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Discovery and development of diagnostic biomarkers for human African trypanosomiasis

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Declaration

I declare that I am the author of this thesis; all references cited have been consulted by myself; the work of which this thesis is a record, unless specifically state, has been done by myself and this work has not been previously accepted for a higher degree.

Lauren Sullivan

I confirm that Lauren Sullivan has performed the research described in this thesis under my supervision and has fulfilled the conditions of the relevant Ordinance and Regulations of the University of Dundee.

Professor Michael A. J. Ferguson

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List of abbreviation

AMP	ampicillin
AP	alkaline phosphatase
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CATT	Card Agglutination Test for Trypanosomiasis
CDC	Centre for disease control
CFG	Consortium for functional Glycomics
CML	Chloramphenicol

CNBr	cyanogen bromide-activated
CRD	Cross-reacting determinant
CSF	Cerebral spinal fluid
CV	Coefficient of Variation
DALYs	Disability adjusted life years
DE52-DEAE	Diethylaminoethyl cellulose
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Electrogenerated chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ESAG	Expression site associated gene
ESI-MS/MS	Electrospray ionization mass spectrometry
FIND	Foundation for Innovative New Diagnostics
GPI	Glycosylphosphatdylinositol
GPI-PLC	Glycosylphosphatdylinositol-Phospohlipase C
GRESAG	Gene related to expression site associated gene
GST	Glutathione-S-transferase
GuHCl	Guanidine Hydrochloride
HAT	Human African Trypanosomiasis
HDL	High density lipoproteins
HRP	Horse radish peroxidase
HSP70	Heat shock protein 70
IgA	Immunoglobulin A

IgG	Immunoglobulin G
IgM	Immunoglobulin M
INF-γ	Interferon-gamma
IPTG	Isopropyl-β-D-thiogalactopyranoside
ISG	Invariant surface glycoprotein
LacNAc	N-acetyllactosamine
LAMP	Loop-Mediated Isothermal amplification
LHD	Liquid handling device
mAECT	mini anion exchange centrifugation technique
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3(N-morpholino)propanesulfonic acid
NiNTA	Nickel-charged nitrilotriacetic acid
nOG	N-octlglycoside
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
ROC	Receiver operator characteristic
SB	Separation buffer
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gle electrophoresis
SDS SB	Sodium dodecyl sulphate sample buffer
SRA	Serum resistance associated gene
sVSG	Soluble VSG
T. b. brucei	*Trypanosoma brucei brucei*

T. b. gambiense	*Trypanosoma brucei gambiense*
T. b. rhodesiense	*Trypanosoma brucei rhodesiense*
T. brucei	*Trypanosoma brucei*
TEV	Tobacco Etch Virus
TL	Trypanolysis test
T_m	Melting temperature
TnBP	Tri(n-butyl)phosphate
Tris	Tris(hydroymethyl)aminomethane
TY	Tryptone Yeast
VSG	Variant surface glycoprotein
WHO	World Health Organisation

Summary

Human African Trypanosomiasis (HAT) or African Sleeping Sickness is a disease prevalent in many parts of Sub-Saharan Africa. HAT is a parasitic infection caused by two species, *Trypanosoma brucei gambiense* and *T. b. rhodesiense.* Clinical diagnosis is not sufficient as symptoms from other endemic diseases, such as Malaria, are similar. Currently the diagnosis of *T. b. gambiense* infection mainly relies on the Card Agglutination Test for Trypanosomiasis (CATT), which has severe limitations. Other diagnostic tests for *T. b. gambiense* and *T. b. rhodesiense* infections require lab based equipment, trained personnel and have varying degrees of sensitivity and specificity. New approaches are needed, firstly to identify new diagnostic biomarkers, and secondly to find a more suitable platform for the test.

Our aim was to develop a lateral flow test based on trypanosome antigens. We used sera from *T. b. gambiense* infected and non-infected patients to identify infection specific diagnostic trypanosome proteins. The trypanosome proteins identified were then cloned into *E. coli* for recombinant expression and purification. The recombinant proteins were then screened by ELISA against 145 patients' sera from the WHO HAT specimen bank. Invariant Surface Glycoprotein (ISG) 65 and soluble Variant Surface Glycoprotein (VSG) 117 were selected for development into a lateral flow format and 80 randomised patients' sera were used to evaluate these prototypes. Here we describe the results showing that un-optimised proto-type lateral flow tests match the reported CATT sensitivity and specificity scores.

1. Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is an infection caused by the single celled eukaryotic parasite *Trypanosoma brucei* (Barrett *et al*., 2003). The two human infectious sub-species, *T. b. gambiense* and *T. b. rhodesiense* are the causative agents of two clinically distinct diseases, collectively defined as HAT (Welburn *et al*., 2001). The parasites are prevalent throughout sub-Saharan Africa and cause approximately 10,000 cases every year (Smith *et al*., 1998, Malvy and Chappuis, 2011) although various estimates predict this figure to be higher and it is more likely to affect 30,000 people annually (Bisser and Courtioux, 2012, Simarro *et al*., 2011, Brun *et al*., 2010).

The *T. b. gambiense* infection is mainly found in West and Central Africa (Figure 1.1) and is the chronic form of the disease (Balmer *et al*., 2011) whereas, the *T. b. rhodesiense* infection is found in East and Southern Africa and mainly causes the acute form. (Balmer *et al*., 2011). *T. b. gambiense* represents 90 to 95% of diagnosed cases, with *T. b. rhodesiense* representing the remaining cases (Simarro *et al*., 2010). Trypanosomes are transmitted mostly via the bite of an infected tsetse fly (*Glossina* genus).

Current diagnosis of *T. b. gambiense* in the field mainly relies on the Card Agglutination Test for Trypanosomiasis (CATT). This has severe limitations which we hope to address in our work by designing a new lateral flow test for the diagnosis of HAT.

1.1. The history and epidemiology of HAT

Trypanosoma brucei belongs to the Salivarian trypanosome group, which diverged from other trypanosomes approximately 300 million years ago and around this time they became gut parasites of early insects (Haag, 1998). It is postulated that the link between the tsetse fly and trypanosomes emerged around 35 million years ago (Steverding, 2008, Lambrecht, 1985). The link between HAT, the tsetse fly and *T. brucei* was relatively recently established. The physicians, John Aktins (1734) and Thomas Winterbottom (1803), identified the symptoms associated with HAT during the slave trade (Cox, 2004). The links between the causative agent, the cattle disease nagana, sleeping sickness and the transmission vector were collectively established by researchers from a range of fields including; David Livingston, David Bruce, Robert Michael Forde, Joseph Everett Dutton and Friedrich Karl Kleine, among others (Steverding, 2008).

The *T. b. gambiense* form of the disease was identified and described by Joseph Everett Dutton, in the country known at the time as Gambia, which is now several different countries (Steverding, 2008). It was recognised to present with chronic symptoms and was mainly localised to West and Central Sub-Saharan Africa. Conversely the *T. b. rhodesiense* form was recognised in Northern Rhodesia, now known as Zambia in 1910 by Stevens and Fantham (Cox, 2004) . The Rhodesian form of the disease was recognised to be acute and aggressive and mainly located in distinct foci in areas of East and Southern Africa (Gibson, 2005). *T. b. gambiense* is more likely to be involved in epidemics due to its long human host cycles and vector feeding habits, which are the major determinants of transmission of the parasite (Pépin and Méda, 2001). Conversely,

the aggressive and acute nature of the rhodesiense trypanosome infections make the potential for epidemics less likely (Pépin and Méda, 2001).

There have been three recorded epidemics of HAT in the 20th Century, with estimates of 300,000 or more infected individuals (Simarro *et al*., 2011). By the 1960s, transmission and the number of cases were very low (Figure 1.2) and today the number of recorded cases has dropped below 10,000 per year, however the prevalence of HAT is predicted to be higher due to under-reporting (Mumba *et al.*, 2011). HAT still continues to be a large burden on individuals and communities in terms of disability-adjusted life years (DALYs) (Simarro *et al*., 2010, Fèvre *et al*., 2008b). DALYs allow for the assessment of disease not only based on death but on morbidity and the impact of the disease on the individual and their community (Lutumba *et al*., 2007, Fèvre *et al*., 2008a).

1.1.1. Control methods

Since the disease's identification the colonial authorities at the time set up extensive control operations, including systematic screening and treatment of *T. b. gambiense* infected individuals; while in areas endemic with *T. b. rhodesiense*, the main control methods included identifying animal reservoirs and attempting to control the vector, *e.g.* with tsetse traps (Simarro *et al*., 2011). With the prospect of elimination in sight during the 1960s and 1970s, the high cost, seemingly few cases and political instability led to the decline in surveillance by public health officials in the affected countries (Steverding, 2008, Simarro *et al.*, 2011). Subsequently HAT numbers have risen and though it appears HAT is currently under control, there is a fear that events, such as

civil unrest or upheaval may serve as a catalyst for future epidemics (Welburn *et al*., 2009, Tong *et al*., 2011b). A large problem of HAT control relates to the geographical distribution of HAT, which is directly linked to the Tsetse fly belt. This currently ranges across many countries with a combined area of 8 million km^2 (Steverding, 2008). The 'HAT atlas' records and makes available locations of HAT foci which can inform screening and control strategies (Simarro *et al*., 2010, Simarro *et al*., 2011).

1.1.2. HAT cases in non-endemic areas

There have been a number of HAT cases diagnosed in non-endemic locations, mainly due to patients spending time in a HAT endemic location (Migchelsen *et al*., 2011, Wolf *et al.*, 2012). In some cases patients were misdiagnosed and mistreated, usually for malaria and *Toxoplasma gondii* infections, because HAT patients have antibodies which cross-react with the diagnostic tests for the other infectious diseases or show inconclusive results (Sahlas *et al*., 2002). Approximately 60 % of cases diagnosed in non-endemic regions are caused by *T. b. rhodesiense*, while 40 % were infected with *T. b. gambiense* (Lejon *et al*., 2003). Travel to HAT endemic areas is likely to increase due to more accessible tourism, so there is a need for physicians and travellers to be made more aware of these diseases (Gautret *et al*., 2009).

Typically HAT occurs in more rural locations however there is a growing concern that tsetse flies are becoming more prevalent in the urban setting, and it has been reported recently that some were carrying *T. b. gambiense* (Simon *et al*., 2012). Another unknown factor is the potential effect of climate change on the tsetse fly belt, which has been predicted to increase the number of people at risk of HAT (Moore *et al*., 2012).

1.2. Trypanosoma brucei

T. brucei is a unicellular protozoan parasite that belongs to the genus Trypanosoma, in the Trypanosomatidae family and it is a member of the order Kinetoplastida. It has a single flagellum that emerges from the posterior of the cell and which extends to the anterior attached to the membrane (Figure 1.3). The flagellum enables the organism to move which is essential for its survival (Engstler *et al*., 2007). The flagullum is associated with the kinetoplast, a large organelle containing the DNA of the cell's single mitochrondion. The flagellar pocket is the only site for endo- and exocytosis and is the site where receptors are located, such as the transferrin receptor (Steverding, 2006b).

T. brucei has a complex lifecycle which is exclusively extracellular and alternates between the insect vector and the mammalian host (Figure 1.4). Metacyclic trypanosomes are injected from the salivary gland of an infected tsetse fly into the mammalian host when it takes a blood meal. Trypanosomes multiply at the site of injection and then enter the blood and lymphatic system. The trypanosomes proliferate into the long, slender dividing form or the short, stumpy non-dividing form (Seed and Wenck, 2003, MacGregor *et al*., 2011). The latter is able to continue onto the next phase of the lifecycle, if taken up by a tsetse fly in a subsequent blood meal. In the tsetse fly the stumpy trypanosomes transform into procyclic trypamastigotes, which further develop into epimastigotes, then into metacyclic forms which migrate to the salivary gland of the tsetse fly, ready to be injected into another mammalian host (MacGregor *et al*., 2012).

1.2.1. Sub species

T. b. brucei is morphologically indistinguishable from *T. b. gambiense* and *T. b. rhodesiense*. *T. b. brucei* is susceptible to lysis in normal human serum due to innate trypanosome lytic factors, e.g. High Density Lipoproteins (HDLs), while *T. b. gambiense* and *T. b. rhodesiense* are resistant to HDLs and are able to persist in the blood and cause disease (De Greef *et al*., 1989, Vanhamme and Pays, 2004, Pays *et al*., 2006).

A proposed mechanism for how *T. b. gambiense* survives in normal human serum is due to expression reduction of haptoglobin receptor gene (Hp/Hb) leading to a reduced uptake of Trypanosome Lytic Factor-1 (TLF-1). The TLF-1 is found on has been shown to be taken up by the haptoglobin receptor and by reducing expression of this gene *T. b. gambiense* confers resistance to normal human sera (Kieft *et al*., 2010).

T. b. rhodesiense has been shown to express the serum resistance-associated (SRA) gene which is the cause of the parasites resistance to normal human sera (De Greef and Hamers, 1994, Gibson, 2005). The SRA protein is similar to a truncated variant surface glycoprotein (VSG) and has been postulated to interact with apolipoprotein L1 that is associated with HDLs, preventing parasite cell lysis (De Greef and Hamers, 1994, Gibson, 2005, Pays *et al*., 2006).

1.2.2. Clinical presentation

Symptoms alone are not sufficient to confirm diagnosis of HAT, therefore additional diagnostic testing is required, as some symptoms of HAT are similar to other diseases found in the same region, *e.g.* malaria or typhoid (Chappuis *et al*., 2005). In some cases, patients present with a chancre, a swelling and sometimes ring-like mark that is caused by localised inflammation at the site of inoculation (Sternberg, 2004). This is more common in *T. b. rhodesiense* infections and in non-Africans (Jelinek *et al*., 2002). Cervical lymph nodes can appear swollen in up to 50 % of cases when the host is infected and this is known as Winterbottoms sign (Chappuis *et al*., 2005). Patients also present with symptoms, including; general malaise, anaemia, headache, pyrexia, weight loss and weakness. In addition there may be neurological symptoms, including psychiatric, motor, sensory and sleep abnormalities (Sternberg, 2004). The swelling of the lymph nodes and other non-specific symptoms are not exclusive to HAT but may be due to other causes (Chappuis *et al*., 2005). However, the reversal of sleep-wake cycle is typical to HAT, with day time somnolence and nocturnal insomnia (Lundkvist *et al*., 2004, Rodgers, 2010).

1.2.3. Disease progression

Trypanosomes are transmitted mostly via the bite from an infected tsetse fly but infections caused by blood exchange and congenital infections have been reported (Rocha *et al*., 2004, Welburn *et al*., 2009).

The trypanosomes progress from the site of infection through the blood and lymphatic system of the host known as the haemolymphatic stage or the first stage of the disease. Eventually the disease can progress, over a period of time ranging from a number of weeks to years, to the neurological stage, otherwise known as the second stage (Checchi *et al*., 2008). In *T. b. rhodesiense* infections the disease progresses from first to the second stage within three weeks to two months, and death occurs within six months in >80 % of cases, while *T. b. gambiense* infections can take up to many years to progress to the second stage (Odiit *et al*., 1997, Chappuis *et al*., 2005). However there is growing evidence of individuals that remain asymptomatic for years and others that can self-cure (Kaboré *et al*., 2011, Jamonneau *et al*., 2012, Sternberg and MacLean, 2010).

The colonisation of the brain requires the trypanosomes to enter the hostile environment of the Cerebal Spinal Fluid (CSF) and the stroma of the choroid plexus (Wolburg *et al*., 2012). In mice, trypanosomes localise near the gila limitans, where they can re-populate blood vessels and disrupt the sleep wake cycle (Wolburg *et al*., 2012). Typically, if no treatment is administered infected individuals die from severe wasting, dysfunctional immune system, deep coma and seizures. Often death is hastened by multiple other infections (Chappuis *et al*., 2005, Rodgers, 2010).

1.2.4. *T. brucei* and the immune response

Trypanosomes have the ability to evade the host's adaptive immune system enabling them to live in the blood of patients. In the mammalian host the blood stream form trypanosomes are covered by a dense monolayer of identical glycoproteins called VSG

(Vickerman, 1978). The VSG coat protects the trypanosome plasma membrane from elements of the host's innate immune system, e.g. complement (Ortiz-Ordonez *et al*., 1994), and the hosts adaptive immune system, e.g. antibodies (Figure 1.5) (Schwede *et al*., 2011). There are over 1000 genes that encode for different, immunologically distinct VSG proteins that share similar tertiary protein structures (Weirather *et al*., 2012, Carrington and Boothroyd, 1996). Each VSG monomer is attached to a glycosylphosphatidylinositol (GPI) anchor which is then incorporated into the cell surface membrane as a homodimer (Ferguson *et al.*, 1985, Ferguson *et al*., 1988, Schwartz and Bangs, 2007). Periodically a different VSG clone will emerge (antigenic variation) leading to the evasion of a small number of trypanosomes from the host adaptive immune system these 'switched' parasite with the new VSG then dominate the trypanosome population (Oladiran and Belosevic, 2012, Gjini *et al*., 2010, Horn and McCulloch, 2010, Rudenko, 2011, Jackson *et al.*, 2012).

The host immune system responds to the trypanosome infection by producing a polyclonal expansion of B-cell lymphocytes (Magez *et al*., 2008, Tabel *et al.*, 2008, Bockstal *et al*., 2011). Immunoglobulin (Ig) G and IgM have been shown to bind to the VSG on the trypanosome cell surface where the bound IgG can be rapidly internalised without damage to the trypanosome (O'Beirne *et al*., 1998, Engstler *et al*., 2007). It has been proposed this adds to the parasite's defence against the host immune system to ensure persistence and further transmission (MacGregor *et al*., 2011, Namangala, 2011, MacGregor *et al*., 2012). It has been suggested that trypanosomes may play a role in modulating the host immune system (Vincendeau and Bouteille, 2006, Millar *et al*., 1999, Gjini *et al*., 2010, Paulnock *et al.*, 2010).

Disease severity has also been shown to have links between the parasite genotype and the host immune and inflammatory response (MacLean *et al*., 2004, MacLean *et al*., 2006, MacLean *et al*., 2007) *e.g.* levels of interferon-γ (IFN-γ) and interleukin-10 (IL-10), both pro and anti-inflammatory cytokines which confuse the host immune response (Courtin *et al*., 2007, Sternberg, 2004, Sternberg and MacLean, 2010, Bucheton *et al*., 2011). On balance it appears to be a combination of parasite and host genetic factors that determine the virulence of HAT (Antoine-Moussiaux *et al*., 2009, Garcia *et al*., 2006, Sternberg and MacLean, 2010).

1.3. Diagnosis of HAT

HAT diagnosis in the field faces many difficulties ranging from the medical to the logistical e.g. screening teams attending communities in remote rural locations, and during violent conflicts (Chappuis et al., 2005, Tong et al., 2011a, Van Nieuwenhove et al., 2001). Once they are with the communities, the screening teams must recruit the entire local population into the HAT screening programme, otherwise under-reporting and under-estimations of infection rates will occur (Mpanya *et al*., 2012, Mumba *et al*., 2011, Odiit *et al*., 2005, Robays *et al*., 2004). The identification of infected individuals relies principally on screening teams that visit at-risk communities or from patients seeking medical help (Odiit *et al*., 2004, Deborggraeve and Büscher, 2010). Some patients remain asymptomatic for years so early diagnosis of any infected individuals benefits not only the patient but also the community where these individuals act as parasite reservoirs (Jamonneau *et al*., 2010).

In areas endemic for *T. b. gambiense* individuals are screened using the CATT (section 1.3.1.1), should this test be positive with increasing blood dilutions (1:16), patients are further examined for the presence of trypanosomes. Samples of their blood and/or from their cervical lymph nodes are examined by microscopy. If parasites are found, the disease is staged. Staging requires sampling of the cerebral spinal fluid, where the probability of the parasites penetrating the brain is determined.

In areas endemic for *T. b. rhodesiense*, analysis of body fluids by microscopy is the main diagnosis step, these fluids include, blood samples, lymph node and chancre (if present) aspirations. If trypanosomes are present the disease is staged as described above. In some areas *T. b. rhodesiense* and *T. b. gambiense* infections are in close geographical proximity (Picozzi *et al*., 2005) which raises concerns for future differential diagnosis for each sub-species, which at present can only be determined in a laboratory setting as there is not a field applicable test.

1.3.1. Current diagnostic tools

Diagnostic tools are required, particularly for *T. b. gambiense* infections due to the low density of parasites in the blood, which can be below the detection limits of microscopy (Wastling and Welburn, 2011). Diagnostic tools include procedures to improve microscopy and serodiagnostic tests, the latter is defined as detecting either circulating parasite antigens or antibodies that recognise parasite antigens (Hutchinson *et al*., 2004). Here I shall discuss a selection of diagnostic tools and their relevance for the diagnosis of HAT in the field.

1.3.1.1. Card Agglutination Test for Trypanosomiasis (CATT)

The current gold-standard field diagnostic tool for HAT screening for suspect *T. b. gambiense* cases is the CATT. It is a serological test which detects host antibodies in a suspension of fixed and stained *T. b. gambiense* trypanosomes expressing LiTaT1.3 VSG (Magnus *et al*., 1978). Over the years, the CATT screening tool has been optimised to improve stability, sensitivity (ranging from 87 % to 98 %) and specificity (95%) (Chappuis *et al*., 2005). Such modifications include dilution of the blood samples, the use of multiple trypanosome clones expressing different VSGs and improvements in thermostability (Jamonneau *et al*., 2000, Chappuis *et al*., 2004, Hasker *et al*., 2010, Truc *et al*., 2002b).

Despite the usefulness and wide deployment of the CATT screening tool, it has several widely acknowledged limitations (Radwanska, 2010, Brun *et al*., 2010, Wastling and Welburn, 2011, Penchenier *et al*., 2003). These include varying degrees of sensitivity and specificity, which in part can be attributed to the agglutination test itself, which requires subjective judgements whether a patients sample contains antibodies or not (Figure 1.6). More profoundly, it has been identified in some places that the *T. b. gambiense* strain is not expressing the LiTAT1.3 VSG gene, and therefore patients do not generate detectable antibodies (Dukes *et al.*, 1992). The CATT screening tool cannot be used to detect *T. b. rhodesiense* infections as *T. b. gambiense* specific strains in are used in this test and antibodies from *T. b. rhodesiense* infected patients do not cross-react (Fèvre *et al.*, 2005). On the other hand, cured patients can have antibodies that can persist up to three years, leading to the possibility of false positive tests (Paquet *et al*., 1992), which is a weakness of any test that relies on antibody detection. CATT

also requires trained screening personnel to use it and requires the need to cultivate infectious parasites for manufacture (Robays *et al*., 2004, Truc *et al*., 2002a).

1.3.1.2. Loop-Mediated Isothermal amplification (LAMP) and Polymerase Chain Reaction (PCR) tools

Further methods have been developed to detect parasites or parasite derived products overcoming the limitations of antibody serological diagnosis. The detection of parasite DNA in blood by loop-mediated isothermal amplification (LAMP) of DNA (Wastling *et al*., 2010) methods are under investigation and are summarised in a recent review (Mugasa *et al.*, 2012). While studies have been carried out to assess and validate the performance of LAMP in the ambient temperatures of tropical countries (Thekisoe *et al.*, 2009), the diagnosis procedure still requires lab-based equipment and reagents, such as buffers and primers, similar to PCR amplification based diagnostic tools (Deborggraeve and Büscher, 2010, Becker *et al*., 2004, Mugasa *et al*., 2010). This platform is currently being investigated by the Foundation for Innovative New Diagnostics (FIND) which was commissioned by the World Health Organisation (WHO) to develop diagnostic tests for diseases of poverty, including HAT, tuberculosis and malaria (Wastling and Welburn, 2011).

