24%	XP_011771743.1
unnamed protein product [Trypanosoma congolense IL3000]	72.4	72.4	79%	4.00E-10	23%	CCD15441.1
unnamed protein product [Trypanosoma congolense IL3000]	68.6	68.6	79%	5.00E-09	23%	CCD15440.1
65 kDa invariant surface glycoprotein [Trypanosoma brucei]	68.6	68.6	87%	5.00E-09	25%	AAA30147.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	67.8	67.8	92%	9.00E-09	20%	XP_011771748.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	67.4	67.4	87%	1.00E-08	23%	XP_011771746.1
65 kDa invariant surface glycoprotein, putative [Trypanosoma brucei gambiense DAL972]	67.4	67.4	87%	1.00E-08	25%	XP_011771741.1
65 kDa invariant surface glycoprotein [Trypanosoma brucei brucei TREU927]	65.9	65.9	93%	4.00E-08	20%	XP_951587.1
65 kDa invariant surface glycoprotein, fragment [Trypanosoma brucei gambiense DAL972]	53.5	53.5	43%	2.00E-04	25%	XP_011771737.1

Appendix K:

Multiple sequence alignment of *Tc*ISG, *T. b. gambiense* ISG65 and *T. brucei* ISG65

Multiple sequence alignment of *T. congolense* ISG (TcISG) (Accession CCD12004.1), *T. b. gambiense* (Tbg) ISG65, putative (Accession XP_011771734.1) and *T. brucei brucei* (Tbb) ISG65 using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

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TcISG      -MGMQRIVCVGSLLVAVLLSGGATGNAGNSNGVCRLNENAAGLLCTIAKLVEKAKNITEN
Tbg        MMKYLLVFAIIATRIPVLLVIGSED--NRVPGDKKLTKEEAAALCKMKHLADKVAKERSQ
Tbb        MMKYLLVFAIIATRIPALLVIGSED--NRVPGDKKLTKEGAAALCKMKHLADKVAEERSQ
            *    :..: :  : .**  *: .  ..  *  :*.:: *. **.: :*.:*. :  .:

TcISG      HDYKDIDETWGYVALHKEVVDHRVKNLPDIIETAKAKGTLTVKDAEHLTTLYLDAHNKNT
Tbg        ELKDRTQNFAGYIEFELYRIDYWLEKLNGPKGRKDGYAKLSDSDIEKVKEIFNKAKDGIT
Tbb        ELKDRTQNFAGYIEFELYRIDYWLEKLNGPKGRKDGYAKLSDSDIEKVKEIFNKAKDGIA
           .  .  ::  **: :.   :*: :::* .     .. ..*: .* *::. :: .*::  :

TcISG      QQHNKSKAAMDAHNKTHEDAKNSTALALGEGYVTGNCNMVSSLLGILQCY--VKGEQPHS
Tbg        KQLPEAKKAGEEAGKLHTEVKKAAENARGQDLD-DDTAKSTGLYRVLNWYCITKEERHNA
Tbb        KQLPEAKKAGEEAEKLHTEVKKAAANARGQDLD-EYRQKSTGLYRVLNWYCITKEERHNA
           :*  ::* * :   * * :.*:::  * *:.         :.*  :*: *  .* *: ::

TcISG      NLNVETLCKEKNYNLDESQHTLLTNCNKIGNRKTYCNGTGAALKVALDKWNGMDKKKAA-
Tbg        TPNCDGIQFRKHYL---SVNRSAIDCSSTGYKEDYD-WSANALQVALNSWENVKPKKLES
Tbb        TPNCDGIQFRKHYL---SVNRSAIDCSSTGYEENYD-WSANALQVALNSWENVKPKKLES
           . * : :  .*:*    * :    :*.. * .: *   :. **:***:.*:.:. **

TcISG      -------------DNGNCEVKKDWEERTKKAQEHMSRLDEHVQIIHDAKLLTTAYFSIVD
Tbg        AGSDKNCNIGQSSESHPCTMTEEWQTPYKETVEKLRELEDAYQRGKKAHDAMLGYANTAY
Tbb        AGSDMNCNIGQSSESHPCTMTEEWQTPYKETVEKLRELEDAYQRGKKAHDAMLGYANTAY
                        :.  * :.::*:   *:: *:: .*::  *  :.*:    .* . .

TcISG      KIKTGVENGKPMKVIVANAREAGQKGAKVVVEKLSVNTENDPHNTTKP----------LV
Tbg        AVNTKVEQEKPLTEVIAAAKEAGKKGAKIIIPAAAPATPTNSTKNEDSASTEHVDRGIAT
Tbb        AVNTKVEQEKPLTEVIAAAKDAGKKGAKIIIPAAAPATPTNSTKNEDSASTEHVDRGIAT
            ::* **: **:. ::* *::**:****:::   :  * .:  :. .           .

TcISG      EEEE--VNVNVQLDGLKFDEDENGPAHSKESFPKIYIYLLSILLPLFCLVMGITLYCLIS
Tbg        NETQVEVGIDADFDSLLD-A--TEAAEVTRRHQRTAMIILAVLVPAIILVVTAVAFFIMV
Tbb        NETQVEVGIDADFDSLLD-A--TEAAEVTRRHQRTAMIILAVLVPAIILVVTAVAFFIMV
           :* :  *.::.::*.*      .  *. .. . :  : :*::*:* : **:  . : ::

TcISG      KPRSSHPEKSIPVDGATMNKAGDTQAHF--420
Tbg        KRRRNNSQD---VDTGKA-EGGVSSVKVVM
Tbb        KRRRNSSKD---VDTGKA-EGGVSSVKVVM
           * * .  :.   ** ..  :.* :..:.
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