Despite the limitations of potential use as a field screening tool, the LAMP platform has been shown to detect down to 1 fg of trypanosome DNA, however this was achieved with purified DNA as the starting material (Thekisoe *et al*., 2007).

1.3.1.3. Microscopy

Microscopy is used in the field to identify parasites in bodily fluids, for example, blood and CSF (Matovu *et al*., 2012). Microscopy is, at present, the ultimate proof that a patient is infected, however this technique lacks sensitivity and requires trained personnel and laboratory equipment. The lack of sensitivity is more relevant to *T. b. gambiense* infections where parasite density ranges from 10,000 to below 100 parasites per ml, the latter being below the threshold of microscopy (Brun *et al*., 2010). Examination of blood from a finger prick has a detection limit of 10,000 trypanosomes per ml (Büscher and lejon, 2004). Developments to concentrate trypanosomes from infected blood and fluids, such as haematocrit centrifugation (HCT) and the mini anion exchange centrifugation technique (mAECT) have improved detection limits to less than 100 trypanosomes per ml (Biéler *et al*., 2012, Büscher *et al*., 2009, Camara *et al*., 2010). However these methods still require lab based equipment and trained personnel.

1.4. Staging the infection and treatments

As described earlier in section 1.2.3 (disease progression), staging the infection is necessary to predict whether the trypanosomes have penetrated the brain (Kennedy, 2008). This will determine which drug the patient is treated with as some drugs are effective only in the first stage of the disease. Staging takes place by sampling the CSF by a lumbar puncture and counting the number trypanosome and/or lymphocyctes per µl and/or measuring the IgM concentration of the CSF (Chappuis *et al*., 2005). The classification/determination of second stage HAT is based on the number of the afore-

mentioned measured properties which are set as guidelines in each country (Kennedy, 2006b, Chappuis *et al*., 2005).

Treatment is administered after the patient has been diagnosed and staged. Many of the drugs in current use have been reported to have severe limitations (Fairlamb, 2003). First stage *T b. gambiense* may be treated using Pentamidine and Suramin but first stage *T. b. rhodesiense* is only responsive to Suramin (Bouteille *et al*., 2003). These drugs are reasonably well tolerated but side effects may be experienced *e.g*. liver and kidney damage (Welburn and Maudlin, 2012). These compounds do not penetrate the blood-brain barrier therefore different second stage drugs are required. Treatment of second stage *T. b. gambiense* is mainly by Nifurtimox-Eflornithine combination therapy (NECT) and Melarsoprol is now only used for *T. b. rhodesiense* (Priotto *et al*., 2009, Opigo and Woodrow, 2009, Kennedy, 2012). Reactive encephalopathy occurs in 5-10% of patients treated with Melarsoprol and approximately half of those cases die (Kennedy, 2006a, Fairlamb, 2003). Clearly new, safe, affordable treatments that are effective in both stages and against both sub-species of trypanosomes are urgently needed.

1.5. Lateral flow tests

Lateral flow devices are simple tests that can rapidly detect nanogram amounts of antibodies or antigens in finger-prick blood samples without the need for any ancillary equipment (Posthuma-Trumpie *et al*., 2009, Bandla *et al*., 2011). Lateral flow tests have been adopted for diagnosis use in other diseases such as malaria, Leishmaniasis

and *Theileria annulata* infections (Bell and Perkins, 2008, Abdo *et al.*, 2010, El-Moamly *et al.*, 2011). These lateral flow tests (point of care tests) are relatively stable at ambient temperatures and require limited training for use (Robert, 1997, Posthuma-Trumpie *et al.*, 2009). The premise for most of these tests is a known diagnostic protein candidate(s), these are usually recombinant proteins if the availability of native diagnostic antigens is too challenging. The candidate antigens are assessed and validated, usually by enzyme linked immunosorbent assay (ELISA), leading to the adaptation and production of lateral flow assays.

Due to the very low parasitemia levels in patients infected with *T. b. gambiense*, a lateral flow test that detects host antibodies (rather than parasite antigens) was considered more likely to have the necessary sensitivity and specificity.

1.6. Summary

There have been many calls for new diagnostic tools and treatments to be designed and made available, due to the severe limitations of the current tools (Brun and Blum, 2012, Welburn *et al.*, 2009). In particular a new test should have greater specificity to avoid false positive results (Radwanska, 2010). Recently the FIND has invested in developing new diagnostic tests for HAT (Steverding, 2006a). Their emphasis is on 'piggy-backing' on diagnostic platforms such as LAMP although they are investigating other platforms (Ndung'u *et al.*, 2010, Matovu *et al.*, 2012). However, these diagnostic methods require relatively sophisticated laboratory equipment (Robert, 1997).

In summary, there is well accepted case for developing a simple, low-cost diagnostic device with greater sensitivity and specificity than the current field tests (Matovu *et al.*, 2012, Simarro *et al.*, 2011). With this aim in mind, we set out to identify novel diagnostic antigens for our prototype lateral flow test device. We chose to use a non-biased (proteomics) approach to potential biomarkers rather than the candidate antigen approach used in previous tests.

We are extremely grateful to Professor Philippe Büscher (Institute of Tropical Medicine, Antwerp) for sending us the initial human serum samples, without which none of this work would have been possible. We are also extremely grateful to the WHO and the HAT specimen bank, firstly for granting us use of these very precious human serum samples but also for all their assistance, in particular for sending clinical details of the patients sampled. The lateral flow assays would not have been created without the help of Dr. Steven Wall and Richard Lamotte (British Biocell International, Dundee), to whom we are extremely grateful.

2. Aims

2.1. The overall aim of this project was to develop a field compatible lateral flow diagnostic test for human African Trypanosomiasis.

The specific aims were:

- To discover potential diagnostic antigens for HAT.
- To recombinantly express and purify a selection of antigens.
- To assess and validate recombinant, synthetic or native antigens for diagnostic potential with clinically assessed infection and control human serum samples.
- To develop the most promising candidate antigen(s) into lateral flow format for screening and assessment with human sera.

3. Materials and Methods

3.1. Reagents

3.1.1. Chemicals and reagents

All general chemicals were purchased from Sigma (St Louis, USA) or VWR (Leven, Belgium) unless otherwise stated. All media for *E. coli* cell cultures, including antibiotics solutions, were made and supplied by the Media Kitchen service in the College of Life Sciences, University of Dundee. Phosphate Buffered Saline (PBS) was supplied by the Media Kitchen service as a ten times concentrated solution.

3.1.2. Serum samples

Two sets of samples were used the first, (Identification sera set) were kindly provided by Philippe Büscher (Institute of Tropical Medicine, Antwerp) and consisted of nine sera from *T. b. gambiense* infected patients and nine from matched non-infected patients (0.5 ml to 1 ml aliquots). The second (Validation sera set) were sera from 145 patients (200 µl aliquots) and were obtained from the WHO Human African Trypanosomiasis specimen Bio-Bank. Serum samples were aliquoted and stored at either -80 ^{o}C for long-term storage or in 50 % (w/v) glycerol at -20 ^{o}C when prepared for ELISA analysis. Freeze-thawing was kept to a minimum; samples from P. Büscher and WHO were freeze-thawed three times and twice, respectively, prior to use in ELISA tests.

3.1.2.1. Human serum samples ethics

Ethical approval was in place for all serum samples used in this study, whereby the samples were taken with the informed consent of the patient. The WHO HAT specimen Bio-Bank samples ethnics included the purpose of distribution of serum samples for the

purpose of developing diagnostic tests. The local ethics board (NHS Tayside) also reviewed and approved the study retrospectively.

3.2. Trypanosome preparations

3.2.1. Ethics

The animal procedures were carried out according the United Kingdom Animals (Scientific Procedures) Act 1986 and according to specific protocols approved by The University of Dundee Ethics Committee and as defined and approved in the UK Home Office Project License PPL 60/3836 held by MAJF.

3.2.2. Preparations of *T. b. brucei* lysate

Six BalbC mice were injected with between 10^5 and 10^6 *T. b. brucei* strain 427 variant MITat 1.4 cells. After three days, infected mouse blood was harvested with sodium citrate anticoagulant, adjusted to 10^7 parasites per ml with phosphate buffer saline (PBS) (2.7 mM KCl, 1.5 mM KH_2PO_4, 136.9 mM NaCl, 8.9 mM $NA_2HPO_4 \bullet 7H_2O$, pH 7.4) and aliquots of 0.5 ml were injected into the peritoneal cavity of 12 Wistar rats. The rat blood was harvested after 3 days with citrate anticoagulant and centrifuged at 1000 x *g* for 10 min at 4 ^oC (4K15 Sigma Centrifuge). Plasma was removed and the buffy coat which contained the trypanosomes was resuspended in separation buffer plus glucose (SB + glucose; 57 mM Na_2HPO_4, 3 mM KH_2PO_4, 44 mM NaCl, pH 8, 10 g/l glucose) and applied to a DE52 DEAE-cellulose (Whatman, GE Healthcare, Maidstone, England) column that had been pre-equilibrated with SB + glucose. The trypanosomes were washed through the column with SB + 1 % (w/v) glucose, counted, centrifuged (900 g, 15 min, 4 ^oC), resuspended in 1 ml PBS and then adjusted to $1x10^9$ parasites/ml in ice-cold lysis buffer (50 mM Na_2PO_4, pH 7.2, 2 % (w/v) *n*-octyl β-D-glucopyranoside

(nOG) detergent, 1 mM phenylmethsulfonyl fluoride (PMSF), 1 mM tosyl-L-lysine chloromethyl ketone (TLCK), 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 x Roche protease inhibitor cocktail minus ethylenediamine tetraacetic acid (EDTA) (Mannheim, Germany). The lysate was incubated for 30 min on ice and then centrifuged at 100,000 *g* for 1 h at 4^{o}C (Beckman Ultracentrifuge, 50.2 Ti rotor).

3.2.3. Preparation of soluble Variant Surface Glycoprotein from *T. b. brucei* lysate

The sVSG purification method is a well-established protocol that has been used in the lab and is described in Mehlert *et al*, 2002. The sVSGs were further purified by gel filtration using a Sephacryl S200 column (4x90 cm) equilibrated with 0.1 M NH_4HCO_3. The samples were run on an SDS PAGE gel to check for purity and were considered >95 % pure (data not shown). The sVSGs were kindly purified and prepared by Angela Mehlert.

3.3. Molecular methods for identification of antigens

3.3.1. Virus deactivation of sera

The samples provided by Professor P. Büscher underwent virus inactivation using a modified procedure that retains antibody reactivity (Burnouf et al., 2006). Briefly, 1 % (w/v) Tri(n-butyl)phosphate (TnBP) (Fluka, UK) and 1 % Triton X-45 (w/v) (Tx-45) (Sigma, USA) were each added to a final concentration of 1 % to thawed serum samples. The sera were vortexed for 2 min and were incubation in a waterbath at 31 ^{o}C for 4 h. The sera were inverted every half hour. To extract the TnBP and TX-45, 2 ml of sterile castor oil (Riedel-de Haën, Germany) was added to each serum sample, inverted and centrifuged at 3800 x *g* for 30 min (4K15 Sigma Centrifuge). TnBP and

TX-45 partitions in the oil layer. The oil-extraction was repeated three times and the virus-inactivated sera (lower phases) were aliquoted and stored at -80 ^{o}C.

3.3.2. IgG purification from serum

Following virus deactivation, 125 µl of sera from four infected and four uninfected (control) patients were pooled. Each pool was applied to a 1 ml protein G column (GE Healthcare, Sweden) equilibrated in PBS (Table 3.1). The columns were washed with 10 ml of PBS and the bound IgG antibodies were eluted with 50 mM sodium citrate pH 2.8, and collected in 1 ml fractions into tubes containing 200 µl of 1 M Tris-HCl, buffer pH 8.5, to immediately neutralise the pH. Peak fractions containing IgG were combined and dialysed for 16 h against coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3).

3.3.3. Protein concentration determination

Aliquots of protein solutions were transferred directly, or diluted then transferred, to cuvettes (Eppendorf) and the absorbance at 280 nm was measured on Eppendorf BioPhotometer. Cuvettes containing only the buffered solutions were used as blanks.

3.3.4. Coupling of IgG to cyanogen bromide-activated (CNBr) Sepharose beads™

CNBr-activated Sepharose™ beads (Sigma, Sweden) were weighed to give a gel volume of 0.5 ml or 0.75 ml, for first immunoprecipitation and 2nd immunoprecipitation respectively. The beads were hydrated in 1 mM HCl then equilibrated in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). The beads were mixed with purified infection IgG or purified control IgG (4 mg of each for the 1st immunoprecipitation and 7.2 mg for the 2nd immunoprecipitation) in a final volume of 3 ml coupling buffer for 16

h at 4 ^{o}C. The coupling of IgG to the gel was confirmed by measuring the absorbance of the supernatant at 280 nm (A_{280} < 0.05) (10 min, 4^{o}C, 500xg in 4K15 Sigma Centrifuge). The Sepharose^{TM}-IgG conjugates were centrifuged at 500 x *g* (10 min, 4 ^{o}C, 4K15 Sigma Centrifuge) and the beads were resuspended in 15 ml 1 M ethanoamine, pH 9, for 2 h at room temperature. Following this, the IgG-Sepharose^{TM} beads were washed with three cycles of 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl followed by 0.1 M sodium acetate buffer, pH 6.0, 0.5 M NaCl and finally washed and stored in PBS containing 0.05% (v/v) NaN_3.

In the first immunoprecipitation experiment, two further control resins were also prepared following the same procedure as above. These were 'Non-matched control' IgG from purified IgG from serum originated from a person that has never been exposed to Trypanosomes. 'Beads only' are beads that have no IgG, this was to address whether CNBr-activated Sepharose^{TM} beads could bind to trypanosome derived products.

3.3.5. Immunoprecipitation

For the 1st immunoprecipitation experiment, aliquots of *T. b. brucei* lysate (10^{10} cell equivalents) were incubated with the Sepharose^{TM}-IgG (infection and non-infection/control) gels, rotating for 1 h at 4 ^{o}C. The Sepharose^{TM}-IgG were centrifuged 10 min at 4 ^{o}C, 600 x g (4K15 Sigma Centrifuge) and the supernatent cell lysate were removed. The gels were washed with 14 ml 50 mM Na_2PO_4 pH 7.2, 2 % (w/v) nOG, 0.5 M NaCl for 10 min at 4 ^{o}C then centrifuged at 600 x g, for 10 min at 4 ^{o}C. This wash step was repeated three times. The gels were washed with 14 ml 10 mM Na_2PO_4, pH 7.2, 1 % (w/v) nOG for 10 min at 4 ^{o}C then centrifuged at 600 x g, for 10 min at 4 ^{o}C. This second wash step was repeated three times. Trypanosome proteins that bound to

the Sepharose^{TM}-IgG were eluted with three 500 µl elutions of 50 mM Sodium Citrate pH 2.8, 1% (w/v) nOG, collected in tubes containing 110 µl 1 M Tris pH 8.5. The three elutions were combined and freeze-dried (machine). The lyophilised sample was resuspended in 200 µ MilliQ H_2O plus 1 ml ice cold 100 % Ethanol and stored at –20 ^{o}C for 16 h. The ethanol precipitation was centrifuge at 16,000 x g for 15 min at 4 ^{o}C (5415 R Eppendorf Centrifuge). The pellet was washed with 500 µl cold MilliQ H_2O and centrifuged at 16,000 x g for 10 min at 4 ^{o}C (5415 R Eppendorf Centrifuge). This was repeated twice. The pellet was then resuspended in 30 µl Sodium dodecyl sulphate (SDS) Sample buffer (SB) (Invitrogen, Carlsband, CA, USA) containing 100 mM dithiothreitol (DTT) (Formedium, England). The samples incubated at 100 ^{o}C for 7 min and then sent to the proteomics facility for tryptic digestion and protein identification.

In the 2nd immunoprecipitation experiment, aliquots of *T. b. brucei* lysate (10^{10} cell equivalents) were incubated with the Sepharose^{TM}-IgG (infection and non-infection/control) gels, rotating for 3 h at 4 ^{o}C. The gels were then packed into disposable 10 ml columns and washed with 10 ml of 10 mM Na_2PO_4, pH 7.2, 200 mM NaCl, 1 % (w/v) nOG, followed by 10 ml of 5 mM Na_2PO_4 pH 7.2, 1 % (w/v) nOG. The trypanosome proteins bound to the Sepharose^{TM}-IgG gels were eluted three times with 750 µl of 250 mM sodium citrate, pH 2.8, 1% (w/v) nOG and the eluates were pooled and neutralised with 1.5 M Tris-HCl, pH 9 and further concentrated to 140 µl using a centrifugal concentrator (Millipore, 0.5 ml capacity with 3 kDa MW cut off membrane (Bedford, MA, USA)). To remove eluted IgG, this fraction was mixed with 60 µl PBS-equilibrated Protein G agarose beads (Pierce, Rockford, USA) which were incubated for 10 min and removed by centrifugation (600 x g, 10 min, in a 5415 R Eppendorf Centrifuge). The supernatant which contained the trypanosome proteins, were then

transferred to low binding Eppendorf tubes and the proteins were precipitated by adding 1 ml ice-cold ethanol and incubated for 34 h at -20 ^{o}C. The ethanol precipitation was followed by centrifuge at 16,000 x g for 15 min at 4 ^{o}C (5415 R Eppendorf Centrifuge). The pellet was washed with 500 µl cold MilliQ H_2O and centrifuged at 16,000 x g for 10 min at 4^{o}C (5415 R Eppendorf Centrifuge). This wash step was repeated twice. The pellet was then resuspended in 30 µl SDS SB (Invitrogen, Carlsbad, CA, USA) containing 100 mM DTT. The samples incubated at 100 ^{o}C for 7 min and then sent to the proteomics facility as before.

In both immunoprecipitation experiments, 10 % of the resuspended protein in SDS SB elutions were loaded on a SDS polyacrylamide gel electrophoresis (PAGE) gel (Invitrogen, Carlsbad, CA, USA) and silver stained to estimate protein concentration and how much to load for the SDS PAGE gel run by the proteomics facility (see Section 3.5 (Silver stain) and Section 3.4 (Proteomics)).

3.3.6. SDS PAGE

Samples were run on pre-cast Novex 4-12 % Bis-Tris SDS-PAGE gels with MOPS running buffer system (Invitrogen) in a XCELL-II mini-tank (Novex) at 200 V using a BioRad Power Pac Junior. SDS-PAGE gels were also cast by hand for methods that did not require downstream proteomics analysis, using the recipe in Table 3.2. The assembled gels were run in a Tris-Glycine buffer (25 mM Tris base, 250 mM glycine, 0.1 % (v/v) SDS) in a MiniPROTEAN Tetra cell (BioRad) at 180 V using a Power Pac 300 (BioRad). Samples were prepared with 4x SDS Sample Buffer (Invitrogen) and DTT (0.1 M) as a reducing agent. Before loading samples were placed in a heating block set at 100 ^{o}C for 5 to 10 min.

Table 3.2: SDS-PAGE recipe for hand cast gels.

	Stacking gel	Resolving gel	
		10 %	12 %
30 % Bis-acrylamide (BioRad)	0.4 ml	1.67 ml	2 ml
1 M TrisHCl pH 8.8	-	1.2 ml	1.2 ml
1.5 M TrisHCl pH 6.8	0.6 ml	-	-
10 % (v/v) SDS	25 µ	50 µl	50 µl
Water	1.4 ml	2.1 ml	1.7 ml
10 % (v/v) APS (VWR)	35 µ	70 µl	70 µl
TEMED (VWR)	3.4 µ	7 µl	7 µl

3.3.7. Coomassie and Silver staining

Proteins on the SDS PAGE gels were subsequently stained with 0.1 % Coomassie brilliant blue stain in 40 % methanol, 10 % acetic acid for 1 h. The excess stain was removed and gels are washed with 40 % methanol, 10 % acetic acid (Destain solution) or with 10 % acetic acid only. For protein mass fingerprinting, the gel was stained for 30 min with SimplyBlue^{TM} Safe Stain (Invitrogen, Carlsbad, CA, USA) and destained with water. When protein quantities were too low to be detected by Coomassie staining, gels were stained with silver using the SilverQuest ^{TM} kit (Invitrogen) following the manufacturer's instructions.

3.4. Proteomic analysis

Following ethanol precipitation, the proteins eluted from the infection IgG and control IgG gels were dissolved in SDS sample buffer, reduced with 100 mM DTT and run on a precast 4-12% BisTris gradient SDS-PAGE gel (Invitrogen) using the MES running system. All mass spectrometry was carried out by the proteomics facility (University of Dundee). The gels were stained with colloidal Coomassie blue and equivalent regions of the infection and control lanes were cut out and underwent in-gel alkylation with

iodoacetamide and were digested with trypsin. The tryptic peptides were analysed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) on a Thermo Orbitrap XL system.

3.4.1. MASCOT Software

MASCOT daemon software was used to match peptides to the predicted trypanosome protein databases (combined GeneDB and UniProt predicted protein sequences).

3.5. Molecular Biology Protocols

3.5.1. *In silico* analyses and searches of DNA and protein sequences

The identified protein sequences were used to BLASTp search the *T. b. brucei* predicted protein database (from the GeneDB database, http://www.genedb.org) (Logan-Klumpler *et al*., 2012). Protein sequence multiple alignments were assembled using CLUSTALW2 (Larkin *et al*., 2007) and Jalview (Waterhouse *et al*., 2009). *In silico* plasmid construction were assembled using GCK (Texcto BioSoftware) or CLC Main Workbench (CLC Bio). Protein extinction coefficients for each recombinant protein were calculated by ProtParam (Gasteiger *et al*., 2005). Recombinant constructs were designed using predictors for signal peptide, SignalP (Petersen *et al*., 2011) for transmembrane domains using TMpred (Hofmann and Stoffel. 1993). When construct design required smaller domains to be cloned out of a larger domain, GLOBplot was consulted to identify protein sequences with high degrees of disorder, which may to be joining domains together (Linding *et al*., 2003). Rare codon predictions were calculated using Prof. Charles Bond's rare codon predictor (University of Melbourne, Australia), which recommended that all recombinant proteins be expressed in *E. coli* cell lines that contained additional rare codon tRNA plasmids.

3.5.2. Primers

Primers used are listed in Table 3.3. Where possible primers were designed to have a similar melting point (T_m) of 60 ^oC and to contain ~20 bp of complementary sequence. The T_m was calculated according to the following formula: T_m = (G+C)x 4 ^oC + (A+T)x 2^oC. Only nucleotides in the complementary sequence of the primer were included in the calculations.

Table 3.3: List of primers

Name	Sequence	Tm (^oC)	Restriction sites
F-GRESAG4-MG-46-Nco1	CATGCCATGGGCTACAGTGGCAAGATTTCAGCA	60	Nco1
R-GRESAG4-836-Xho1	CCGCTCGAGATTTATTAGTCGAATCCATTTTGCTT	60	Xho1
F-GRESAG4-KTIITGTN	AGACAATAATAACAGGGACTAAT	60	For sequencing
R-GRESAG4-TPSLHIV	TTGGTGACAGGTGAATCACAT	60	
F-GRESAG4-50-Nde1	TAATTACATATGATTTCAGCAAAGGTATATGATCCTATTACTGCA	58	Nde1
R-GRESAG4-840-Xho1	TTATATTAACTCGAGTAAAGCCAGCTGCCACAGCACCAATATGCT	63	Xho1
F-GRESAG4-46-Nde1	ATAATATCATATGTACAGTGGCAAGATTTCAGCAA	60	Nde1
R-GRESAG4-407-Xho1	TATTATACTCGAGTAATTGGCACTCACCACCAAAGT	60	Xho1
R-GRESAG4-401-Xho1	TAATTACTCGAGTTAGTCACCAACCACAAGATCATCAATTACATAAC	59	Xho1
F-GRESAG4-401+-Nde1	TAATATATTCATATGGACTTTGGTGGTGAGTGCCAAGGCAT	61	Nde1
F-ISG64-24-Xho1	TATAATTACTCGAGAATGCAAAGTTGACCAAAGATGGTGCGTT	59	Xho1
R-ISG64-363-BamH1	TATAATAGGATCCTTAATCACTAGTCTCCAGGAGGTCACCGAA	61	BamH1
F-ISG75-30-HindIII-M	TAATAATGAAGCTTCTATGAACTTACCTGTCGCATATAAGCAGTATG	57	HindIII
R-ISG75-295-BamH1	TAATTAGGATCCTTATTCCAGATACTGTGCAAGAGACTCCCTT	60	BamH1
F-ISG75-pL2-30-BamH1-M	TATAATGGATCCATGAACTTACCTGTCGCATATAAGCAGTATG	57	BamH1
R-ISG75-pL2-295-Not1	TTAAATGCGGCCGCTTATTCCAGATACTGTGCAAGAGACTCCCTT	60	Not1

All primers were synthesized by the Oligonucleotide Synthesis Service in the University of Dundee (http://www.lifesci.dundee.ac.uk/services/oligo/index.php).

3.5.3. Plasmid vectors

Plasmids used in this project include sub-cloning plasmids and plasmids used with Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible expression for recombinant protein purification.

Table 3.4: Plasmid description

Plasmid	Purpose	Source
pCR2.1	Sub-cloning from PCR product for DNA sequencing	(TOPO vectors) Invitrogen
pET15b-TEV	*E. coli* overexpression plasmid containing N-terminal His tag, with a modified multiple cloning site and TEV protease cleavage site.	Kind gift from Dr. Scott Cameron (AHF group, University of Dundee)
pGEX-TEV	*E. coli* overexpression plasmid containing an N-terminal GST tag and a modified multiple cloning site (to the same as pET15b-TEV) and a TEV protease cleavage site.	Kind gift from Jennifer Fleming (WNH group, University of Dundee)

3.5.4. PCR

All polymerase chain reactions (PCRs) were performed with High Fidelity Platinum(R) Taq DNA polymerase (Invitrogen, USA) in a 50 µl volume using a PTC-225 Peltier Thermal Cycler (MJ Research). Conditions and programs are in Table 3.5. PCR products were analysed by agarose gel electrophoresis.

Table 3.5: PCR reaction conditions

Amplification	Reaction conditions	Primers	Template DNA	
			Genomic DNA	Plasmid
GRESAG4, ISG64, ISG75	1x High Fidelity PCR buffer	0.2 µM	150 pg	50 pg
	2 mM Mg_2SO_4			
	0.2 mM dNTPs			
	1 U Platinum Taq			

Table 3.6: Amplification program

Cycle	Temperature (^oC)	Time
1	94	2 min
2-34	94	30 s
	55-65 (gradient on plate)	30 s
	72	1 min 30
35	72	10
	4	1 h

3.5.5. Agarose gel electrophoresis

For DNA separation, 1 % (w/v) agarose gels in 1x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer containing 0.4 µg/ml ethidium bromide were used. The gels were run at 80 V using BioRad Mini-Sub Cell GT tanks connected to Power Pac 300 power packs. All DNA samples were run in presence of 1x Blue/Organge Load dye (Promega, Madison, USA) and 1 Kb or 100 bp DNA ladders (Promega, Madison, USA) which were used to estimate DNA fragment size. The gels were imaged using a GelVue UV Transilluminator with a UGenius gel documentation system (Syngene).

3.5.6. Gel extraction of DNA

PCR derived DNA fragments of plasmid DNA were purified post separation by agarose gel by using the QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany). This was

followed by the microcentrifuge protocol and the DNA concentration was estimated by A_{260} in a spectrophotometer (Bio Photometer, Eppendorf) using UVettes (Eppendorf) with a 1 cm path length.

3.5.7. Restriction endonuclease digestion

Endonucleases were purchased from Promega (Madison, USA) or New England Biolabs (NEB, USA). DNA to be digested was incubated with the restriction enzyme and the required digestion buffer at the recommended optimal temperature (usually 37 ^{o}C) for 1 to 2 hours (Table 3.3). Small aliquots of the DNA were analytically (included only one enzyme digestion) were digested in parallel with the preparative digestion (usually included both restriction enzymes). The digestion mix from the negative control, analytical digestions and the preparation were analysed by agarose gel electrophoresis.

3.5.8. *E. coli* strains and cultures

E. coli cells used for transformations post ligations were the high-efficiency TOP10 cells (Invitrogen, USA) while DH5α (DSTT, Dundee) strain was used for plasmid propagation and sub-cloning. These cells were grown in Luria Broth (LB) medium (Table 3.7) containing 50 µg/ml ampicillin at 37 ^{o}C, 200 RPM in Infors HT incubators. As described earlier, in *in silico* predictions (Section 3.5.1), of tRNA codon bias occurred in all of the trypanosome sequences selected, overexpression cell line BL21 CodonPlus (DE3)RIPL (Stratagene) was used as it compensated by containing plasmids for rare tRNA codons, encoding for Arginine, Isoleucine, Proline and Leucine. For overexpression of recombinant proteins cells were grown in 2x Tryptone Yeast extract (TY) medium (Table 3.7). For bacterial stabilates, 100 µl of 80 % (w/v) glycerol

(AnalaR, VWR, France) were added to 800 µl of cells grown from a single colony in for 16 h at 37 ^{o}C, 200 RPM and snap-frozen on dry-ice for storage at -80 ^{o}C.

Table 3.7: Media

Medium	Chemical substance	Amount per 1 L (g)
LB (Adjusted to pH 7)	NaCl	10
	Tryptone	10
	Yeast extract	5
2x TY (Adjusted to pH 7)	NaCl	5
	Tryptone	16
	Yeast extract	10

3.5.9. Ligation

Concentrations of purified inserts and digested plasmid vectors were estimated by spectrophotometer (A_{260}). A range of different ratios of vector to insert were used, including 1:1, 1:3 and 1:9 (vector:insert), also vector only controls were performed in parallel. The ligation reactions were set up to a 10 µl final volume using the Rapid DNA ligation kit (Roche, Mannheim, Germany) (Table 3.8).

Table 3.8: Ligation reaction for expression plasmids (Roche)

Reaction component	Volume or amount
Insert	Ratios as above
Vector	Ratios as above
5 x buffer	1.10 µl
2x buffer	6 µl
T4 ligase	0.5 µl

Table 3.9: TOPO-TA ligation

Reaction component	Volume or amount
PCR product	4 µl
TOPO-TA vector	1 µl
Salt solution	1 µl

The reaction mixture was incubated at room temperature for 20 min, 3.5 µl of each ligation was transformed into TOP10 competent cells (Invitrogen, USA).

3.5.10. Transformation

Aliquots of chemically competent cells (50 µl) were incubated with ~10 ng of plasmid DNA or 1/3 of a ligation mixture on ice for 5 min. Once cells were fully defrosted cells were heat shocked at 42 ^{o}C for 20 to 35 s depending on the strain of cells, and then were cooled on ice for 2 min. SOC medium was added to the cells, which were then grown at 37 ^{o}C for 45 min at 200 RPM (Infors HT incubator) before being plated on LB + agar plates containing ampicillin and other antibiotics if required.

3.5.11. Purification of plasmid DNA

Plasmid DNA from transformed DH5α or TOP10 *E. coli* strains were derived from purified and prepared 5 ml cell cultures (minipreps) grown at 37 ^{o}C, 200 RPM for 16 h using the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany).

3.5.12. DNA sequencing

DNA sequencing was performed by the Sequencing Service at the University of Dundee, using the Applied Biosystems 3370 DNA analyzers. Regions of interested were sequenced twice in each direction, with the consensus sequence built in CLC Main

Workbench (CLCbio). Comparison between the consensus and the *in silico* sequences were performed using CLC Main Workbench and ClustalW2 (Larkin *et al.*, 2007).

3.6. Protein biochemistry for recombinant antigens

3.6.1. Cell density measurement

Cell density was monitored by measuring the density by Absorbance at 600 nm BioPhotometer (Eppendorf).

3.6.2. Protein expression optimisation strategy

A single colony was picked from a freshly transformed cell line, grown overnight on LB with antibiotics (AMP and CML), into 50 ml 2xTY media. Cultures were incubated at 37 ^{o}C until the Absorbance at 600 nm (Abs $_{600}$) reached 0.3 or 0.8 and induce expression with IPTG (Formedium, England)

Table 3.10. Optimisation of expression conditions:

Condition	IPTG Induction (Abs_{600})	Length of induction (h)	Temperature of Induction (^{o}C)
A	0.3	3	37
B	0.8	3	37
C	0.8	16	18

Table 3.10: Optimisation of expression conditions, description:

The first condition (A) is where the culture was induced with 1 mM IPTG at Abs_{600} at 0.3 and was expressed at 37 ^{o}C for 3 h.

The second condition (B) is where the culture is induced at Abs_{600} of 0.8 and expression temperature was 37 ^{o}C for 3 h.

Finally the third condition (C) uses the Abs $_{600}$ at 0.8 where the culture is cooled before being induced for 16 h at 18 ^{o}C.

3.6.3. Sample resuspension for SDS PAGE loading

1 ml Uninduced and Induced samples were collected, the cell density was measured at Abs_{600} and the sample was centrifuged in a microcentrfuge (Eppendorf) for 17 seconds to pellet the cells. The supernatant was removed and the cell pellet was resuspended in corresponding volumes from the pre-made stocks (Table 3.11). Samples were boiled for 5 to 10 min in a heating block and loaded on an SDS PAGE gel or frozen for use in other SDS PAGE gels.

Table 3.11: Suspension of 1 ml cell culture pellet for relative loading for SDS PAGE gels.

OD	1 M DTT (µl)	4 x SDS SB (µl)	mq H_2O (µl)	total (µl)
0.3	6	15	24	60
0.4	8	20	32	80
0.5	10	25	40	100
0.6	12	30	48	120
0.7	14	35	56	140
0.8	16	40	64	160
0.9	18	45	72	180
1	20	50	80	200
1.1	22	55	88	220
1.2	24	60	96	240
1.3	26	65	104	260
1.4	28	70	112	280
1.5	30	75	120	300
1.6	32	80	128	320
1.7	34	85	136	340
1.8	36	90	144	360
1.9	38	95	152	380
2	40	100	160	400
2.2	44	110	286	440
2.4	48	120	312	480
2.6	52	130	338	520

2.8	56	140	364	560
3	60	150	390	600
3.2	64	160	416	640
3.4	68	170	442	680
3.6	72	180	468	720
3.8	76	190	494	760

3.6.4. Large scale over-expression and purification of recombinant proteins from *E. coli*

Typically, cells containing the desired expression plasmid were selected for by streaking the stabilate cells or by re-transformation on LB containing amplicillin and chloramphenicol plates. Each selected colony was grown in 5 ml 2 x TY media, 37 ^{o}C, 200 RPM (Thermos shakers), containing ampicillin and chloramphenicol, until cloudy. Each culture was inoculated into 0.5 L 2 xTY (with antibiotics) (in a 2L conical flask) and incubated at 37 ^{o}C, 200 RPM, until the cell density has reached desired Abs_{600}. At this point 5 ml 2 % (v/v) IPTG was added to the media to induce expression of the recombinant sequence. Induction continues for the appropriate duration and temperature as described in Table 3.10. Cells were harvested by centrifugation (J6-MC Beckmann Centrifuge) at 4200 x g, 20 min at 4 ^{o}C. The supernatent was removed and the cell pellet is resuspended in buffer A (Table 3.x) (without protease inhibitors). The resuspension was either stored at -80 ^{o}C or processed immediately, see below.

Table 3.12: Buffers used in Ni NTA purification

Buffer	Chemical	Concentration
Buffer A	NaH_2PO_4 pH8 with NaOH	50 mM
	NaCl	300 mM

Lysis buffer (buffer A +)	Imidazole	10 mM
Wash buffer (buffer A+)	Imidazole	20 mM
Elution buffer (buffer A+)	Imidazole	250 mM

3.6.4.1. Cell lysis

A Roche protease inhibitor cocktail tablet was added to lysis buffer/cell suspension. The cells were lysed by French Press above 10,000 psi. The cell lysate was clarified by centrifugation at 12,600 x g, 15 min, 4 ^{o}C (J2-21 Beckmann centrifuge).

3.6.4.2. Sepharose 6B purification

Sepharose 6B resin (Sigma, USA) was washed with H_2O and then buffer A (Table 3.12) by centrifuging and replacing the supernatant with the next wash buffer and centrifuged at 500 x g, 10 min, at 4 ^{o}C in a 4K15 Sigma Centrifuge. The lysate was incubated with the resin for 10 min at 22 ^{o}C, rotating end over end, and centrifuged at 500 x g, 10 min, at 4 ^{o}C in a 4K15 Sigma Centrifuge.

3.6.4.3. Ni NTA purification

Nickel-charged nitrilotriacetic acid (NTA) cross linked to agarose was used to purify 6xHis-tagged proteins under native and denaturing conditions (QIAGEN, Hilden, Germany). Eight ml 50 % slurry was washed with H_2O and equilibrated with buffer A (Table 3.12) (centrifuge at 500 x g, 10 min, at 4 ^{o}C in a 4K15 Sigma Centrifuge). The lysate post Sepharose B6 incubation was incubated for 1 h at 22 ^{o}C and then centrifuged at 500 x g, for 10 min, at 4 ^{o}C in a 4K15 Sigma Centrifuge. The Ni NTA agarose was then washed three times with 50 ml buffer A (centrifuging at 500 x g, 10 min, at 4 ^{o}C in a 4K15 Sigma Centrifuge in between to exchange washes). On the final wash the Ni

NTA agarose was transferred to a column with filter to prevent any Ni NTA passing through. The Ni NTA agraose was washed with 20 ml Wash buffer, 20 ml Wash buffer with 20 mM Imizadole and the 10 ml Elution buffer. The eluted material was dialysed using 3.5 KDa Slide-A-Lyzed dialysis cassettes (Thermo, Rockford, USA) or SnakeSkin Tubing (Thermo, Rockford, USA) against three 1L changes of buffer A, at 4 ^{o}C. During the purification protocol samples for SDS PAGE were taken to monitor the presence of recombinant proteins.

3.6.4.4. Cleavage of His tag for rISG65-1

1.4 mg of His-rISG65 was incubated with 100 µl of 1 mg/ml His-TEV protease (a kind gift from Professor William Hunter, University of Dundee) at 22 ^{o}C for 3 h. Aliquots of the supernatant was taken every 1 h and were run on a SDS PAGE gel to gauge the cleavage efficiency.

3.6.4.5. Size exclusion chromatography

Purified recombinant proteins were concentrated, if necessary, to 2-3 mg/ml (final volume 0.5 ml) by using Vivaspin20 concentrator (Sartorius, Goettingen, Germany) with a molecular weight cut-off of 10 kDa. The Superose 12 10/300 GL (GE Healthcare, Sweden) size exclusion chromatography column was calibrated in buffer A with standard proteins, Bovine serum albumin (BSA) (4 mg/ml) and Cytochrome C (0.5 mg/ml). Concentrated recombinant proteins were loaded on the pre-equilibrated Superose 12 column via an AKTA Purifier system (GE Healthcare). The column was pre-equilibrated with buffer A (Table 3.12) and the column was eluted with 1.5 column volumes buffer A at a flow rate of 0.5 ml/min. Aliquots from the fractions of interest

were loaded on a SDS-PAGE gel and the proteins content staining assessed by Coomassie.

3.6.4.6. GST tagged recombinant proteins

The protocols used are the same recombinant expression as those used in (Sections 3.6.4 to 3.6.4.1). Following from cell lysis, clarified supernatant lysate was applied to pre-equilibrated, with Buffer A, Sepharose Glutathione Fast Flow beads (GE Healthcare) rotating for 2 h at 4 ^{o}C. The material was applied to a 10 ml disposable column (BioRad) and the beads washed with 25 column volumes of buffer A. GST tagged recombinant proteins were eluted with 10 ml buffer A containing 10 mM reduced glutathione. 1 ml fractions were collected and aliquots were loaded on SDS PAGE and protein was quantified by Absorbance at 280 nm.

3.6.5. Protein concentration determination

Protein concentrations were estimated using a BioRad assay using BSA as a standard and following the manufacturer's instructions (BioRad). Protein solutions were also estimated by Absorbance at 280 nm and calculating protein concentration based on protein's predicted extinction coefficient.

3.7. Enzyme linked Immunosorbent Assay (ELISA) plates

White, un-treated 96 well plates (Costar, Corning, USA, #3370) were coated with 2 µg/ml protein solution at 50 µl/well for 16 h at 4 ^{o}C. The proteins (recombinant or native) were diluted in coating buffer (0.05 M $NaHCO_3$, pH 9.6). Coating solution was removed and wells were blocked with 200 µl/well PBS containing 5% (w/v) BSA, for 3 h at 22 ^{o}C or 16 h at 4 ^{o}C. Plates were stored at 4^{o}C and used within 24 h. ELISA

measurements were made with both pooled and individual serum samples. The pooled sera were diluted to 1:60 in 50 % (w/v) glycerol, PBS and 1 % (w/v) BSA and stored at -20 ^{o}C. For the ELISA assay used for the individual sera, the 1:60 diluted samples were further diluted to 1:1000 immediately before use.

3.7.1. Serum storage

Individual patients' serum and pooled serum samples were diluted to 1:60 and stored at -20 ^{o}C in a 96 well 0.5 ml block (Greiner, Germany) containing PBS, 1% (w/v) BSA and 50 % (w/v) glycerol (5 µl serum aliquot diluted in 295 µl solution). Only the first three columns of each 96 well block was occupied due to the program used for liquid handling (Section 3.3.2).

3.7.2. Adaption for liquid handling device (LHD)

The LHD transferred the 1:60 diluted sera to the dilution block for further serial dilutions (Bio-Tek, Precision). Aliquots (50 µl) of serial serum dilutions were transferred in triplicate to the ELISA antigen plates.

3.7.3. Pooled serum dilution for ELISA plates

Serum pools were made by combining patients sera from: 1st stage *T. b. gambiense* patients (n=10), 2nd stage *T. b. gambiense* patients (n=40) and matched uninfected patients (n=50); and from 1st stage *T. b. rhodesiense* patients (n=5), 2nd stage *T. b. rhodesiense* patients (n=20) and matched uninfected patients (n=25). The 1:60 diluted pooled sera were further diluted to 1:1000 in PBS, 0.1 % (w/v) BSA and then serially diluted (doubling dilutions) to 1:32,000 by LHD into a 0.5 ml 96 well block (Greiner, Germany). Aliquots of the dilutions (50 µl) are then transferred to antigen plates (n=3).

3.7.4. Individual sera dilution for ELISA plates

Individual serum samples were diluted to 1:60 in the serum blocks. The LHD further diluted the 1:60 sera to 1:1000 in PBS, 0.1 % (w/v) BSA and then serially diluted, in doubling dilutions, to 1:8000 or carried out a further one dilution step to 1:5000. Aliquots of 50 µl were transferred to the antigen plates (n=3).

3.7.5. ELISA

Sera were incubated for 1 h at room temperature, aspirated and 150 µl ELISA wash buffer (PBS, 0.1 % (w/v) BSA) was added to each well by the LHD, left for 10 min and aspirated. This wash cycle was performed three times. Biotinylated goat anti-human-IgG (Jackson Immunoresearch, USA) (re-constituted to 1 mg/ml) was diluted to 1:5000 and 50 µl aliquots were applied to each well. After 1 h incubation at room temperature the secondary antibody solution was removed and wells were washed three times, as described above. Horseradish peroxidase (HRP) conjugated to NeutrAvidin (Sigma, St Louis, USA) was diluted to 1:4000 and applied to the wells (50 µl/well) for 1 h at room temperature. Wells were washed as before. Finally, electrogenerated chemiluminescence (ECL) Femto substrate (Pierce, Rockford, USA) containing was diluted 1:5 according to the manufacturer's instructions (*i.e.*, 0.5 ml solution A, 0.5 ml solution B with 4 ml PBS) and 50 µl/well was added. Plates were read using an Envision plate reader after 2.5 min substrate incubation at 22 ^{o}C.

3.7.6. Quantification of IgG in human serum

IgG quantification kit was used and manufacturer's instructions were followed (Mabtech, Sweden). Briefly, monoclonal anti-human IgG were diluted to 2 µg/ml in PBS and coated in wells for 16 h at 4 °C (Coatar clear 96 well plates). Coating solution

was removed and wells were washed 3 times with PBS 0.05 % (w/v) Tween 20 and blocked for 16 h at 4°C with PBS, 1 % (w/v) BSA. Human serum samples were diluted 1:60,000 and 1:80,000 and applied in duplicate to the ELISA plates. Serum dilutions were incubated with the plate for 1 h and wells were washed as before. A second monoclonal anti-human IgG conjugated with Alkaline Phosphatase (AP) was diluted 1:1000 in PBS and incubated with wells for 1 h. Colourmetric AP substrate (SigmaFAST, Sigma, St Louis, USA) was added to the wells and the plate was read after 30 min at 595 nm wavelength.

3.8. Lateral flow assay

Steven Wall at British Biocell International (BBI) was responsible for the design and manufacture of the lateral flow test prototypes.

3.8.1. Lateral flow pooled sera optimisation

Serum aliquots (0.625 to 20 µl) were diluted, if necessary, to a final volume of 20 µl PBS and applied to the sample pad. Chase buffer (80 µl of PBS) was added to the sample pad and the test was allowed to develop for 30 min. The test line was visually scored, based on band intensity, and the device was opened and the sample pads (at the top and bottom of the nitrocellulose membrane) were removed to prevent backflow. The lateral flow tests were photographed and scanned using a densitometer (CAMAG, at BBI facility).

3.8.2. Lateral flow buffer optimisation

Serum aliquots (5 µl) were diluted in 15 µl PBS and applied to the sample pad. One of three chase buffers was then applied to the sample pad; options included, i) PBS, ii)

PBS, 0.05 % (w/v) Tween 20, and iii) PBS, 1 % (w/v) BSA. The test was processed as described in Section 3.8.1.

3.8.3. Randomisation of sera

Sera were randomised by a member of the University of Dundee Tissue Bank. Forty *T. b. gambiense* infected patients' sera and forty *T. b. gambiense* uninfected patients' sera were randomly selected from the fifty *T. b. gambiense* infected and fifty uninfected WHO patient sera. These eighty serum samples were then randomised and coded.

3.8.4. Randomised lateral flow trial

The coded randomised sera aliquots (5 µ) were diluted in 15 µl PBS and were applied to the sample pad, followed by 80 µl PBS, 0.05 % (w/v) Tween 20. The tests were processed as described in Section 3.8.1.

3.8.5. Cattle sera source

There were two groups of cattle sera used the first were kind gifts from Onderstepoort Veterinary Institute (OVI) (South Africa) and contained non-infected calves (n=3), calves infected with a highly virulent strain of *T. congolense* (n=5) and calves infected with a low virulent strain of *T. congolense* (n=8). The second group of cattle sera were kind gifts from Philippe Büscher (ITM) and represent samples taken pre-infection and post-infection of 5 calves infected with *T. congolense* and the same for 5 calves infected with *T. vivax*.

3.8.6. Cattle sera lateral flow

Pooled sera aliquots (10 µ) were diluted in 10 µl PBS and were applied to the sample pad, followed by 80 µl PBS, 0.05 % (w/v) Tween 20. The tests were processed as described in Section 3.8.1.

3.9. Carbohydrate antigens

3.9.1. Consortium for Functional Glycomics (CFG)

200 µg of protein G purified IgG from pooled infected and control sera (see section 3.X) were sent to the CFG. A glycol-array was performed with six technical replicates. CFG provided us with Excel spreadsheets were returned showing the glycol-array output. Infection IgG data were normalised to control IgG data. Structures with a normalisation ratio above 5 were considered to indicate an infection specific response.

3.9.2. Synthetic biotinylated structures

Dmitry Yashunsky synthesised a range of biotinylated Galβ1-4GlcNAcβ1-6Galβ1-4GlcNAc, structures, ranging from two LacNAc (Galβ1-4GlcNAc) repeats (β1-6 linked) to five LacNAc repeats.

3.9.3. Carbohydrate ELISA – plating the compound

To anchor carbohydrate structures to the plate, NeutrAvidin (Thermo, Rockford, USA) was diluted to 2 µg/ml in plating buffer and coated at 50 µl/well for 16 h at 4 ^{o}C (Section 3.x) (Costar, Corning, USA #3370, white non-treated 96 well plates). The plates were quickly washed with PBS, 0.1 % (w/v) BSA. The biotinylated-LacNAc compounds were diluted in PBS to 1.25 µg/ml and 50 µl aliquots were applied to the NeutrAvidin plates for 1 h at 22 ^{o}C. The solutions were removed and the wells blocked

with 200 µl/well PBS, 0.1 % (w/v) BSA and 0.5 µg/ml biotin for 1 h at 22 ^{o}C. Plates were further blocked with PBS, 5 % (w/v) BSA for 1 h at 22 ^{o}C. Plates were used immediately or stored at 4 ^{o}C for up to 16 h.

3.9.4. Pooled and individual sera dilutions for carbohydrate ELISA

Similar to Section 3.7.3 and 3.7.4, however sera were diluted from 1:60 to 1:500 and serially diluted to 1:16,000 (doubling dilutions). For pooled sera ELISAs, the ELISA plates were washed 3 times with PBS 0.1% (w/v) BSA. Goat anti-human IgM+ IgG+IgA conjugated HRP (Jackson ImmunoResearch, USA) was diluted to 1:5000 in PBS 0.1% (w/v) BSA, and was incubated with the wells for 1 h. Wells were washed 3 time as describe above, and ECL ELISA substrate was applied to the plates as described in section 3.7.5. For the individual sera ELISA screen, sera were diluted 1:500 in PBS 0.1% (w/v) BSA and applied to plates for 1 h. Wells were washed 3 times with PBS 0.1 % (w/v) BSA. Either; goat anti-human IgG+IgM+IgA conjugated to HRP, goat anti-human IgG conjugated to HRP (Jackson ImmunoResearch, USA) or goat anti-human IgM conjugated with HRP (Jackson ImmunoResearch, USA) were diluted to 1:5000 and applied to wells. Plates were processed as described in 3.7.5.

3.10. Statistics

Bar graphs and scatter plots (x by y) were generated by Microsoft Excel. Box plots, Receiver Operator Characteristic (ROC) curves, antigen scatter plots (y axis only) were generated by SigmaPlot 12. Statistical analysis included Mann-Whitney (Rank Sum Test) and Dunn's post-hoc (Analysis of Variance (ANOVA) on rank) in SigmaPlot 12. The P values were recorded for Mann-Whitney with <0.05 set as the cut off for statistical significance. The Q and P values were recorded for Dunn's post-hoc test

rests. Higher ‘Q’ values indicates the difference between the two groups is statistically significant, and less than P=0.05 gives confidence of the statistical significance of the Q value, in that the median values among the treatment groups are greater than would be expected by chance.

4. The identification of immunodiagnostic protein biomarker antigens for African trypanosome infections in man.

4.1. Background

The primary tools available to us for the initial identification of immunodiagnostic biomarker antigens were a small number of serum samples from patients infected with *T. b. gambiense* and uninfected (control) patients from the same region. We refer to these initial sera as the "identification set". In this chapter I will describe how this set was used to identify protein antigens recognised by infection sera. An important step in this process was to de-select those antigens that were also recognised (and/or non-specifically bound) by antibodies in the sera of uninfected patients. The remaining infection-specific proteins were then assessed as potential disease-specific diagnostic candidates (Chapter 5).

We chose to select the IgG fraction to immunoprecipitate parasite antigens for several reasons: Firstly, this is the major antibody fraction of human serum (approximately 75% of immunoglobulins) and it is easy to purify on immobilised protein-G. Secondly, IgG antibodies tend to have relatively high affinity for their target antigens and relatively low non-specific binding, compared to IgM antibodies. Thirdly, preliminary discussions with British Biocell International (BBI) (lateral flow specialists) about the prospective set-up of a lateral flow test for HAT led to the recommendation that we should make a test based on antigens recognised by IgG antibodies rather than IgM antibodies.

As a source of trypanosome antigens for immunoprecipitation, we chose to use *T. b. brucei* total detergent lysate. This was a pragmatic choice, based on the ease with which

large quantities of bloodstream form *T. b. brucei* parasites can be propagated in rodents. This choice was also informed by the high degree of similarity between *T. b. brucei* and the human pathogens *T. b. gambiense* and *T. b. rhodesiense* and the coverage and quality of the *T. b. brucei* genome sequence (upon which the proteomic identification of proteins depends).

4.2. Immunoprecipitation

Two immunoprecipitation experiments were carried out.

4.2.1. IgG purification, immobilisation and immunoprecipitation

The IgG fractions were purified by a standard protocol using protein G (Materials and Methods), from sera pooled from four *T. b. gambiense* infected (Table 4.1) and four matched uninfected (control) patients (Table 4.2) and from one unmatched control serum (Male, age 33). These IgG fractions were then coupled to CNBr-activated Sepharose^{TM} (3.3.4). Middle aged male sera were selected for pools and with only four individuals matching this description in the control sample selection, the infection individuals for pool were chosen based on age similarity with chosen control individuals.

In the first immunoprecipitation experiment, aliquots of trypanosome detergent lysate (equivalent to 1 x 10^{10} parasites) were immunoprecipitated with either infection- or control-IgG attached to Sepharose 4B and with Sepharose 4B alone. Aliquots (95% of eluted proteins corresponding to 9.5 x 10^{9} cell equivalents of trypanosome lysate) were analysed by SDS-PAGE (Figure 4.1). The results indicated the elution contained significant amounts of IgG heavy and light chain which had leached from the antibody

columns, at 55 kDa and 25 kDa, respectively, (lanes A-C) and that the amounts of trypanosome protein antigens specifically adsorbed and eluted from the infection-IgG Sepharose (lane A) were relatively small. Nevertheless, the bands marked *1 to *5 were excised for in-gel tryptic digestion and proteomic analysis. The identities of the main components of these bands are shown in Table 4.3, including keratin, which is a common contaminant of SDS PAGE gels and proteomics and was disregarded. These data suggested that three *T. brucei* antigens might be recognised by infection-IgG, namely, VSG MITat.1.4, ISG75 and ISG65.

In the second immunoprecipitation experiment, we increased the amounts of immobilised IgG from 4 mg to 7.5 mg. We also introduced a step to remove eluted IgG heavy and light chains prior to SDS-PAGE analysis. In this experiment, we saw a significant increase in the amount of antigens immunoprecipitated (Fig 4.2), suggesting that the amount of immobilised antibody had been a limiting factor in the first experiment.

4.3. Proteomics

4.3.1. Identification of tryptic peptides

The gel slices, marked on Figure 4.2, were alkylated, digested with trypsin and loaded on ESI-MS/MS for peptide identification. Peptides were matched to predicted protein sequences using MASCOT daemon software, which ranked the identified predicted protein sequences with a score, known as the MASCOT score. This ranking algorithm takes into account many criteria, including, the peptide coverage of the predicted protein sequence and combines with the probability of identifying the peptide compared to randomly identifying the peptide. In many cases proteins were identified with the

highest MASCOT score in one gel slice, with smaller MASCOT scores found for the same protein in the gel slices above and below.

4.3.2. Protein identification

A MASCOT score cut off of 50 was used to reject potentially ambiguous protein identifications *e.g*. those identified from a single peptide per protein and/or those with relatively low quality MS/MS spectra. We then compared the lists of proteins found within each gel slice (Figure 4.3 and for full list see Appendix Figures A1 and A2) and selected the 24 protein identifications that occurred only in the infection-IgG eluate (Table 4.4). The de-selection of those antigens that also bound to the control-IgG Sepharose was crucial in this process.

Of the 24 proteins identified uniquely from the infection-IgG elutions, 11 of them were ‘hypothetical proteins’ and 7 of the twelve highest MASCOT scored proteins (>200 MASCOT score) were cell surface proteins, *e.g.,* the transferrin receptor, (composed of expression site associated gene (ESAG) 6 and ESAG7 proteins), ESAG2 protein, gene-related to ESAG (GRESAG)4 protein and members of the Invariant Surface Glycoproteins (ISG) families ISG64, ISG65 and ISG75.

5. Expression, purification and ELISA of recombinant protein antigens

Diagnostic kits require multi-milligram to gram quantities of diagnostic biomarkers for industrial scale manufacture and distribution. A challenge for any immunodiagnostic test is the ability to make or synthesise enough material for mass production. Thus, there may be excellent candidate antigens, as identified by immunoprecipitation, which may not prove feasible for recombinant protein expression. Proteins that are easy to express in a soluble form and that are stable and well characterised are needed to manufacture tests. Therefore, our aim was to first assess the ease of protein expression of a number of the potential diagnostic antigens identified in chapter 4 and then to assess their promise for diagnostic use.

5.1. Protein antigen selection criteria and overview

The trypanosome proteins that were identified uniquely in infection-IgG immunopurified fractions (Table 4.4) were considered for recombinant expression. Of these, the proteins with high MASCOT scores were prioritised. The rationale for this selection was that, by using an excess of trypanosome lysate in the affinity purification step, the amount of an eluted antigen should reflect, to a first approximation, the relative amount of antigen-specific immobilised IgG. The latter should, in turn, correspond to the immune response to that antigen in infected patients. Using this criterion, the protein antigens selected for study were ISG75, ESAG7, GRESAG4, ISG65, ISG64 and ESAG6, respectively.

Next, we looked into the likely ease of protein expression of these antigens in *E. coli*. At this stage, we de-selected ESAG6 and ESAG7 because they form a heterodimer (adding

the complication of dual-expression) and because successful (but low level) protein expression has only been reported in a eukaryotic baculovirus expression system (Chaudhri *et al*., 1994). On the other hand, *E. coli* recombinant expression of domains of ISG75, ISG65 and ISG64 have been reported, either in the literature (Tran *et al*., 2008) or by personal communication (Professor Mark Carrington, University of Cambridge). Consequently, we selected all three ISGs for protein expression trials. Finally, we performed expression trials on the putative extracellular domain of GRESAG4, for which there are no literature precedents.

5.2. Recombinant ISG75 (rISG75) expression

ISG75 is a cell-surface glycoprotein with a large N-terminal extracellular domain, a single transmembrane domain and a short cytoplasmic domain. It was identified by cell-surface biotinylation (Ziegelbauer and Overath, 1992) and it is estimated to be present at about 50,000 copies per cell (Ziegelbauer *et al*., 1992). The function(s) of ISG75 are unknown but it has recently been suggested that it is the specific receptor for the drug Suramin and that it shuttles between the cell surface and the endosomal system of the parasite (Alsford *et al*., 2012).

The proteomics experiments described earlier (4.2.1 and 4.3.2) identified *T. b. brucei* ISG75 (Uniprot_Q26769). We took the predicted amino acid sequence for this gene and identified the closest relative in the *T. b. gambiense* protein database (Tbg972.5.320; 99 % identity). This sequence is, in turn, very similar (92% identical) to another ISG75 from the EATRO 1125 *T. b. brucei* strain (gene ID cannot be included at present due to the incomplete genome sequencing of the EATRO 1125 *T. b. brucei*). Figure 5.1 shows the recombinant protein sequence (for resides 32-419) which was already available from

our collaborator, Professor Mark Carrington and we therefore elected to use this recombinant antigen (rISG75-1) for further studies.

However, since the trypanosomatid ISG75 family falls into 2 subgroups (Tran *et al*., 2006), we also attempted to express a similar part of another ISG75 belonging to the other subgroup (gene identifier Tb927.5.350). The sequence of this ISG75 variant is aligned with that of Tbg972.5.330 in (Figure 5.2) and shares 41% identity. Primers were designed to PCR amplify part of the Tb427.5.350 gene corresponding to amino acids 30 to 295 and the PCR product was cloned into a pET15b-TEV vector and then transformed into BL21(DE3)RIPL, *E. coli* cells (3.5-3.6).

Three expression conditions were investigated (Figure 5.3, A) and condition B was chosen for a 1L expression and purification trial. Unfortunately, the rISG75-2 was not expressed in a soluble form and was found mostly in the insoluble pellet fraction shown in Figure 5.3, B. Therefore, we solubilised the pellet material in 6 M Guanidine Hydrochloride (GuHCl) buffer to attempt to re-fold it during purification. However the protein precipitated when the denaturing agent was removed, either by on-column buffer exchange or by overnight dialysis (Figure 5.3, C).

A final attempt was taken to obtain soluble rISG75-2 by sub-cloning it into a modified pGEX vector with tobacco etch virus (TEV) protease cleavage site located in between the glutathione S-transferase (GST) tag and the protein (kind gift from Jen Fleming). The GST-rISG75-2 plasmid was transformed into *E. coli* and expressed by IPTG induction. A 60 kDa protein product was observed by SDS-PAGE and in-gel digestion

and proteomics identified it as rISG75-2 sequence (MASCOT score of 459) (Figure 5.4).

While the GST tag did marginally improve the solubility of rISG75-2, there was not enough protein to attempt a TEV proteolysis or for the preparation of ELISA plates. Therefore, we decided to discontinue work on rISG75-2 as a potential recombinant diagnostic candidate.

5.3. Recombinant GRESAG4 (rGRESAG4) expression

Gene Related to Expression Site Associated Gene (GRESAG)4 is found on the trypanosome cell surface. It has a large N-terminal extracellular domain, a single transmembrane domain and a cytoplasmic domain which contains the adenylyl cyclase activity (Gould and de Koning, 2011, Bieger and Essen, 2000, Bieger and Essen, 2001). GRESAG4 encodes for an adenylyl cyclase that converts adenosine tri-phosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Seebeck *et al*., 2001). Recombinant constructs described in the literature represented the catalytic domain (Bieger and Essen, 2000, Bieger and Essen, 2001) but not the N-terminal extracellular domain which may contain many infection specific epitopes. In contrast some mammalian adenylyl cylases lack the extracellular domain and have twelve transmembrane domains while others have no transmembrane domains (Seebeck *et a*l., 2004, Dessauer, 2009, Bieger and Essen, 2000).

The second proteomic experiment identified *T. b. brucei* GRESAG4 (Tb927.7.7530) with a MASCOT score of 1456. We took the predicted amino acid sequence for this gene and identified the closest relative in the *T. b. gambiense* protein database

(Tbg972.4.4530; 87% identity) (Figure 5.5). Primers were designed to PCR amplify the part of Tb427.7.7530 corresponding to amino acids 46 to 840 and the PCR product was cloned into pET15b-TEV and transformed into BL21(DE3)RIPL *E. coli* cells (Materials and Methods).

5.3.1. Expression and purification trials of rGRESAG4

Three expression conditions were investigated (Figure 5.6, A) and condition B was chosen for 1L expression and purification trial of the protein (Figure 5.6, B). Unfortunately, the rGRESAG4 (50-840) protein was not expressed in a soluble form and the IPTG-induced 80 kDa protein in the insoluble pellet (Figure 5.6, B, lane 3) was identified as GRESAG4 by proteomics (MASCOT score 642).

The GLOBplot program was used to predict regions of disorder in the GRESAG4 full length protein sequence and to predict protein sub-domains (Linding *et al*., 2003). This led to the identification of two predicted sub-domains, 'A' and 'B' (Figure 5.7) within the extracellular domain of Tb427.7.7530 GRESAG4 and various constructs of these domain were amplified by PCR (Figure 5.7). The PCR products were then cloned into either pET15b-TEV or pGEX-TEV vectors and transformed into *E. coli* cells (BL21(DE3)RIPL). Attempts to express soluble protein representing GRESAG4 part a (corresponding to residues 46-407) and GRESAG4 part b (corresponding to residues 401-836 and 401-840) were unsucessful in initial expression trials (data not shown). However, GRESAG4 part a (G4a) corresponding to residues 50-401 was cloned into pGEX-TEV, transformed into BL21(DE3)RIPL cells and was successfully expressed. Expression condition C was used for the 1L expression and purification trial of this clone. The co-expressed N-terminal GST tag improved the solubility of G4a (Figure

5.8) however there were other protein impurities ranging from 17 to 25 kDa and the GST tag could not be cleaved from this G4a fusion protein (data not shown).

There was not enough G4a fusion protein (<100 µg/ L) to attempt further purification by size exclusion. However, despite the 17-25 kDa impurities, we decided there was enough protein to make some ELISA plates with which to screen the 'identification serum set'.

5.4. Recombinant ISG65 (rISG65) expression and purification

ISG65 is a cell surface glycoprotein with a large N-terminal extracellular domain, a single transmembrane domain and a short cytoplasmic domain. Similar to ISG75, Invariant Surface Glycoprotein 65 (ISG65) was isolated and identified from the trypanosome cell surface, and estimated to be present at approximately 70,000 copies per cell (Ziegelbauer and Overath, 1993, Ziegelbauer and Overath, 1992). The function(s) of ISG65 are unknown, but recent studies show that ubiquitylation of the C-terminal intracellular domain is a general mechanism to regulate some trans-membrane cell surface proteins (Chung *et al.*, 2008, Leung *et al*., 2011).

5.4.1. Recombinant ISG65-1

The proteomics experiments, described in chapter 4, identified *T. b. brucei* ISG65 (Uniprot_Q26712) as a potential biomarker. The predicted amino acid sequence was taken for this gene and the closest relative in the *T. b. gambiense* protein database was identified (Tbg972.2.1720, 98 % identity). This sequence is very similar (93 % identical) to another *T. b. brucei* EATRO1125 ISG65 gene 1125d (Figure 5.9) for which

a DNA clone (residues 20 to 366) was already available from our collaborator, Professor Mark Carrington.

Three expression conditions were trialled (Figure 5.10, A) and condition C was chosen for 1L expression and purification (Figure 5.10, B). Purification of rISG65-1 was successful with a good yield of soluble protein (14 mg/ L) (Figure 5.10, B). The amounts of impurities in the eluate from the first NiNTA purification (Figure 5.10, B, lane 7) could be reduced by applying the eluate to a Sepharose 12 size exclusion column (Figure 5.10, D), or by TEV proteolysis followed by purification on a second NiNTA column (Figure 5.10, E). Materials from both of these second round purifications (*i.e.,* the materials represented in Figure 5.10, D, lanes 8 and 9, and Figure 5.10, E, lane 6) were used interchangeably for making rISG65-1 ELISA plates (3.7).

5.4.2. Recombinant ISG65-2

The ISG65 family also falls into 2 subgroups, so we expressed a second recombinant ISG65 (rISG65-2) sequence from a similar part of another ISG65 that belongs to the other subgroup. This sequence is derived from another *T. b. brucei* EATRO 1125 ISG65 sequence that was made available to us as a DNA clone (residues 31 to 354) by our collaborator, Prof. Mark Carrington. The sequence of this rISG65 is aligned with that of Tbg972.2.1720 (its nearest neighbour in the *T b. gambiense* genome) with which it has 65 % identity (Figure 5.11).

The second rISG65 (rISG65-2) construct was transformed into BL21(DE3)RIPL *E. coli* cells. Three expression conditions were carried out (Figure 5.12, A) and condition C was chosen for 1L expression and purification trials (Figure 5.12, B). The purification

of rISG65-2 was successful with most of the rISG65-2 protein being found in the soluble fraction (Figure 5.12, B) and a reasonably high yield of 5 mg/ L of culture was obtained. The purity of rISG65-2 was enhanced by dialysis and performing a second round of NiNTA chromatography using a pre-packed 1 ml NiNTA column and an imidazole gradient for elution (Figure 5.12, C).

5.5. Recombinant ISG64 (rISG64) expression and purification

ISG64 is a cell surface glycoprotein with a large N-terminal extracellular domain but it is smaller than ISG65 and ISG75. ISG64 has a single transmembrane domain and a short cytoplasmic domain but as yet it has no known function.

5.5.1. Recombinant ISG64-1

The second proteomic experiment identified *T. b. brucei* ISG64 (Tb927.5.1410) with a MASCOT score of 582. We took the predicted amino acid sequence for this gene and identified the closest relative in the *T. b. gambiense* protein database (Tbg972.5.6550; 98% identity) (Figure 5.13). The *T. b. brucei* sequence (Tb427.5.1410) corresponding to amino acids 24 to 363 was amplified by PCR and the product was cloned into pET15b-TEV and transformed into BL21(DE3)RIPL *E. coli* cells (3.5-3.6).

Three expression conditions were trialled (Figure 5.14, A) and condition C was chosen for 1L expression and purification (Figure 5.14, B). Purification resulted in a yield of 6.5 mg/L. The lower band in the SDS PAGE profile of the purified rISG64 (Figure 5.14, B, lane 8) is similar to that found in the rISG65-1 purification (Figure 5.10) and it was a proteolysis product as confirmed by proteomics (data not shown). Attempts to separate

the 30 kDa component from the 40kDa protein were unsuccessful, as shown by SDS-PAGE analysis of fractions collected after a Sepherose 12 size exclusion column (Figure 5.14, D). The rISG64-1 material used for making ELISA plates therefore contained both the 40 and 30 kDa components.

5.5.2. Recombinant ISG64-2

Similar to the ISG75 and ISG65 families, ISG64 also falls into 2 subgroups. We therefore expressed and purified similar parts of another ISG64 belonging to the other sub group (gene identifier Tb927.5.1390). This sequence is derived from another *T. b. brucei* EATRO 1125 ISG64 sequence which was made available to us as two DNA clones (residues 20 to 355, and 20 to 292) by our collaborator, Prof. Mark Carrington. The sequences of these rISG64-2 and rISG64-3 constructs were aligned with that of Tbg972.5.6550 and have 70% and 66% identity, respectively (Figure 5.15).

Three expression conditions were trialled for rISG64-2 (Figure 5.16, A) and condition A was chosen for 1L expression and purification (Figure 5.16, B). Purification of rISG64-2 was successful with a large soluble yield of 5.7 mg/ L (Figure 5.16, B). The NiNTA column elution (Figure 5.16, B lane 7) was applied to a Sepharose 12 size exclusion column (Figure 5.16, C), and fractions containing protein were analysed by SDS-PAGE (Figure 5.16, D).

5.5.3. Recombinant ISG64-3

The final recombinant protein we chose to make was rISG64-3. Three expression conditions were trialled (Figure 5.17, A) and condition B was chosen for 1L expression and purification (Figure 5.17, B). Purification of rISG64-3 was successful with a large soluble yield of 8.6 mg/ L obtained (Figure 5.17, B). The elution (Figure 5.17, B, lane 7) was applied to a Sepharose 12 size exclusion column (Figure 5.17, C), and the fractions containing protein were analysed by SDS-PAGE (Figure 5.17, D).

5.6. Recombinant protein biomarker assessment

While recombinant protein domains have the same primary amino acid sequence as the equivalent part of the native protein, there is no guarantee that a recombinant protein will bind antibodies raised to the whole native protein. For example, the recombinant protein may not be appropriately folded to present native conformational epitopes and/or the recombinant protein domain may exclude the immunodominant part of the native molecule. Therefore, before any recombinant proteins are considered for lateral flow device development, they are first assessed for immunoreactivity with patient sera by a complementary method. The most commonly used method to quantify antibody titres directed to different proteins is the Enzyme Linked Immunosorbent Assay (ELISA).

A representative and founder of the lateral flow company (now called BBI - Richard Lamotte) advised us in the early stages of this project to aim to exploit patient's IgG rather than IgM responses. This is due to the principle that, in general, IgGs are bivalent molecules of relatively high affinity whereas IgMs are decavalent low affinity molecules that may cause high background signals in lateral flow assays. Therefore, our

immunoprecipitation experiments utilised the IgG fraction from sera to identify potential immunodiagnostic biomarker antigens and the ELISAs described in this chapter were set up to measure the patient's IgG response to the potential recombinant biomarkers.

The ELISA method and principles are described in more detail in the Materials and Methods section (3.7 - 3.7.5). Briefly, typically 200 ng of recombinant protein are adsorbed to each well and the plates are then blocked with PBS containing 5% BSA, to reduce non-specific antibody binding. Patient's sera are diluted in PBS, 0.1 % BSA and added to the plate for 1 h. The plates are washed three times with PBS containing 0.1% BSA. The human antibodies that bound to the plate are then detected using enzyme-linked (horse radish peroxidase, HRP) binding molecules and a chemiluminescence substrate, with the resulting signal measured by a plate reader (Envision). Some optimisation of the detection system was performed.

5.7. Assessment of diagnostic potential of recombinant antigens by ELISA using pooled human sera.

Recombinant antigens; rISG64, rISG65 and rISG75 all gave high infection specific ELISA signals, with low non-infected signals at serum dilutions of 1:1000 (Figure 5.18). GST-G4a was abandoned at this stage because it had poor infection versus non-infection discrimination (Figure 5.18). From these results, all the recombinant ISG proteins were taken forward to be screened against the individual sera (Chapter 6).

6. Immunodiagnostic biomarker antigen validation

6.1. Validation human serum sample set

In addition to the 'identification serum set' (n=18) used in Chapter 5 to select potential recombinant immunodiagnostic antigens for further study, we obtained a larger set of human serum samples (n= 145) from the WHO HAT specimen bank. We refer to this set as the 'validation serum set'. These samples contained sera from 1st stage *T. b. gambiense* patients (n=10), 2nd stage *T. b. gambiense* patients (n=40) and matched uninfected patients (n=50); and from 1st stage *T. b. rhodesiense* patients (n=5), 2nd stage *T. b. rhodesiense* patients (n=20) and matched uninfected patients (n=25). Anonymised clinical data are also available on these samples (Appendix, Tables A3-A10). In this Chapter, I describe how these sera were used with the recombinant antigens selected in Chapter 5 and some native VSG antigens to select antigens for the development of lateral flow devices.

6.2. Adaptation of the ELISA procedure to a liquid handling device

The general ELISA procedure was adapted for a liquid handling device, which made the procedure much more time efficient, reduced pipetting error and improved inter-plate and intra-plate consistency. A number of liquid handling devices were available for trial. Assessment of each liquid handling device was carried out by simulating the serial dilution step used in the ELISA using OrangeG dye in 50 % glycerol stored at – 20 ^{o}C and measuring the variation by Coefficient of Variation expressed as a percentage (% CV). CV is calculated by dividing the standard deviation by the mean and multiplying by 100 to give the % CV. Assays or systems that have a low variation will have a low % CV. The Precision liquid handling device performed consistently well within a CV of

8-10 % for both intra-plate and inter-plate variation, while the other liquid handling devices (Rainin and Hydra) were either no better or worse by this criterion. The Precision liquid handling device was also compatible with use in a laminar flow hood, providing protection from potential exposure to human serum aerosols. For these reasons, we adopted the Precision liquid handling device, which was kindly made available for us to use by Professor Alan Fairlamb (Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee).

6.3. IgG quantification of human serum samples

Alongside the individual sera screens against recombinant antigens, the IgG in each serum sample was quantified using a commercial ELISA kit to check whether infection and control sera contained comparable levels of total IgG (3.7.6). The ELISA data were expressed as box plots (Figure 6.1) and were further statistically analysed by Mann-Whitney Rank test for comparison between infection and control IgG levels and by Dunn's post-hoc for comparison between 1st stage with 2nd stage and with control.

There was no statistical significance for the comparison between *T. b. gambiense* infection and control IgG concentration by the Mann-Whitney Rank test (p=0.110); however there was statistical significance when comparing the IgG concentration from *T. b. rhodesiense* infected and control groups (p=<0.001). Further statistical analysis was carried out by Dunn's post-hoc to see if there was any significance between three groups (1st stage, 2nd stage and control). As inferred by the *T. b. gambiense* box plot (Figure 6.1, a) the 1st stage patients (Q=2.807; P=<0.05) have statistical significant higher IgG levels than compare with control and 2nd stage groups. There was no statistical significance difference between 2nd stage and control groups from the *T. b. gambiense* sera set. Conversely, the 2nd stage *T. b. rhodesiense* patient group have

higher IgG concentrations (Q=3.720; P=<0.05) compared with patients from the 1st stage and control groups (Figure 6.1, b).

Larger values of 'Q' indicate the difference between the two groups being compared is statistically significant, with the P values less than 0.05 indicating the likelihood of being incorrect of the significant difference is less than 5%. These statistical results indicate a significant difference of the IgG concentration of the 1st stage *T. b. gambiense* and 2nd stage *T. b. rhodesiense* patient groups compared to their respective control groups; however this is data from a single experiment and needs to be repeated before assigning biological significance. The purpose of this experiment was to assure us the infection-specific IgG titres to recombinant proteins are due to specific antibodies and not simply to increased total IgG concentrations in infected patients. The ratio of highest of infection serum IgG concentration to lowest control serum IgG concentration is less than 2-fold, which is unlikely to significantly bias ELISA and other immunodiagnostic read-outs with respect to determining a positive or negative serology.

6.4. Pooled sera screen with recombinant proteins

Before performing ELISA measurement with all of the individual sera, we looked at the relative performance of the different recombinant antigen ELISA plates using pooled sera from the validation set. Purified recombinant proteins (described in Chapter 5) were applied to ELISA plates (3.7) and screened against pooled *T. b. gambiense* and *T. b. rhodesiense*, 1st and 2nd stage infection and control patient sera.

6.4.1. *T. b. gambiense* pool results

The pooled *T. b. gambiense* 1st stage and 2nd stage infection sera and pooled matched control sera were diluted to 1:1000 and applied to ELISA plates coated with the different recombinant antigens. These experiments were carried out in triplicate and the mean signal ± 1 standard deviation are shown in Figure 6.2. All of the recombinant rISG65 and rISG64 protein constructs showed good separation of signal between the infection and control pools, for both 1st and 2nd stage disease. The rISG75 results were disappointing and we considered de-selecting this antigen for *T. b. gambiense* infection detection at this point. However we were aware that ISG75 has been described as a potential diagnostic antigen by others (Tran *et al.*, 2009, Tran *et al.*, 2008) and we elected to retain it for further analysis.

6.4.2. *T. b. rhodesiense* pool results

Identical experiments to section 6.4.1 were performed using the pooled *T. b. rhodesiense* 1st stage and 2nd stage infection sera and pooled matched control sera. None of the antigens performed well with 1st stage *T. b. rhodesiense* sera, with the exception of rISG75 (Fig 6.2). However, we found later that this was a due to single individual with an extraordinarily high antibody titre for rISG75 and we therefore consider this to be an anomalous result. Overall, the rISG65 constructs performed better than the rISG64 constructs with 2nd stage sera but the results were not as good as with the *T. b. gambiense* infection sera.

6.5. Individual sera screen with recombinant proteins

A good potential diagnostic biomarker is one that elicits antibody response in all infected patients. To test for this, all 163 available individual sera were serially diluted from 1:1000 to 1:8000 (doubling dilutions) or diluted 1:000 to 1:5000 in a single dilution step and applied in triplicate to ELISA plates coated with the recombinant proteins selected for further assessment (*i.e.*, rISG64-1, rISG64-2, rISG64-3, rISG65-1, rISG65-2 and rISG75). Data shown below are for the results with 1:1000 dilutions of the sera and are considered separately with respect to diagnostic potential for *T. b. gambiense* and *T. b. rhodesiense* infections.

6.5.1. Results with *T. b. gambiense* infection and matched control sera

The individual patient sera were screened against the six selected rISG proteins and the results are shown in bar charts representing the ELISA read outs of each patient's antibody titres to each recombinant protein (Figure 6.3 and 6.4). These data illustrate that there is heterogeneity in the way that individual sera react with the recombinant antigens. For example, a few patient sera react strongly to both rISG65s but only weakly to the ISG64s (Figure 6.4). It is clear from all of the data representations that rISG75 gives many false positives (that are in some cases are higher than the control signals (Figure 6.4)) confirming the data using the pooled sera and suggesting that this recombinant antigen is not suitable for the development of an immunodiagnostic test.

These data are also broken down into 1st stage and 2nd stage infection versus control data as box plots (Figure 6.5, a-f) to provide a more convenient way of assessing the ELISA data for each recombinant protein. As inferred by the box plots the Dunn's Post-Hoc test supports the observation that in all cases, apart from ISG75, 1st stage and 2nd

stage sera contain statically significant more IgG to the recombinant antigens compare to control (data not shown). In the case of ISG75, only the 2nd stage group of patients had statistically significant higher levels of IgG directed to rISG75-1 compared to control (Q=4.616, P=<0.05) and there was no statistical significance between 1st stage and control. The potential of antigens to stage the infection was tested by looking for statistical significance between the ELISA signals of 1st and 2nd stage groups. For all recombinant antigens there was no significant difference between IgG titres between the 1st and 2nd stage groups as determined by Dunn's post-hoc test (data not shown).

The heat map (Figure 6.5, g) is a convenient way to give an overall impression of all the ELISA data, emphasising the heterogeneity of each individual's antibody response to each protein. The results are also collated as scatter plots of signals for all (stage 1 and stage 2) infection versus control sera against recombinant antigen type (Figure 6.6).

A Receiver Operator Characteristic (ROC) curve was performed to calculate sensitivity on the y axis and specificity on the x-axis (Table 6.1). The area under the ROC curve can then be used to identify the best diagnostic candidate, an area under the ROC curve of 1 is the best possible outcome, which corresponds to 100 % sensitivity and specificity.

As predicted, the rISG75 protein performs badly as a potential diagnostic biomarker with an area under the ROC curve of 0.75, whereas rISG65-1 and rISG64-1 performed very well with areas under the ROC curve of 0.99 and 0.98, respectively (Figure 6.7). As a result of this data, and because we had a high recombinant yield of this protein, we selected rISG65-1 to be developed in lateral flow format test for *T. b. gambiense* infections.

6.5.2. Results with *T. b. rhodesiense* infection and matched control sera

The individual ELISA data for *T. b. rhodesiense* patients are displayed in bar graphs (Figure 6.8 and 6.9), as box plots, combined in a heat map (Figure 6.10) and as a scatter plot (Figure 6.11). The bar graphs show each individual's IgG antibodies to each recombinant protein. The box plots show the disease stage-specific effects where, in the cases of rISG65-1 and rISG64-3, there appears to be little difference between 1st stage and control patient sera. Indeed this observation is supported by statistical analysis (Dunn's post-hoc) where only the 2nd stage group for all antigens were statistically significant (data not shown). In addition to this Dunn's post hoc test is unable to prove statistical significance for the 1st stage group with control group, emphasising there is no antigen that can discriminate between infection with control with regards to this group 1st stage patients (data not shown).

The heat map shows the patient's antibody/antigen profile, with most patients in 2nd stage displaying IgG reactivity to at least two or more recombinant antigens.

Recombinant ISG65-2 was the only antigen that produced a measurable infection-specific IgG signal in most patient sera; however rISG65-2 is also recognised by a small number of non-infected patients. Two non-infected patients' sera, CR41 and CR50, displayed similar IgG infection-antigen profiles by recognising two or more antigens which raised the question as to whether these patients were really uninfected at the time of sampling. In any case, if we assume the clinical metadata with the serum samples is correct, we can only conclude that for *T. b. rhodesiense* infections there is no obvious candidate for a diagnostic antigen among those tested here, with the limited number of serum samples we had available.

The ROC analysis, in particular the area under the ROC curve, indicates that the best distinguishing recombinant antigen is rISG64-3 (A=0.87) (Figure 6.12), closely followed by rISG65-2 and rISG64-2, both of which have areas of A=0.86 with the sensitivity and specificity scores recorded in Table 6.2.

6.6. Summary of differences between *T. b. gambiense* and *T. b. rhodesiense* patient serum immunoreactivities

Comparison of the immunoreactivities of *T. b. gambiense* and *T. b. rhodesiense* infection sera indicate some significant differences. Thus, *T. b. gambiense* infected patient sera appear to show greater response to rISG65-1 and rISG64-1, whereas the *T. b. rhodesiense* infected patient sera appear to show greater response to rISG65-2 and rISG64-3. The *T. b. gambiense* first stage patients generally show antibody response towards many of the recombinant antigens, whereas in *T. b. rhodesiense* first stage patients appear to have fewer specific antibodies in these tests. As *T. b. rhodesiense* is thought to be essentially genetically identical to *T. b. brucei* (with the notable exception of the serum resistance antigen (SRA) in the *T. b. rhodesiense* genome (De Greef and Hamers, 1994)) the relatively poor immunoreactivity of the *T. b. rhodesiense* infection sera to the recombinant ISG antigens presumably reflects other factors. Indeed, the greater immunoreactivity in *T. b. gambiense* infection sera may reflect the significantly more chronic nature of these infections. This greater temporal exposure may lend itself to higher IgG titres against the parasite antigens. The results encouraged us to pursue rISG65-1 for lateral flow device development as it had the best properties for *T. b. gambiense* detection. Also it is the easiest to express and purify and has some diagnostic potential for *T. b. rhodesiense.*

6.7. Native *T. brucei* Variant Surface Glycoprotein (VSG) screen

In addition to the recombinant protein screens, we screened four native *T. b. brucei* variant surface glycoproteins (VSGs). For these studies, we used the conveniently purified soluble form VSG (sVSG) that is formed upon trypanosome lysis by the action of endogenous GPI-PLC on the VSG glycosylphosphatidylinositol (GPI) membrane anchor (Ferguson *et al*., 1985). Part of the rationale for this study was that a carbohydrate epitope called the cross-reacting determinant (CRD) (Cross, 1979), revealed by GPI-PLC mediated release of the VSG coat from dying trypanosomes (Zamze *et al.*, 1988), might elicit an anti-CRD response in all infected patients regardless of the VSG variants experienced in individual infections. We therefore wanted to test if a collection of different sVSGs available in our laboratory, each containing the CRD, could react with antibodies in our human host sera samples. As will be described, we did not find evidence of significant anti-CRD antibody responses but we did find very high titres of IgG antibody against one of the VSGs

6.7.1. Screens for anti-CRD and anti-VSG antibodies in infection sera.

Four purified sVSG variants (117, 118, 121 and 221) were kindly provided by Dr Angela Mehlert. ELISA plates were created for each one and serial dilutions of the pooled validation sera were analysed for IgG immunoreactivity to them (Figure 6.13). None of the infection sera showed significant reactivity to sVSG121, compared to the matched control sera, whereas sVSG221, sVSG117 and to a lesser extent sVSG118, showed reasonable reactivity to the pooled *T. b rhodesiense* infection sera (Figure 6.14). The same profile was seen for the pooled *T. b gambiense* infection sera, except that the immunoreactivity to sVSG117 was exceptionally strong. From these data, we concluded

that the weak reactivity to sVSG121 indicates that the majority of the immunoreactivity to the other sVSGs must be due to anti-peptide antibodies rather than anti-CRD antibodies. We followed up these results by investigating the potential of sVSG117 as a diagnostic biomarker antigen for *T. b. gambiense* infections.

6.7.2. Screen of individual sera against sVSG117 and combined sVSG117 and rISG65-1 ELISA plates.

Soluble VSG117 was further screened against the individual *T. b. gambiense* patient sera to assess whether the high antibody titres in the pooled *T. b. gambiense* infection sera were due to a small number of hyper-reactive individuals or whether immunoreactivity was common to all or most infected patients. We also created ELISA plates coated with both sVSG117 and rISG65 to assess whether these might out perform the individual antigen ELISA plates. The results of the means of triplicate analyses are shown first as bar graphs (Figure 6.15, A) and scatter plots (Figure 6.15, B) for the ELISA signals obtained with 1:1,000 diluted serum samples. These results suggest that the sVSG117 plates out performs the rISG65-1 plates and are at least equivalent to the combined sVG17 + rISG65 plates. The data are also presented as bar graphs, where the error bars represent 1 standard deviation from the mean value.

To calculate the sensitivity and specificity of sVSG117 as a potential immunodiagnostic biomarker, the data were analysed and showed that the sVSG117 ELISA plate test has an area under the ROC curve of 1 (Figure 6.16). The combined (dual antigen) sVSG117 + rISG65-1 ELISA plate had an area under the ROC curve of >0.99 and for ELISA plate with rISG-65 alone the value was 0.99. The sensitivities and specificities of the three ELISA plates were also calculated from these data (Table 6.3). This showed

that sVSG117 out-preforms rISG65-1 and combined sVSG117 + ISG65-1 as a diagnostic antigen for *T. b. gambiense* infections.

6.8. Summary

The key findings up to this point are:

- The ECL ELISA was shown to be a good assay to validate potential diagnostic biomarkers for lateral flow development as the assay is sensitive and reproducible.
- Patients infected with *T. b. gambiense* have IgG antibodies to many of the selected recombinant proteins validating the use of the trypanosome derived proteins identified by the immunoprecipitation experiment (chapter 4).
- Patients infected with *T. b. gambiense* and *T. b. rhodesiense* show different IgG infection profiles when tested with the recombinant antigen panel.
- Patients infected with *T. b. gambiense* have extraordinarily high antibody titres towards sVSG117, whereas *T. b. rhodesiense* patients do not. This may be useful when assessing which species patients are infected in areas where *T. b. gambiense* and *T. b. rhodesiense* co-exist. This is becoming an increasingly likely scenario in some areas in Uganda (Picozzi *et al*., 2005), or for travellers who may have visited areas endemic for both species.

7. Lateral flow Test

The ELISA data suggested that rISG65-1 was the best recombinant antigen to differentiate infection and control sera. Therefore, recombinant HIS_6-cleaved ISG65-1 (2 mg) was supplied to Steven Wall at BBInternational (Dundee MediPark), who carried out the production of the rISG65 prototype lateral flow test. One hundred un-optimised lateral flow prototypes were generously provided for us to evaluate.

While these prototypes were being made a second antigen, sVSG117, was screened and found to give better ELISA sensitivity and specificity scores (6.6.2). The sVSG117 glycoprotein was therefore also sent to BBI for development in the lateral flow format. We received one hundred un-optimised sVSG117 lateral flow prototypes containing just sVSG117 for testing against the human sera. We also received sixteen dual test line lateral flow test prototypes that contained both sVSG117 and rISG65 test lines.

7.1. Lateral flow test description

The lateral flow approach utilised is not animal species-specific and was identical for the rISG65 and sVSG117 lateral flow formats (Figure 7.1). Here, for simplicity, only the rISG65-1 lateral flow test is described in detail. The rISG65-1 antigen was both immobilised in a band on a nitrocellulose membrane (test band) and coupled to colloidal gold and suspended in the sample pad. When the test serum and chase buffer are applied to the sample pad, the rISG65-colloidal gold conjugate is resuspended. The liquid is drawn across the nitrocellulose membrane towards the receiving sample pad at the top of the lateral flow device. During this time, anti-rISG65 antibody in the serum binds to the rISG65-gold conjugate and when the antibodies reach the rISG65 test band, one Fab arm of the IgG binds to the immobilised rISG65 while the other Fab domain bridges to

the rISG65-gold-conjugate. Accumulation of this specific antibody sandwich generates a visible test line. The control line is an internal positive control for the lateral flow test and does not relate to the infection status of the patient. The final reading of this test should be as follows, one band for non-infected sera and two bands for infected sera.

7.1.1. Optimisation

All optimisation experiments were carried out with the rISG65-1 lateral flow prototype, with the final conditions transferred for the sVSG117 lateral flow test.

7.1.1.1. Volume of sera

To assess the sensitivity and specificity of the lateral flow assay, different volumes (20 µl, 10 µl, 5 µl, 2.5 µl and 1.25 µl) of pooled *T. b. gambiense* infection sera (2^{nd} stage patients, n=40) and matched *T. b. gambiense* non-infection (control) sera (n=50) were made up to a final volume of 20 µl with PBS and applied to the sample pads. Chase buffer (80 µL) was subsequently applied to sample pads and the lateral flow tests were left for 30 min, the tests were photographed and scanned by a laser densitometer (Camag) (Figure 7.2).

The test line was still visible using 1.25 µl of pooled infection (2^{nd} stage) *T. b. gambiense* sera, indicating that the rISG65 lateral flow test is very sensitive. However, at higher serum concentrations, non-specific binding was observed for the non-infected patient pooled sera. Therefore, 5 µl of serum was used for all future lateral flow tests as it provided a good compromise between absolute sensitivity and signal to noise ratio.

7.1.1.2. Chase buffer optimisation

There are limited optimisation procedures that can be carried at this stage in the lab with the prototype device, as optimisation should really carried out by the manufacturer during product optimisation and feasibility stages. However, we performed some attempts to reduce the non-specific binding of the test line by the pooled control sera by testing different chase buffers. Aliquots of pooled control sera (5 µl) were added to the sample pads followed by 80 µl of either: (1) PBS, (2) PBS, 0.05 % Tween 20 (PBS-Tw20) or (3) PBS, 0.1 % BSA (PBS-BSA). The lateral flow test is principally a visual test so, to assess the background signal at the rISG65-1 test line, 25 people were asked to rank the intensity of the test band. Most people chose the lateral flow test with PBS, 0.05 % Tween 20 chase buffer as having the least signal at the test line. This subjective assessment was consistent with the Camag scan results which showed that PBS-BSA chase buffer increased the non-specific signal compared to PBS alone, while PBS-Tw20 reduced it (Figure 7.3). The different chase buffers had negligible effects on the development of the control line.

7.1.1.3. Chase buffer assessment with pooled infection sera

To check whether the new Tween 20-containing chase buffer compromised the positive test line signal with pooled *T. b. gambiense* infection sera, the PBS-Tw20 chase buffer experiment was repeated with the pooled *T. b. gambiense* infection and control sera. The presence of Tween 20 in the chase buffer did not significantly affect the positive or control test lines for the *T. b. gambiense* infection sera but it did improve the signal to noise ratio for the test (Figure 7.4).

7.1.2. Comparison of the rISG65-1 and sVSG117 lateral flow devices with individual sera using visual scoring

To prevent bias when interpreting test line intensities on the lateral flow tests, we used randomised sera. Eighty randomly selected serum samples representing 40 *T. b. gambiense* infected and 40 control sera from the validation sera set (6.1) were randomised and re-labelled by a member of the University of Dundee Tissue Bank. Serum aliquots (5 µl) were diluted with 15 µl PBS and applied to the sample pads of both rISG65-1 and sVSG117 lateral flow devices, followed by the addition of 80 µl PBS, 0.05 % Tween 20 and the test line intensities were ranked by eye. Visual scoring took place by comparing and ranking the test lines based on the interpretation by a single eyewitness. The scores were recorded and tests were decoded and grouped by infection status. After visual inspection and scoring, the device cases were broken open, the sample pads at both ends were discarded and the nitrocellulose strips containing the test line were mounted on squared paper for Camag scanning. The Camag scanning was important as it validated the visual interpretation. The visual scores and Camag scores were plotted against each other in a scatter plot to assess the relationship between the scores. It shows a strong correlation between the visual interpretation and the densitometer of rISG65-1 which had a R^2 value of 0.959 and sVSG117 that had a R^2 value of 0.957 (Figure 7.5).

The rISG65-1 lateral flow tests showed good sensitivity. Using a visual cut-off score of 2.5, all of the *T. b. gambiense* infection sera were deemed positive (Figure 7.6 A). However, five control sera gave high (≥4) or borderline (≥3) scores, suggesting a weakness in the specificity of the rISG65 lateral flow assay. On the other hand, using a visual cut-off score of 1, the sVSG117 test showed excellent sensitivity and specificity

(100 %) with all control sera having a low signal (≤ 0.5) and all *T. b. gambiense* infection sera producing test line intensities ≥1 (Figure 7.6 B). By adding the rISG65-1 and sVSG117 visual scores and using a combined cut-off of 6, the combined data also gave 100% sensitivity and selectivity (Figure 7.6 C).

7.1.3. Comparison of the rISG65 and sVSG117 lateral flow devices with individual sera using densitometer scoring

Following visual assessment of the test lines on the lateral flow devices, the test and control lines were measured using Camag laser, a densitometer, offering less subjective analyses of the line intensities (Figure 7.7). For the rISG65-1 devices, using a cut-off of 255 AU, two of the five false-positive the tests in (Figure. 7.6 A) were resolved providing 100 % sensitivity and 92.5 % specificity. Using a cut-off score of 110 AU, the sVSG117 gave a single false-negative result or using a cut-off of 75 AU, a single false-positive result. By adding together the rISG65-1 and sVSG117 scores and using a combined cut-off of 450 AU, the combined data give only a single false-positive result, suggesting that a combined antigen test of some sort might improve specificity.

The mean densitometry scores for the positive sera (Table 7.1) were lower for the sVSG117 device than the rISG65-1 device, despite the fact that these same sera gave higher titres against sVSG117 than rISG65-1 ELISA plates (6.6.2). In addition, the sVSG117 test line appears to be wider and less uniform than the rISG65-1 test line (Figure 7.8). This is likely to be a manufacturing issue, since the antigen application conditions (*e.g.*, antigen concentration and application buffer), have not yet been optimised this far.

7.1.4. Comparison of lateral flow and ELISA methods

Signal-to-nosie ratios were calculated for the ELISA mean values and lateral flow densitomery scores for infected and control sera (Table 7.1). Despite the isssues with the unoptimised application of the sVSG117 test lines we found that sVSG117 out performed rISG65-1 in both tests. The mean infection signal >10 times the mean control signal for sVSG117 by ELISA and lateral flow, compared to >4 times the difference between the mean values for infected and control signals. One possible explanation for some of the variation between the ELISA and lateral flow device data may be that the former was set up to detect only IgG responses to the antigens whereas the latter will detect any antibody subtype (*e.g.* IgG and IgM) to the antigens. While the results described here suggest that sVSG117 is a good antigen for the diagnosis of *T. b. gambiense* infections, the fact that it is a variant surface glycoprotein suggests to us that it is not suitable on its own because VSG genes can be lost from telomeric expression sites during duplicative transpostion events in antigenic variation.

7.2. Preliminary dual band (rISG65-1 plus sVSG117) lateral flow prototype test results

A limited number (n=16) of dual test line lateral flow devices were manufactured, in order to carry out a proof-of-concept experiment using pooled *T. b. gambiense* and *T. b. rhodesiense* sera with matched pooled controls. As expected, the *T. b. gambiense* infection pooled sera resulted in 3 lines, corresponding to (bottom to top) the rISG65 test line, the sVSG117 test line and the control line (Figure 7.9). However, the *T. b. rhodesiense* infection pooled sera only resulted in 2 lines, the rISG65 test line and the control line (Figure 7.9). This may be due to *T. b. rhodesiense* not expressing VSG117,

so that patients do not have antibodies against this VSG. While this could be seen as a weakness, it also opens up the potential for a dual test line diagnostic that can not only diagnose *T. b. gambiense* and *T. b. rhodesiense* infections but also differentiate between the trypanosome sub-species in geographical areas where the trypanosome species overlap. However, considerably more *T. b. rhodesiense* infection sera need to be screened before the potential for anti-sVSG117 immunoreactivity in differential diagnosis can be assessed.

7.3. Preliminary single (rISG65-1) and dual band (rISG65-1 plus sVSG117) lateral flow prototype test results with bovine sera

Because the *T. congolense* genome contains genes with reasonable homology (up to 46% sequence similarity) to the *T. brucei* ISG65 gene family, aliquots of pooled high-virulence (n=5) and low-virulence (n=8) (Savannah strain) *T. congolense* infection and control bovine sera (n=3) were applied to the rISG65-1 single lateral flow assays. Aliquots of pooled *T. congolense* infection and control (n=5 for each) and pooled *T. vivax* infection and control bovine sera were applied to the dual band (rISG65-1 and sVSG117) lateral flow assays. The serum aliquots were mixed with 10 µl PBS before application to the sample pads and 80 µl of PBS-Tween 20 chase buffer were used.

The results from the single rISG65 test line suggested cross-reactivity of the *T. congolense* infection sera to the *T. brucei* rISG65 protein (Figure 7.10). However, the test line signal was also rather high for the control sera. Furthermore, the double test lines lateral flow devices showed cross-reactivity of the *T. vivax* and *T. congolense* infection sera to the *T. brucei* rISG65 protein (Figure 7.11). However the *T. congolense* non-infection (control) signal for the rISG65 test line was also high. The densitometer

readings suggest that for *T. vivax* and *T. congolense* infection and control sera there was a small amount of immune-reactivity for the *T. brucei* sVSG117 test line (Figure 7.11). From these very preliminary data, we conclude that we need to screen a much larger number of *T. congolense* infection and control sera and optimise the chase buffer to reduce non-specific binding to the rISG65-1 test line before we can assess the potential of rISG65-1 to detect *T. congolense* infections in cattle. Indeed, it may be better to select potential diagnostic antigens by unbiased proteomics methods to move towards a bespoke test for *T. congolense* cattle infections.

8. Results II: Identification and preliminary assessment of carbohydrate antigens

The African trypanosomes synthesise complex cell-surface glycoconjugates, several of which are essential to parasite survival and/or infectivity. Examples for the infective bloodstream form of *T. brucei* include the variant surface glycoprotein (VSG), the ESAG6/ESAG7 heterodimeric transferrin receptor (Mehlert *et al.*, 2012, Mehlert and Ferguson, 2007, Cross, 1996, Pays and Nolan, 1998, Steverding *et al.*, 1995); the p67 lysosomal glycoprotein (Peck *et al.*, 2008) and the membrane-bound histidine acid phosphatase *Tb*MBAP1 (Engstler *et al.*, 2005). In addition, bloodstream form *T.brucei* synthesise a family of extremely large and complex poly-N-acetlyllactosamine (poly-LacNAc) containing N-linked glycans that occur in the flagellar pocket and throughout the endosomal/lysosmal system (although the identities of the proteins that carry them is still unknown) (Atrih *et al.*, 2005). While most of the N-linked glycan structures made by the parasite are identical or similar to those that occur in the mammalian host (Izquierdo *et al.*, 2009b, Izquierdo *et al.*, 2009a), and are therefore unlikely to elicit an immune response, the giant poly-LacNAc N-linked glycans are extremely unusual. Further, we do not yet know the complete repertoire of African trypanosome glycan structures, for example novel O-linked and/or phosphate-linked glycans may exist in *T. brucei,* as they do in the related *T. cruzi* and leishmania parasites (de Macedo *et al.*, 2010, Mendonça-Previato *et al.*, 2005). We therefore decided to look for evidence of anti-carbohydrate antibodies in infected sera.

8.1. Glyco-array studies

A glyco-array is similar to a DNA microarray except that the spots carry specific glycan structures and instead of DNA sequences. Such a glyco-array has been created by The Consortium for Functional Glycomics (CFG), and it displays 456 different glycan structures. We sent the same IgG fractions pooled from infection and control sera that we used in for the proteomics-based antigen identification experiments described in Chapter 4 to the CFG. The glyco-arrays were performed as six technical replicates, on two separate occasions with different aliquots of the pooled infected (n=4) and matched control (n=4) antibodies. The data were provided to us as Excel files and the immunoreactivity (mean ± 1 standard deviation) of control IgG were taken away from the infection IgG signals and show infection and control specific glycans (Figure 8.1).
On initial inspection, the infection IgG appeared to have very low titres to most of the carbohydrate structures compared to the control IgG. However, we surmised that the control IgG had a particularly high titre against the anti-blood group A antigen (Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-3Gal). The data were therefore normalised by simply dividing the infection sera signals by the corresponding control sera signals and those with values over 5 were selected for further study (Figure 8.2).

Of the ten glycan structures selected in this way, nine (90%) contained at least one Galβ1-4GlcNAc (LacNAc) substructure. This represented a significant enrichment of LacNAc-containing structures specifically recognised by infection IgG over those on the CFG V4.1 glyco-array, where 156 glycan out of 461 glycans (34%) contained Galβ1-4GlcNAc in the core structure or in a side chain. This result stimulated our interest in assessing whether the unusual giant poly-LacNAc N-linked glycan structure

in *T. brucei* might be eliciting an immune response that cross reacts with some of the LacNAc-containing structures available on the glyco-array.

An unusual (and therefore potentially immunogenic feature) of bloodstream form *T. brucei* poly-LacNAc structures is that the inter-LacNAc linkage is predominantly β1-6 (as in -6Galβ1-4GlcNAc**β1-6**Galβ1-4GlcNAcβ1-) rather than the more common β1-3 linkage (as in -6Galβ1-4GlcNAc**β1-3**Galβ1-4GlcNAcβ1-) found in mammals (Atrih *et al*., 2005). To test the hypothesis that these unusual β1-6 poly-LacNAc structures of *T. brucei* might be responsible for anti-carbohydrate antibody responses in the infected host, we commissioned the chemical synthesis of β1-6 linked poly-LacNAc structures by our collaborators, Dr Andrei Nikolaev and Dmitry Yashunsky of the University of Dundee and Moscow.

8.2. Poly-LacNAc structures and ELISA development

Synthetic LacNAc structures (inter-linked by β1-6 glycosidic bonds) and linked to a biotin moiety at the reducing terminus by a spacer group were provided with 2, 3 4, and 5 LacNAc repeats (Figure 8.3). The biotin group was included to facilitate anchoring the LacNAc structures, via NeutrAvidin^{TM}, to the ELISA plates.

Unlike in the protein ELISAs described previously, the LacNAc ELISAs did not use the two-step biotinylated anti-human IgG followed by HRP-conjugated NeutrAvidin detection system because of the biotin present in the synthetic carbohydrate antigens. In this case, bound antibodies were detected with HRP conjugated directly to the anti-human Ig secondary antibodies. The ELISA system was optimised by varying plate

antigen concentrations, sera dilutions, secondary antibody dilutions and the chemiluminescence substrate solution.

8.3. Immobilised poly-LacNAc ELISA screens with pooled sera

Pooled *T. b. gambiense* and *T. b. rhodesiense* infection and control sera were diluted to 1:500 in PBS 0.1% BSA and applied in triplicate to NeutrAvidin coated plates which had been loaded with the synthetic biotinylated LacNAc repeat structures (3.9.3). The validation set of pooled *T. b. gambiense* 1st and 2nd stage infection sera showed discriminating anti-LacNAc antibody titres with the $LacNAc_4$ and $LacNAc_5$ structures compared to control sera (Figure 8.4, A). The pooled *T. b. rhodesiense* infection sera showed no significant reactivity to the synthetic LacNAc structures (Figure 8.4, B). The control *T. b. rhodesiense* sera pool reacted with NeutrAvidin (biotin) plate control suggesting there are antibodies directed towards the NeutrAvidin carrier protein.

Based on these results, we decided to investigate the diagnostic potential of the synthetic $LacNAc_5$ structure for *T. b. gambiense* infection.

8.4. Screening of the $LacNAc_5$ structure with individual *T. b. gambiense* infection and control sera.

The next step was to screen, in triplicate, the $LacNAc_5$-biotin-NeutrAvidin plates with individual patients' sera (Figure 8.5). Bound antibodies were detected with HRP conjugated to anti-human IgG+IgM+IgA, anti-human IgG or anti-human IgM. The ELISA results (mean ± 1 standard deviation) are shown in (Figure 8.5). These results suggest that the total (IgG+IgA+IgM) antibody response to the $LacNAc_5$ structure is significantly higher in infected patients. Interestingly, the data using specific IgG

detection is less impressive whereas the IgM response appears to be more robust. This trend can be seen more clearly is the associated box plots shown in Figure 8.5, (D-F).

As inferred by the box plots the Dunn's Post Hoc test supports the observation that 1st (Q=4.004, P<0.05) and 2nd stage (Q=8.429, P<0.05) sera contain statistically significant higher IgM to the lacNAc5 plate compared to the control sera (Table 8.1). There was no statistical significance between 1st stage and 2nd stage groups for any antibody response to LacNAc5.

Table 8.1: Statistical results for *T. b. gambiense* LacNAc5 ELISA data.
Statistical significance determined by Mann-Whitney Rank test or Dunn's Post-Hoc rank test with Q values and Probability (P) values shown, statistical significance is shown in bold (P=<0.05).

Antibody response to LacNAc5	Mann-Whitney	Dunn's Post-Hoc		
	Infection vs. Control	1st stage vs. 2nd stage	1st stage vs. Control	2nd stage vs. Control
IgG, IgA, IgM	**P=<0.001**	Q=0.735 P>0.05	**Q=3.738** **P<0.05**	**Q=7.911** **P<0.05**
IgG	**P=<0.001**	Q=0.475 P>0.05	**Q=3.727** **P<0.05**	**Q=5.726** **P<0.05**
IgM	**P=<0.001**	Q=0.762 P>0.05	**Q=4.004** **P<0.05**	**Q=8.429** **P<0.05**

The same ELISA data from Figure 8.5 was also analysed by Receiver-operator characteristics (ROC) curve (Figure 8.6) to assess whether infection sera can be discriminated based on antibody response to LacNAc5 plates. The IgM response in *T. b. gambiense* infection sera to the $LacNAc_5$ glycan has an area under the ROC curve (AUC) of 0.93 whereas the IgG response had an AUC 0.83. The antibody class responses measured here showed infection discrimination between infection and control groups (Figure 8.6, B) were statistically significant (P<0.001; Mann-Whitney rank test) (Table 8.1).

8.5. Re-analysis of the diagnostic potential of $LacNAc_5$ for *T. b. rhodesiense* infection.

Based on the results with the *T. b. gambiense* sera, we decided to screen the individual *T. b. rhodesiense* infection and control against the $LacNAc_5$-biotin-NeutrAvidin plates. The raw ELISA data show an individual (CR41) who has a high IgG specific anti-NeutrAvidin response, which is not amplified by anti-human-IgM-HRP secondary antibody (Figure 8.7, A-C). The IgM response to lacNAc5 shows an infection-specific basis as expressed by the box plots (Figure 8.7, D-F), where only the 2nd stage infected group (Q=4.876, P<0.05, Mann-Whitney Rank (MWR) test) was statistically significant from control (Table 8.2). The only other statistical significant difference between infection and control sera was the IgG, IgM, IgA ELISA comparing 2nd stage infected group (Q=3.575, P<0.05, MWR) with control group.

Table 8.2: Statistical results for *T. b. rhodesiense* LacNAc5 ELISA data.
Statistical significance determined by Mann-Whitney Rank test or Dunn's Post-Hoc rank test with Q values and Probability (P) values shown, statistical significance is shown in bold (P=<0.05).

Antibody response to LacNAc5	Mann-Whitney	Dunn's Post-Hoc		
	Infection vs. Control	1st stage vs. 2nd stage	1st stage vs. Control	2nd stage vs. Control
IgG, IgA, IgM	**P=0.002**	Q=1.919 P>0.05	Q=0.343 P>0.05	**Q=3.575** **P<0.05**
IgG	P=0.118	Not a statistically significant difference (P=0.279)		
IgM	**P=<0.001**	Q=0.891 P>0.05	Q=2.193 P>0.05	**Q=4.876** **P<0.05**

The same ELISA data from Figure 8.7 was also analysed by Receiver-operator characteristics (ROC) curve (Fig. 8.8) to assess whether infection sera can be

discriminated based on antibody response to LacNAc5 plates. The IgM response in *T. b. rhodesiense* infection sera to the $LacNAc_5$ glycan has an area under the ROC curve (AUC) of 0.92 whereas the IgG response had an AUC 0.64. The IgM antibody response to LacNAc5 between infection and control groups (Figure 8.8, B) was statistically significant ($P<0.001$; Mann-Whitney rank test) (Table 8.2).

8.6. Summary of findings. Evaluation and comparison of *T. b. gambiense* and *T. b. rhodesiense* anti-LacNAc-5 antibody response.

This preliminary data suggest there are specific antibody reactions to the biotinylated synthetic $LacNAc_5$ structure found in both *T. b. gambiense* and *T. b. rhodesiense* infected patients. The IgM anti-$LacNAc_5$ was found to be higher in infected patients compared with controls, and based on these results, $LacNAc_5$ could be considered a potential diagnostic antigen for both *T. b. gambiense* and *T. b. rhodesiense* infections (Table 8.3).

Table 8.3: Sensitivity and specificity scores for both *T. b. gambiense* (shaded) and *T. b. rhodesiense* (non-shaded) from ELISA experiments detecting different antibody classes from the serum (IgG+IgA+IgM, IgG and IgM) to the $LacNAc_5$ plate antigen. For *T. b. rhodesiense* data, R047 patient's results are not included in statistical analyses.

Sera	Antibody class	Cut off >	Sensitivity (%)	95% CI	Specificity (%)	95% CI
T. b. gambiense	IgG+IgA+IgM	2628000	86.4	75 to 94	86.2	74.6 to 93.9
T. b. rhodesiense	IgG+IgA+IgM	1073000	75	53.3 to 90.2	75	50.9 to 91.3
T. b. gambiense	IgG	1307000	74.6	61.6 to 85	74.1	61 to 84.7
T. b. rhodesiense	IgG	274807	62.5	40.6 to 81.2	75	50.9 to 91.3
T. b. gambiense	IgM	7553000	93.2	83.5 to 98.1	86.2	74.6 to 93.9
T. b. rhodesiense	IgM	3578000	87.5	67.6 to 97.3	80	56.3 to 94.3

In comparison with the most promising protein antigen candidate for *T. b. rhodesiense* infections, rISG64-3, which had sensitivity and specificity scores of 92 % and 85 %, the $LacNAc_5$ antigen (IgM) scored 87.5 % and 80 %, respectively. Therefore further optimisation and validation needs to be carried out to fully assess the diagnostic potential for $LacNAc_5$ antigen. Further studies would include quantifying total IgM concentration in all sera, to see if these results are due to a higher overall IgM rather than specific anti-$LacNAc_5$ antibodies.

9. Discussion

The overall goal of this project was to develop a field-based diagnostic assay (lateral flow test) that could replace the CATT screening tool. The specific aims of this project were, i) to identify new potential diagnostic candidates for HAT, ii) to investigate if the candidates could be solubly expressed and purified, and, finally iii) to assess their diagnostic potential with patients sera. We took an un-biased approach to identify potential diagnostic biomarkers, and identified several cell surface glycoproteins such as the ISGs, ESAG 6 and 7, ESAG 2 and ESAG 11. A selection of these were then cloned, recombinantly expressed and purified from *E. coli*. These expressed proteins were assessed by ELISA with serum from *T. b. gambiense* and *T. b. rhodesiense* infected and control individuals and rISG65-1 and sVSG117 were selected to be developed into lateral flow assays for re-testing with randomised sera. Based on this initial assessment the ELISA and lateral flow prototypes match the CATT sensitivity and surpass the specificity scores (Table 9.1)(Truc *et al*., 2002a).

Table 9.1: Sensitivity and specificity of CATT, rISG65-1 and sVSG117 ELISAs and lateral flow scores. CATT(1) values from (Truc *et al*., 2002a).

Test/Antigen	Sensitivity (95 % CI)	Specificity (95% CI)
CATT	**87-98 %[1]**	**93-95 %[1]**
rISG65 ELISA	97 % (88 to 100%)	93 % (83.5 to 98%)
sVSG117 ELISA	100 % (93.9 to 100%)	100 % (93.9 to 100%)
rISG65 lateral flow (visual score)	88 % (73 to 96%)	93 % (79.6 to 98%)
sVSG117 lateral flow (visual score)	100 % (91 to 100%)	100 % (91 to 100%)
rISG65 lateral flow (Camag score)	100 % (91 to 100%)	93 % (79.6 to 98%)
sVSG117 lateral flow (Camag score)	97.5 % (86.8 to 99.9)	97.5 % (86.8 to 99.9)

9.1. Immunoprecipitation and the selection criteria

Identification of new potential diagnostic markers was necessary due to limited options of potential diagnostic markers that could be used in an lateral flow assay (Manful *et al.*, 2010, van Nieuwenhove *et al.*, 2011, Radwanska *et al.*, 2000). In our study potential diagnostic biomarkers were identified by proteomic analysis of proteins that adsorbed to IgG columns derived from either infected or uninfected pools of patient sera. Many of the proteins identified were common to both infected and uninfected control elutions. Some of the antigens found in both samples might be specifically recognised by normal human IgG through, for example, cross-reacting antigens from other infections, while others might bind to IgG-Sepharose non-specifically. Heat Shock Protein 70 (HSP70) was identified by both *T. b. gambiense* infected and control patients' IgG with high MASCOT scores of 2325 and 2194, respectively, suggesting it is a poor diagnostic candidate (Appendix Table A1 and A2). Heat shock protein (HSP) 70 has been proposed to be potential diagnostic candidate in cattle infections (Bossard *et al*., 2010) and for *T. b. rhodesiense* infections in man (Manful *et al.*, 2010), although both studies acknowledged limitations in using HSP70 exclusively. Soluble VSG117 was identified by both *T. b. gambiense* and control patients IgG with MASCOT scores of 2271 and 859, suggesting limited potential as a diagnostic marker (Appendix Table A1 and A2). However, the ELISA and lateral flow data show this is not the case. A potential explanation for the presence of sVSG117 in the control elution may be due to the high abundance of VSG which may have non-specifically bound to the control IgG column and was not washed away before elution. Another explanation may be that the antibodies may have recognised the CRD rather than the protein sequence. VSG117 has been found to be expressed by other trypanosome species, such as, *T. evansi* (Jia *et al.*,

2012) which infects camels, horses, cattle and buffaloes, so there may be potential for the *T. b. brucei* sVSG117 to be utilised for diagnosis in these infections.

While our approach succeeded in the identification of novel biomarkers there were limitations. A major limit was caused by the decision to use only four patients' sera in the pools for the infection and non-infection IgG columns. Ideally a pool of many more patients' IgG would be preferable as this may allow for more biomarkers. However this was avoided due to the uncertain provenance of the serum used. Alternatively, immunoprecipitation with each individual patient's IgG could have been done and the proteins identified for each could allow for common antigens to be identified, however this would be very resource intensive and the amount of sera available was limited. Another limitation is the fact that *T. b. brucei* lysate was used rather than the infection relevant strains *T. b. gambiense* and *T. b. rhodesiense,* which, at the time was not available for use as we did not have clonal isolates. Use of these lysates would have been more appropriate and potentially may have allowed for additional strain specific biomarkers.

9.2. Recombinant or native biomarkers?

Recombinant expression is an efficient method for obtaining large quantities of diagnostic antigen, providing the antigen can be solubly expressed and purified. The recombinant proteins were selected based on their sequence similarity to the ISG and GRESAG4 sequences identified in the immunoprecipitation and therefore will contain antibody epitopes present during an infection. However, there were difficulties in the expression and purification of ISG75 and GRESAG4. The problems with ISG75 are likely to be due to the multiple disulphide bonds, which need to be correctly aligned for correct folding (Ziegelbauer *et al.,* 1992). In the trypanosome, ISG proteins are

translated in the Endoplasmic reticulum, which is a highly oxidative environment containing chaperone and other proteins which facilitate the correct tertiary structure of proteins containing disulphide bonds and remove incorrectly folded proteins. Future experiments would include utilising bacterial expression systems that target recombinant proteins into the periplasmic space, this could be useful as recombinant proteins are processed through the secretory system (Arredondo and Georgiou, 2011). This strategy could also be employed for recombinant GRESAG proteins. Re-assessment of ISG75 and GRESAG proteins should be carried out once expression and purification conditions have been further optimised, as their poor performance in ELISA may be due to incorrect folding of the proteins.

Recombinant and synthetic antigens, such as rISG65-1 and biotinylated-$LacNAc_5$ (IgM) antigens have very good diagnostic potential as shown by the ELISA data. Analysis of this ELISA data by ROC curve showed AUC of 0.99 for rISG65-1 and AUC of 0.96 for LacNAc5 (IgM) indicating discrimination between *T. b. gambiense* infection and control sera. For *T. b. rhodesiense* individuals the analysis of ELISA data for rISG65-1 gave an AUC of 0.84 and LacNAc5 (IgM) antigen an AUC of 0.92, suggesting these antigens are able to discriminate between infection and control sera. The native sVSG117 had greater diagnostic potential for the *T. b. gambiense* infection (defined by ELISA) with sera showing an area under ROC curve score of 1. The sVSGs can be relatively easily purified from total *T. brucei* lysate and there is no concern about contaminating *E. coli* proteins and correct folding of the protein. In addition, the native sVSG contains N-linked glycan structures and the CRD, which can be the sites of many antibody epitopes, in particular at the interface between glycan and peptide sequence (Galanina *et al.*, 2003). There are many examples of the use of either recombinant and

native antigens, to which each have limitations (Akyar *et al.*, 2010, Abdo *et al*., 2010, Martínez-Sernández *et al*., 2011, El-Moamly *et al*., 2011, Widmer *et al.*, 1999), however a test that combines native and recombinant antigens may improve diagnostic outcomes (Busse *et al*., 2008).

9.3. Validation for diagnostic assays

As a validation assay the ELISA worked very well. The sensitivity of the ELISA matched the sensitivity of the lateral flow assays (Table 9.1). ELISA does have limitations, as it is limited by which class of antibody amplified, as shown in the LacNAc ELISA, where patients had more IgM anti-LacNAc antibodies than IgG. A new validation approach is being investigated is not limited by which antibody class. This relies on the AlphaLISA technology where antigens, coupled to donor and acceptor beads, are brought into close proximity with each other by any antibodies in the sera, the beads are then excited by a specific wavelength where emission signals allows for quantification (Bielefeld-Sevigny, 2009, Modi *et al.*, 2011). Alternatively, validation of prospective diagnostic antigen should be assessed by lateral flow principles, for instance, using dot blots with colloidal gold conjugated antigen to amplify the signal, rather than conventional secondary and tertiary antibody amplification. This could better assess the potential of new antigens by not taking account of which antibodies are responsible in the first assessment.

The prototype lateral flow tests in their present state are not suitable for use in the field as they require further optimisation of the test antigen, the addition of a blood pad, and rigorous validation against a greater number of sera. Further research is also required to measure the half-life of antibodies in patients after they have been treated for HAT, as

persistent antibodies may lead to false positives. Lateral flow tests, whilst having limitations would potentially be more suitable for use in the field because of their stability and the fact that they can be used by non-specialists (Bandla *et al.*, 2011). In addition the lateral flow format is versatile, in that the HAT test lines could be could be incorporated into a test which also screens for other diseases such as malaria, which often co-exists in HAT endemic areas (Bell and Perkins, 2008).

9.4. Sera

This work is only as good as the resources available and the WHO HAT sera were well characterised. Further validation of the antigens with sera from non-endemic regions may be necessary, such as the well characterised sera from the TB sera bank (Nathanson *et al.*, 2010) and other organisations holding patient's sera. The false positives observed in the lateral flow studies may be due to cross-reacting antibodies, a previous infection or an undiagnosed self-curing infection (Jamonneau *et al.*, 2012). It has been described that antibodies can persist up to 3 years post cure, however it is not known which class of antibodies persist or which antigens they recognise (Paquet *et al*., 1992). A longitudinal study should be carried out to gain a greater insight into this and how it could affect the diagnostic potential of any future lateral flow test relying on antibodies. Also the lateral flow assay and other recombinant antigens described here, should be investigated with patients who tested positive with the Tryanolysis Test (TL) (Jamonneau *et al*., 2010). This test which is similar to the CATT and uses crude preparations of total trypanosome suspensions which may reveal any cross-reactive antigens. In addition, a longitudinal study may allow for the identification of antigens that are recognised by active infections and those antigens that induce longer lasting immunological memory (Jamonneau *et al*., 2012, Ilboudo *et al*., 2011) .

Due to the limitations of parasite identification in infected blood, other serodiagnostic methods are required, with each test having limitations and advantages. Antibody based techniques are very sensitive and specific, however the current field screening tool (CATT) lacks assay sensitivity, compared to ELISAs. There is also the issue of antibody detection that is not suitable for checking efficacy of treatments due to persistent circulating anti-trypanosome antibodies (Paquet *et al.*, 1992, Radwanska, 2010). On the other hand, many methods to detect parasite derived products, such as PCR based methods and centrifuge methods, are not suitable for the field, due to the requirement of centrifuges, PCR cyclers, electricity for storage of reagents and other lab based equipment and trained personnel (Deborggraeve and Büscher, 2010).

There were differences in the infection profiles between the trypanosome sub species, the antibody responses to different biomarkers depending on the infection species. This may be attributed to the differing nature of the infections; *T. b. rhodesiense* infection has a faster progression which may not allow enough time for antibody production in the host. Whereas *T. b. gambiense* infected patients may have had months if not years of exposure to the parasite and this could in turn lead to a greater diversity of antibodies present in their sera.

9.5. Staging

The staging of the disease is currently problematic and is still necessary until a new drug that is safe and active in both stages of the disease, in that instance staging will not be so crucial (Bisser and Courtioux, 2012). However it is not known when a new drug will be discovered so diagnostic tests that can reliably identify infected patients are needed (Matovu *et al.*, 2012). There are precedents in the literature for combining diagnosis of innate and adaptive immune response to infections, *e.g.* in mycobacterial

diseases where IL-10 levels and anti-phenolic glycolipid (PGL-I) antibodies can be simultaneously detected (Corstjens *et al.*, 2011). In HAT this may be possible for markers such as matrix metalloproteinase-9, CKCL10 or anti-galactocerebroside antibodies (Courtioux *et al.*, 2005, Hainard *et al.*, 2009, Hainard *et al.*, 2011), however these all still require CSF sampling.

9.6. Summary

An ideal scenario would be to have complementary diagnostic tests, drawing on the strengths of each approach. The use of a more sensitive antibody screening tool to identify suspected patients, followed by parasite confirmation either with microscopy, LAMP or PCR methods maybe possible. This strategy could also be used when checking for the efficacy of treatment. The CATT screening tool needs to be updated and lateral flow tests could provide a new platform for the diagnosis of HAT. Hopefully the work carried out here can help further the progress of developing a field compatible diagnostic tool. Ultimately, this tool would be used to identify patients infected with Trypanosome infection enabling them to be treated, thus saving and improving the lives of those affected by HAT.

Appendix 1: Detailed results from the second immunoprecipitation and proteomic experiments described in Chapter 4.

Table A1: *T. b. brucei* proteins identified in the infection-IgG elution. Proteins ordered in ascending MASCOT score.

Description	Identifier	MW	MASCOT score	number of peptides matched
75kDa Invariant surface glycoprotein	Uniref100_Q26714	58661	2584	97
ALD fructose-bisphosphate aldolase, glycosomal	Tb10.70.1370	41045	2490	81
HK1 hexokinase	Tb10.70.5820	51262	2346	62
heat shock protein 70	Tb11.01.3110	75320	2325	72
Variant surface glycoprotein MITAT 1.4A precursor	UniRef100_P02896	56282	2271	88
glucose-regulated protein 78	Tb11.02.5450	71391	1750	50
Receptor-type adenylate cyclase GRESAG 4	Tb927.7.7530	137920	1456	67
ESAG 7	Tb427 telo10 v1 145	38433	1152	31
65kDa Invariant surface glycoprotein	Uniref100_Q26712	48192	1098	39
gPGK phosphoglycerate kinase	Tb927.1.700	47558	1026	37
Phosphoglycerate kinase, glycosomal	uniref100_P07378	47431	997	35
Glycosomal membrane protein	uniref100_O60944	24210	936	29
chaperone protein DnaJ	Tb927.2.5160	44786	832	23
heat shock protein 83	Tb10.26.1080	80712	821	26
Hypothetical protein	Tb427_telo10_v1_145	38889	811	28
GAPDH glyceraldehyde 3-phosphate dehydrogenase, glycosomal	Tb06.26G9.1050	43841	772	23
hypothetical protein	Tb927.4.1300	41988	765	26
TEF1 elongation factor 1-alpha	Tb10.70.5670	49474	754	33
Hypothetical protein	Tb427_telo56_all_533	38611	739	19
hypothetical protein	Tb09.160.5530	37993	737	19
Hypothetical protein	Tb427_telo15_all_453	39034	729	16
AK arginine kinase	Tb09.160.4570	41571	708	27
RNA-binding protein, putative	Tb927.8.6440	20529	664	16
ATP synthase F1, beta subunit	Tb927.3.1380	55741	645	16
TIM triosephosphate isomerase	Tb11.02.3210	26973	640	23
2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase	Tb11.01.3550	41516	601	21
64 kDa invariant surface glycoprotein	Tb927.5.1410	46867	582	23
gim5B Gim5B protein; glycosomal membrane protein	Tb09.211.2740	26529	524	23
DRBD3 RNA-binding protein	Tb09.211.0560	36961	519	19
glucose-regulated protein 78/luminal binding protein 1 (BiP)	Tb11.02.5450	71505	519	15
Polyubiquitin	Tb11.01.1680	76556	510	10

kinetoplastid membrane protein KMP-11	Tb09.211.4511	11069	489	23
protein disulfide isomerase	Tb927.7.1300	41913	445	16
dynein light chain	Tb11.50.0007	10422	422	9
alpha tubulin	Tb927.1.2340	50383	418	17
AOX; TAO alternative oxidase	Tb10.6k15.3640	37738	408	23
(H+)-ATPase G subunit	Tb927.8.2310	12741	393	9
hypothetical protein	Tb927.6.3020	32198	368	8
RPS12 40S ribosomal protein S12	Tb10.6k15.2050	16279	319	8
Hypothetical protein	Tb11.02.0010/ uniref100_Q386R9	24706	301	7
hypothetical protein	Tb09.160.5200	32552	288	10
14-3-3-like protein	Tb11.01.1290	30291	282	10
tGLP1 Golgi/lysosome glycoprotein 1	Tb927.8.1870	67555	274	8
variant surface glycoprotein (VSG)-related	Tb11.02.1566	40752	270	7
Hypothetical protein	Tb10.61.2850/ Uniref100_Q388J6	50899	270	9
Hypothetical protein	Tb10.70.1130/ Uniref100_Q38B23	48342	269	6
ESAG6 and hypothetical protein	Uniref100_Q8WPU1	44221	256	10
Hypothetical protein	Tb427_telo51 (&59)_v2_533	44959	256	10
hypoxanthine-guanine phosphoribosyltransferase	Tb10.70.6660	26515	248	8
LA La protein; RNA-binding protein	Tb10.70.5360	37636	243	8
Hypothetical protein	Tb427_telo15 (&4)_all_528(&157)	45097	241	9
Hypothetical protein	Tb427_telo10_v1_217	45096	241	14
expression site-associated gene 2 (ESAG2) protein	Tb927.1.4890	53686	235	7
PEX14 peroxin 14	Tb10.100.0130	39970	226	8
Flagellar calcium-binding protein TB-17	UniRef100_P17882	25477	220	6
Vacuolar ATP synthase subunit B	Tb11.01.3560	55927	220	5
hypothetical protein	Tb927.6.4440	37695	218	6
Hypothetical protein	Tb427_telo_v1_217	45096	214	10
Hsc70-interacting protein (Hip),	Tb927.3.5340	42173	211	5
Hypothetical protein	Tb11.01.0210/ Uniref100_Q386P9	51028	209	3
Hypothetical protein	Tb927.7.2120	46341	202	6
60S ribosomal protein L18a	Tb10.70.3510	21119	198	6
Hypothetical protein	Tb927.6.4440/ uniref100_Q587B0	37923	198	6
hypothetical protein	Tb927.7.180	49781	197	5
GTP binding protein	Tb927.2.5060	44664	185	6
PRS phosphoribosylpyrophosphate synthetase,	Tb10.6k15.0970	40452	185	7
histone H4	Tb927.5.4170	11135	184	4

proteasome alpha 3 subunit			179	
elongation factor 1 gamma	Tb11.01.4660	46274	178	6
adenylosuccinate synthetase	Tb11.02.1120	67317	167	8
TbL18LP 60S ribosomal protein L18			164	
hypothetical protein	Tb927.4.2030	22691	159	7
ESAG11	Tb927.1.4900/ Tb42_telo126_all_1147/ Uniref100_Q4GY70	32032	156	5
PRS phosphoribosylpyrophosphate synthetase,	Tb927.5.2960	42342	156	4
Hypothetical protein	Tb427_telo10_v1_217	45096	156	6
ESAG2	Tb11.01.6230	56132	150	5
eukaryotic translation initiation factor 5, putative	Tb10.70.4880	43431	144	2
hypothetical protein	Tb11.02.2030	12750	143	3
Hypothetical protein	Tb927.6.4180	16317	140	2
HSP60 chaperonin	Tb10.70.0430	59751	139	4
TRYP2 tryparedoxin peroxidase			136	
calpain-like protein fragment	Tb927.1.2260	14021	135	4
thioredoxin	Tb927.4.2450	44748	132	4
hypothetical protein	Tb11.01.7070	23157	132	3
NDPK nucleoside diphosphate kinase			130	
LPG3/heat shock protein 90/glucose regulated protein 94	Tb927.3.3580	87712	128	3
nucleoside hydrolase	Tb927.7.4570	39366	121	4
TCP-1-theta t-complex protein 1	Tb10.6k15.2330	58501	118	2
hypothetical protein	Tb927.3.1300	46058	115	2
Hypothetical protein	Tb927.6.3020	32369	115	3
Glycerol kinase	Tb09.211.3540	57041	113	6
hypothetical protein	Tb927.4.2030	22691	111	2
NHP2 ribosomal protein S6	Tb09.160.3670	13563	109	2
Hypothetical protein	Tb927.6.2860	49790	109	3
lysosomal/endosomal membrane protein p67	Tb927.5.1810	72686	104	2
Hypothetical protein	Tb427_telo15_all_954	428587	102	4
PYK1 pyruvate kinase 1	Tb10.61.2680	39170	100	4
TDP1 high mobility group protein	Tb927.3.3490	30847	97	4
I/6 autoantigen	Tb927.7.3440	27261	95	4
elongation factor 2	Tb10.70.2660	95300	94	4
Hypothetical protein	Tb927.4.2740	16317	94	3
phospholipase A1			93	
beta tubulin	Tb927.1.2330	50413	90	3
Gp63-3 surface protease homology	Uniref100_Q4FKH2	70254	89	2
acidic ribosomal protein			88	
Hypothetical protein	Tb927.6.4140/	13229	87	3

	uniref100_Q586B2			
dynein-associated protein	Tb09.211.4920	11123	85	2
Hypothetical protein	Tb927.3.1300	46343	82	2
TbP34 RNA-binding protein			81	
metalloprotease, putative; cell division protein FtsH homologue	Tb11.01.6360	74429	80	2
acyl carrier protein, mitochondrial precursor	Tb927.3.860	16520	78	2
Hypothetical protein	Tb10.6k15.3950/ uniref100_Q38AS5	35254	76	3
hypothetical protein	Tb927.7.2190	29224	71	2
RBP29 RNA binding protein	Tb10.61.3200	41052	70	5
Hypothetical protein	Tb927.7.2570	52912	70	2
gk glycerol kinase, glycosomal	Tb09.211.3540	56299	67	1
epsin	Tb11.50.0006	55460	66	2
RuvB-like DNA helicase	Tb927.4.1270	50049	62	2
hypothetical protein	Tb11.01.4280	44945	60	2
Hypothetical protein	Tb927.4.2030/ uniref100_Q583I9	22691	60	2
flagellum-adhesion glycoprotein	Tb927.8.4060	64947	59	1
BS2 protein disulphide isomerase	Tb10.6k15.2290	55887	57	1
Hypothetical protein	Tb11.02.4300/ Uniref100_Q385C5	48868	56	2
Hypothetical protein	Tb11.02.1910/ uniref100_Q385Y1	36953	56	2
Hypothetical protein	Tb927.5.2100	49921	52	2

Table A2: *T. b. brucei* proteins identified in the non-infection-IgG elution. Proteins ordered in ascending MASCOT score. Experiment described in Chapter 4.

Description	Identifier	Mol wt.	MASCOT score	number of peptides matched
Hexokinase	UniRef100_Q95PL2	51834	5179	134
Heat shock 70 kDa protein 4	UniRef100_P11145	71676	2194	73
ALD fructose-bisphosphate aldolase, glycosomal	Tb10.70.1370	41558	1866	54
Variant surface glycoprotein MITat 1.8	UniRef100_Q58NS6	50790	1602	49
TEF1 elongation factor 1-alpha	Tb10.70.5670	49474	1430	44
Glycosomal membrane protein	uniref100_O60944	24210	1385	42
glucose-regulated protein 78	Tb11.02.5450	71505	1353	44
Pyruvate kinase 1	UniRef100_P30615	55287	1057	34
chaperone protein DnaJ	Tb927.2.5160	45356	1026	26
gPGK phosphoglycerate kinase	Tb927.1.700	47558	990	36
Phosphoglycerate kinase	uniref100_P07378	47431	932	33
Heat shock protein 83	Tb10.26.1080	81169	878	34
Variant surface glycoprotein MITAT 1.4A precursor	UniRef100_P02896	57081	859	31
2-oxoglutarate dehydrogenase	Tb11.01.3550	41516	852	27
gim5A protein	Tb09.211.2730	26790	821	25
hypothetical protein	Tb09.160.5530	38335	797	23
vacuolar ATP synthase subunit B	Tb11.01.3560	55927	745	22
TIM triosephosphate isomerase	Tb11.02.3210	26973	660	24
AK arginine kinase	Tb09.160.4590	40457	659	23
GAPDH	Tb927.6.4280	44241	653	32
glycerol-3-phosphate dehydrogenase	Tb927.8.3530	38408	643	23
Arginine kinase	UniRef100_Q9U420	40375	638	23
alpha tubulin	Tb927.1.2340	50383	623	16
protein disulfide isomerase	Tb927.7.1300	42256	594	22
14-3-3-like protein	Tb11.01.1290	30462	553	17
gk glycerol kinase, glycosomal	Tb09.211.3570	57117	516	18
elongation factor 1 gamma	Tb11.01.4660	46730	506	17
ATP synthase beta chain, mitochondrial precursor	Tb927.3.1380	55969	503	16
hypothetical protein	Tb927.7.180	50408	490	13
variant surface glycoprotein (VSG)-related	Tb11.02.1566	41493	472	15
ATP synthase	Tb11.01.1190	24944	450	16
(H+)-ATPase G subunit	Tb927.8.2310	12798	428	9
dynein light chain	Tb11.50.0007	10479	400	10
DRBD3 RNA-binding protein	Tb09.211.0560	37018	362	13
S-adenosylhomocysteine hydrolase	Tb11.01.1350	49101	360	12

cytosolic malate dehydrogenase	Tb11.01.3040	35528	349	12
Elongation factor	Tb10.70.2660	95300	334	14
V-type ATPase, A subunit	Tb927.4.1080	68219	330	12
hypothetical protein	Tb927.4.1300	42444	330	10
HK2 hexokinase	Tb10.70.5800	51630	329	12
enolase	Tb10.70.4740	47133	324	11
hypothetical protein	Tb09.160.5200	32837	323	12
Hypothetical protein	Tb11.02.0010/ Uniref100_Q386R9	24706	318	8
aspartyl aminopeptidase, putative; metallo-peptidase, Clan MH, Family M20	Tb927.3.3410	49809	317	9
Thioredoxin	Tb927.4.2450	44748	314	13
TAO alturnative oxidase	Tb10.6k15.3640	37738	309	17
Hsc70-interacting protein	Tb927.3.5340	42173	309	6
kinetoplastid membrane protein KMP-11	Tb09.211.4511	11069	301	11
NHP2 ribosomal protein S6	Tb09.160.3670	13620	300	6
hypothetical protein	Tb927.6.4440	37923	290	6
NRBD2	Tb11.01.5590	30256	268	8
proliferative cell nuclear antign	Tb09.160.3710	32750	267	9
hypothetical protein	Tb927.2.4090	29534	259	5
NDPK	Tb11.01.7800	16904	243	9
hypoxanine-guanine phosphoribosyltransferase	Tb10.70.6660	26515	228	8
ribosomal protein S5	Tb11.02.4170	21487	225	5
hypothetical protein	Tb927.5.1780	49188	225	6
stress-induced protein sti1	Tb927.5.2940	62915	223	7
Protease alpha 3 subunit	Tb927.7.4420	32362	223	9
ADP-ribosylation factor-like protein 3A	Tb927.3.3450	20096	220	5
calpain-like protein fragment	Tb927.1.2260	14192	218	6
RBP29 RNA-binding protein	Tb10.61.3200	41052	216	8
RuvB-like DNA helicase	Tb927.4.2000	52805	216	9
beta tubulin	Tb927.1.2330	50413	215	8
RPS12 40S ribosomal protein	Tb10.6k15.2050	16279	212	7
protein disulphide isomerase	Tb927.7.1300	42256	199	8
Proteasome subunit alpha type 1	UniRef100_O96788	29431	195	7
eukaryotic translation initiation factor 5	Tb10.70.4880	43431	194	5
hypothetical protein	Tb11.02.4120	27640	194	5
reticulon domain protein	Tb927.6.3840	21285	190	9
Hypothetical protein	Tb10.61.2850	50899	187	6
LPG3	Tb927.3.3580	87712	184	5
hypothetical protein	Tb10.26.0680	14433	183	7

hypothetical protein	Tb927.4.2030	22691	183	7
60S acidic ribosomal subunit protein	Tb11.46.0002	34891	179	5
RNA binding protein	Tb927.8.6440	20529	178	4
serine/threonine protein phosphatase	Tb927.6.640	79913	176	5
tGLP1 Golgi/lysosome glycoprotein 1	Tb927.8.1870	68353	176	5
Tb427 telo10 v1 145	Tb427 telo10 v1 145	38889	175	3
hypothetical protein	Tb927.6.2860	49790	175	4
acidic phosphatase	Tb927.5.610	48166	166	4
short chain dehydrogenase	Tb927.5.1210	34083	163	7
dynein-associated protein	Tb09.211.4920	11237	162	6
GTP binding protein	Tb927.2.5060	44664	159	6
MCA4 metacaspase	Tb10.70.5250	39628	157	5
hypothetical protein	Tb11.02.2890	26237	156	4
nucleoside hydrolase	Tb927.7.4570	39708	155	5
ATP synthase alpha chain	Tb927.7.7420	63862	155	5
hypothetical protein	Tb10.6k15.3240	25630	152	4
40S ribosomal protein S4	Tb11.02.1085	30739	150	10
translation elongation factor 1-beta	Tb10.70.1100	21945	145	6
TB-24 flagellar calcium-binding protein	Tb927.8.5440	24580	144	5
adenylosuccinate synthetase	Tb11.02.1120	67317	138	7
small nuclear ribonucleoprotein SmD1	Tb927.2.4540	12324	137	5
acyl carrier protein, mitochondrial precursor	Tb927.3.860	16577	137	4
hypothetical protein	Tb10.61.3130	75312	135	4
nascent polypeptide associated complex alpha subunit	Tb11.01.1465	11456	134	4
calreticulin	Tb927.4.5010	45242	134	3
TRYP2 tryparedoxin peroxidase	Tb927.8.1990	25786	134	4
HGPRT	Tb10.70.6650	23573	133	5
guanine deaminase	Tb927.5.4560	55875	132	2
gnD 6-phosphogluconate dehydrogenase, decarboxylating	Tb09.211.3180	52532	131	5
HSP60	Tb10.70.0430	59751	130	4
profilin	Tb11.01.5350	16340	130	3
adenylosuccinate synthetase	Tb11.02.1120	67317	130	5
hypothetical protein	Tb11.39.0004	45720	130	3
Hypothetical protein	Tb927.4.2740/ uniref100_Q583A1	16317	128	5
LA La protein; RNA-binding protein	Tb10.70.5360	37693	127	6
hypothetical protein	Tb927.1.2970	27339	127	4
hypothetical protein	Tb927.7.4120	52218	126	3
phospholipase A1	Tb927.1.4830	32782	124	3

hypothetical protein	Tb927.7.2190	29338	124	3
Hypothetical protein	Tb927.4.2530/ uniref100_Q584D4	16881	121	3
hypothetical protein	Tb11.01.1980	12901	120	3
hypothetical protein	Tb11.01.7740	33169	119	4
Hypothetical protein	Tb09.160.4440	30870	118	5
VAMP	Tb927.5.3560	24672	111	2
hypothetical protein	Tb927.7.4260	29346	110	2
tyrosyl-tRNA synthetase	Tb927.7.2400	46225	109	3
proteasome regulatory non-ATP-ase subunit	Tb11.01.6030	31375	107	4
Sm-D2 small nuclear ribonucleoprotein SmD2	Tb927.2.5850	12568	106	2
HSP10 10 kDa heat shock protein	Tb927.7.1320	10664	106	1
PRS phosphoribosylpyrophosphate synthetase	Tb10.6k15.0970	41193	104	3
Hypothetical protein	Tb09.211.0410/ uniref100_Q38EB9	32407	103	2
Hypothetical protein	Tb927.3.1680/ uniref100_Q57ZE3	35454	102	3
Tb427 telo10 v1 660	Tb427 telo10 v1 660	61483	101	4
hypothetical protein	Tb11.02.4300	48868	101	3
histone H4	Tb927.5.4170	11135	100	3
hypothetical protein	Tb927.5.2100	49921	100	2
hypothetical protein	Tb10.6k15.0255	11373	98	2
hypothetical protein	Tb927.1.5000	30497	97	3
hypothetical protein	Tb11.01.6715	111121	95	2
autoantigen	uniref100_Q26768	27975	93	5
Hypothetical protein	Tb10.70.1130	48342	92	2
Hypothetical protein	Tb927.8.1170/ uniref100_Q57VN5	36796	90	5
metalloprotease	Tb11.01.6360	74711	87	4
adenine phosphoribosyltransferase	Tb927.7.1780	26185	85	3
nucleosome assembly protein-like protein	Tb09.160.4240	47784	84	3
hypothetical protein	Tb07.22O10.680	52912	84	2
TDP1 high mobility group protein	Tb927.3.3490	30847	83	4
glycosomal phosphoenolpyruvate carboxykinase; glycosomal	Tb927.2.4210	58927	82	3
TCTP	Tb927.8.6750	19367	81	2
succinyl-CoA ligase	Tb10.6k15.3250	55571	80	3
GBP21 mitochondrial RNA binding protein1	Tb11.55.0009	23353	79	2
hypothetical protein	Tb927.4.1610	39892	79	1
TbP34 RNA-binding protein	Tb11.01.5570	28759	78	2
PEX11 glycosonal membrane protein	Tb11.01.3370	24240	73	4
Hypothetical protein	Tb10.6k15.3950/	35254	70	3

	uniref100_Q38AS5			
PEX14 peroxin 14	Tb10.100.0130	39970	69	2
hypothetical protein	Tb927.3.5540	58722	68	1
UBA2 ubiquitin-activating enzyme E1	Tb09.211.3610	136223	67	1
ACS3 fatty acyl CoA synthetase 3	Tb09.160.2810	78603	65	3
zinc metallopeptidase	Tb10.05.0230	62911	65	2
protein kinase	Tb10.70.2070	49824	65	2
hypothetical protein	Tb09.211.3110	44770	64	2
Hypothetical protein	Tb927.8.2070/ uniref100_Q57XG1	22184	64	3
asparate aminotransferase	Tb10.70.3710	44930	62	2
RPN5 proteasome regulatory non-ATP-ase subunit 5	Tb10.70.6360	55375	60	2
casein kinase 2	Tb11.01.2590	33898	58	2
Hypothetical protein	Tb09.211.1150/ uniref100_Q38E40	20679	58	2
TbNT3 adenosine transporter 2	Tb927.2.6200	51584	56	2
GPI transamidase component TTA1	uniref100_Q7YTW4	42139	56	2
Hypothetical protein	Tb11.01.7070/ Uniref100_Q38IP3	23157	55	1
asparaginyl-tRNA synthetase	Tb927.4.2310	85386	54	1
coatomer epsilon subunit	Tb11.01.6530	347858	51	2
Hypothetical protein	Tb11.01.5680	51983	51	2
Hypothetical protein	Tb11.01.7200/ uniref100_Q381M9	52925	51	2

Appendix 2: Bioclinical data for the patients used in the validation sera set, data and samples from the WHO HAT specimen bank.

Table A3: General and clinical information of patients infected with *T. b. gambiense*. Shaded ID codes represent the 1 st stage patients. Table shows where patients were diagnosed, either at a hospital (H) or by mobile team (MT), if the patient were in an at-risk area (Yes) or not a high risk area (No) or unknown (UN). Patient's sex and age were recorded. The patient's general appearance at diagnosis (1, not ill; 2, mildly ill; 3, moderately ill; 4, gravely ill) and the duration of their main symptom (see table A7 for details of the bioclinical information) are shown. Females that were pregnant are shown as '***Female***'.

ID	Hospital (H) or Mobile Team (MT)	Risk area?	Male or Female	Age	General appearance	Signs and symptoms	Duration of main symptom (weeks)
4-01	MT	Yes	Female	13	2	0	
4-02	MT	No	Male	59	2	0	1
4-06	MT	Yes	Female	20	2	3	
5-03	MT	UN	***Female***	33	3	5	
5-04	MT	Yes	Female	48	2	6	
5-05	MT	Yes	Female	46	2	6	
1-13	MT	Yes	Male	30	1	0	4
1-18	MT	Yes	Male	20	3	0	40
1-20	MT	Yes	Male	18	2	7	2
1-21	MT	Yes	Male	20	3	0	50
2-21	H	Yes	Male	23	3	0	72
2-24	H	Yes	Male	28	3	0	48
2-26	H	Yes	Male	27	3	0	40
2-27	H	Yes	Male	32	3	0	48
2-29	H	Yes	Male	66	2	0	48
2-31	H	Yes	Male	20	3	0	48
2-32	H	Yes	Female	28	2	0	36
2-33	H	Yes	Male	59	3	0	41
2-34	H	Yes	Male	27	2	0	29
2-35	H	Yes	Female	65	2	0	48
2-36	H	Yes	Male	32	3	0	52

5-10	MT	Yes	Female	31	2	0	
5-11	MT	Yes	Female	26	3	0	
6-05	H	Yes	Male	34	3	0	8
6-06	H	Yes	Male	30	3	2	20
6-07	H	Yes	Female	22	3	0	12
6-08	H	Yes	Female	34	4	0	32
6-09	H	Yes	Male	42	2	0	16
6-10	H	Yes	Male	30	2	0	28
6-11	H	Yes	Female	23	3	0	40
6-13	H	Yes	Male	32	3	0	24
6-14	H	Yes	Male	34	3	0	20
6-15	H	Yes	Male	18	3	0	18
6-16	H	Yes	Male	26	3	0	28
6-23	H	Yes	Male	34	2	0	16
7-01	H	No	Male	34	3	0	2
7-02	H	Yes	Male	46	3	0	24
7-03	H	Yes	Male	52	2	0	4
7-04	H	Yes	Male	28	3	0	16
7-05	H	Yes	Female	38	1	0	4
7-06	H	Yes	Male	31	3	0	28
7-07	H	Yes	Male	52	2	0	6
7-08	H	No	Male	37	2	0	108
7-09	H	No	Male	23	2	0	1
7-10	H	Yes	***Female***	33	3	0	1
7-11	H	Yes	Male	36	2	0	4
1-04	H	Yes	Male	35	4	0	40
1-05	H	Yes	Male	38	4	0	60
1-06	MT	Yes	Female	15	2	0	50
1-44	MT	Yes	Female	25	3	0	100

Table A4: General and clinical information of patients non-infected with *T. b. gambiense*. Table shows where patients were diagnosed, either at a hospital (H) or by mobile team (MT), if the patient were in an at-risk area (Yes) or not a high risk area (No) or unknown (UN). Patient's sex and age were recorded. The patient's general appearance at diagnosis (1, not ill; 2, mildly ill; 3, moderately ill; 4, gravely ill) and the duration of their main symptom (see table A8 for details of the bioclinical information) are shown. Females that were pregnant are shown as '***Female***'.

ID	Hospital (H) or Mobile Team (MT)	Risk area?	Male or Female	Age	General appearance
2C01	H	Yes	Female	68	1
2C02	H	Yes	Female	29	1
2C03	H	Yes	Male	45	1
2C04	H	Yes	Female	38	1
2C05	H	Yes	Male	22	1
2C06	H	Yes	Female	40	1
2C08	H	Yes	Female	29	1
2C09	H	Yes	Female	25	1
2C20	H	Yes	Female	40	2
2C21	H	Yes	Male	53	1
2C23	H	Yes	Male	40	1
2C24	H	Yes	Female	36	1
2C26	H	Yes	Male	38	1
2C36	H	Yes	Male	28	1
2C57	H	Yes	Female	45	1
2C58	H	Yes	Female	59	1
2C59	H	Yes	***Female***	36	1
2C60	H	Yes	Female	52	1
2C61	H	Yes	Male	67	1
2C62	H	Yes	Male	30	1
3C04	H	Yes	Female	20	1
3C05	H	Yes	Male	27	1
3C17	H	Yes	Female	63	1
3C18	H	Yes	Male	40	1
3C19	H	Yes	Male	51	1

4C05	MT	Yes	Male	19	1
4C15	MT	Yes	Female	29	1
4C16	MT	Yes	Male	42	1
5C01	MT	Yes	Male	40	1
5C06	MT	Yes	Female	45	1
5C28	MT	Yes	Male	33	2
6C03	H	Yes	Male	49	1
6C04	H	Yes	Male	44	1
6C11	H	Yes	Female	30	1
6C15	H	Yes	Female	48	1
6C20	H	Yes	Female	34	1
6C22	H	Yes	Female	38	1
6C27	H	Yes	Female	26	1
6C28	H	Yes	Male	56	1
6C29	H	Yes	Male	47	1
6C40	H	Yes	Male	30	1
7C16	H	No	Female	26	1
7C22	H	No	Female	41	1
7C26	H	No	Male	56	1
7C27	H	No	Female	40	1
7C29	H	No	Female	43	1
7C30	H	Yes	Female	50	1
7C45	H	No	Female	55	1
7C46	H	Yes	Male	45	1
7C52	H	No	***Female***	35	1

Table A5: General and clinical information of patients infected with *T. b. rhodesiense*. Shaded ID codes represent the 1st stage patients. Table shows where patients were diagnosed, either at a hospital (H) or by mobile team (MT), if the patient were in an at-risk area (Yes) or not a high risk area (No) or unknown (NA). Patient's sex and age were recorded. The patient's general appearance at diagnosis (1, not ill; 2, mildly ill; 3, moderately ill; 4, gravely ill; 9, not known) and the duration of their main symptom (see table A9 for details of the bioclinical information) are shown. Females that were pregnant are shown as '***Female***'.

ID	Hospital (H) or Mobile Team (MT)	Risk area?	Male or Female	Age	General appearance	Signs and symptoms	Duration of main symptom (weeks)
R004	MT	NA	Male	19	2	1	
R007	MT	NA	Female	60	1	0	
R035	MT	NA	Male	38	9	0	
R047	MT	NA	Male	32	2	0	
R061	MT	NA	Male	18	1	0	
R009	H	Yes	Male	22	3	7	3
R014	MT	NA	Male	44	2	0	
R020	MT	NA	Male	32	4	1	
R022	MT	NA	Male	26	1	0	
R025	MT	NA	Female	42	2	0	
R039	MT	NA	Male	25	2	1	
R040	MT	NA	Male	37	2	1	
R041	MT	NA	Male	26	1	0	
R049	MT	NA	Male	41	1	0	
R052	MT	NA	Male	25	1	0	
R058	MT	NA	Female	27	4	1	
R060	MT	NA	Male	20	1	0	
R063	MT	NA	Male	24	1	0	
R065	MT	NA	Male	13	1	0	
R066	MT	NA	Male	58	1	0	
R067	MT	NA	Female	70	1	0	
R069	MT	NA	Female	60	1	0	
R070	MT	NA	Female	58	1	0	

R071	MT	NA	Female	18	1	0	
R072	MT	NA	Male	37	1	0	

Table A6: General and clinical information of non-infected patients. Table shows where patients were diagnosed, either at a hospital (H) or by mobile team (MT), if the patient were in an at-risk area (Yes) or not a high risk area (No) or unknown (NA). Patient's sex and age were recorded. The patient's general appearance at diagnosis (1, Nnt ill; 2, mildly ill; 3, moderately ill; 4, gravely ill) and the duration of their main symptom (see table A10 for details of the bioclinical information) are shown. Females that were pregnant are shown as '***Female***'.

ID	Hospital (H) or Mobile Team (MT)	Risk area?	Male or Female	Age	General appearance
CR01	H	Yes	Male		1
CR02	H	Yes	Male		1
CR04	H	Yes	Male		1
CR06	H	Yes	Female	24	1
CR08	H	Yes	Male	20	1
CR09	H	Yes	Female	18	1
CR20	MT	NA	Female	21	4
CR40	MT	NA	Female	22	1
CR41	MT	NA	Female	64	1
CR43	MT	NA	Female	48	1
CR48	MT	NA	***Female***	22	1
CR49	MT	NA	***Female***	39	1
CR50	MT	NA	Male	25	1
CR52	MT	NA	***Female***	21	1
CR59	MT	NA	Male	48	1
CR61	MT	NA	***Female***	28	1
CR62	MT	NA	***Female***	21	1
CR63	MT	NA	***Female***	23	1
CR64	MT	NA	***Female***	20	1
CR68	MT	NA	Female	27	1

Table A7: Bioclinical information of patients infected with *T. b. gambiense*. Shaded ID codes represent the 1st stage patients. Table shows results from the CATT test (1, positive, 0, negative) and the highest blood titration which still gave a positive result (expressed as fraction). Results from microscope examination of the patient's blood for trypanosomes (1, positive; 0, negative, blank, procedure was not carried out), and microscope examination after CTC and mAECT (centrifugation techniques) and lymph node puncture. Cerebrospinal fluid was also examined for trypanosomes (1, positive; 0, negative) and white and red blood cells were counted. Blank spaces represent unknown or not applicable data.

			BLOOD			LYMPH NODE	CEREBROSPINAL FLUID				
ID	***CATT 1/4***	***PATIENTS Highest positive titration***	***WET***	***CTC***	***MAECT***	***Puncture***	***Direct examination***	***Double Centrifugation***	***Modified Simple Centrifugation***	***Number of White Blood cells /µl***	***Number of Red Blood Cells/µl***
4-01	1	1/32				1	0	0		4	0
4-02	1	1/32				1	0	0		2	0
4-06	1	1/32				1	0	0		5	0
5-03	1	1/32			0	1	0			5	0
5-04	1	1/32			1	1	0			1	0
5-05	1	1/32		1	1		0			4	0
1-13	2					1	0			2	0
1-18	2			0		1	0			4	50
1-20	1	1/32		0	1	0	0			1	0
1-21				0	1		0			4	0
2-21	1	1/32	0	1	0		1			1232	0
2-24	1	1/32	0			1	1			435	0
2-26	1	1/32	0			1	1			231	0
2-27	1	1/32	0			1	1			305	0
2-29	1	1/32	0	1			1			180	0
2-31	1	1/32	0	0	0		1			611	0
2-32	1	1/32	0			1	1			352	0
2-33	1	1/32	0	1	1		1			819	0
2-34	1	1/32	0	1			0			7	0
2-35	1	1/32	0	1			1			530	0
2-36	1	1/32	0	0	0	1	1			388	0
5-10	1	1/32		0	1		0			74	0
5-11	1	1/32		1		1	1			147	0
6-05	1	1/16				1	0			575	0
6-06	1	1/32		0	0		0		1	187	0
6-07	1	1/16		0	0	0	0		1	176	0
6-08	1	1/16				1	1			500	0

6-09	1	1/64				1	0			380	0
6-10	1	1/32				1	1			1200	0
6-11	1	1/32				1	1			750	0
6-13	1	1/64		0	1		1			800	0
6-14	1	1/32				1	0			240	0
6-15	1	1/16				1	0			75	0
6-16	1	1/32				1	0			77	0
6-23	1	1/64		0	1	0	1			972	0
7-01	1	1/32		0		1	1		1	369	0
7-02	1	1/8		0	0		0		1	176	0
7-03	1	1/32				1	1			82	0
7-04	1	1/16				1	1			353	0
7-05	1	1/32				1	0			191	0
7-06	1	1/32				1	0		1	128	0
7-07	1	>1/32		1		0	0			16	0
7-08	1	1/16		0	0	0	1			215	0
7-09	1	>1/32				1	0			895	0
7-10	1	1/16		0	1		1			152	3
7-11	1	>1/32				1	0			386	0
1-04	2					1	0		0	400	0
1-05	2			0	0		0		1	100	
1-06	1	>1/32				1	1			20	
1-44	2			1		1	0			13	0

Table A8: Bioclinical information of non-infected patients. Table shows results from the CATT test (1, positive, 0, negative) and the highest blood titration tried which still gave a negative result (expressed as fraction). Results from microscope examination of the patient's blood for trypanosomes (1, positive; 0, negative, blank, procedure was not carried out), and microscope examination after CTC and mAECT (centrifugation techniques) and lymph node puncture. Cerebrospinal fluid was also examined for trypanosomes (1, positive; 0, negative) and white and red blood cells were counted. Blank spaces represent unknown or not applicable data.

	CATT			BLOOD			LYMPH NODE	
ID	***CATT***	***CATT 1/4***	***CONTROLS Lowest dilution tested***	***WET***	***CTC***	***MAECT***	***Puncture***	***Thin Blood Film***
2C01	0	0	0	0	0	0	0	0
2C02	0	0	1/4		0	0		
2C03	0	0	1/4		0	0		
2C04	0	0	1/4	0	0	0		
2C05	0	0	1/4	0	0	0		
2C06	0	0	1/4	0	0	0		
2C08	0	0	1/4	0	0	0	0	
2C09	0	0	1/4	0	0	0		
2C20	0	0	1/4	0	0	0	0	
2C21	0	0	1/4	0	0	0		
2C23	0	0	1/4	0	0	0	0	
2C24	0	0	1/4	0	0	0	0	
2C26	0	0	1/4	0	0	0	0	
2C36	0	0	1/4	0	0	0		
2C57	0	0	1/4		0	0		
2C58	0	0	1/4	0	0	0		
2C59	0	0	1/4		0	0		
2C60	0	0	1/4		0	0		
2C61	0	0	1/4	0	0	0		
2C62	0	0	1/4	0	0	0		
3C04	0	0	1/00		0	0		
3C05	0	0	1/1	0	0	0		
3C17	0	0	1/1	0	0	0		
3C18	0	0	1/1	0	0	0		
3C19	0	0	1/1	0	0	0		
4C05	0	0	0		0	0		
4C15	0	0	0		0	0		
4C16	0	0	0		0	0		
5C01	0	0	0			0		
5C06	0	0	0			0		

5C28	0	0	0		0	0	0	
6C03	0	0	0		0	0		
6C04	0	0	0		0	0		
6C11	0	0	0		0	0		
6C15	0	0	0		0	0		
6C20	0	0	0		0	0		
6C22	0	0	0		0	0		
6C27	0	0	0		0	0		
6C28	0	0	0		0	0		
6C29	0	0	0		0	0		
6C40	0	0	1/2		0	0		
7C16	0	0	0		0	0		
7C22	0	0	0		0	0		
7C26	0	0	0			0		
7C27	0	0	0			0		
7C29	0	0	0			0		
7C30	0	0	0			0		
7C45	0	0	0			0		
7C46	0	0				0		
7C52	0	0	1/2			0		

Table A9: Bioclinical information of patients infected with *T. b. rhodesiense*. Shaded ID codes represent the 1st stage patients. Results from microscope examination of the patient's blood for trypanosomes (1, positive; 0, negative, blank, procedure was not carried out), and microscope examination after CTC and mAECT (centrifugation techniques) and lymph node puncture. Cerebrospinal fluid was also examined for trypanosomes (1, positive; 0, negative) and white and red blood cells were counted. Blank spaces represent unknown or not applicable data. Extra information was included for R035, which had a weakly positive/negative CATT result.

		BLOOD			CEREBROSPINAL FLUID			
ID	***Thin blood film***	***WET***	***CTC***	***MAECT***	***Direct examination***	***Modified Simple Centrifugation***	***Number of White Blood cells /µl***	***Number of Red Blood Cells/µl***
R004	1		1		0	0	3	
R007		1	1		0			
R035	0	0	1		0	0	5	0
R047		1	1		0	0	1	
R061		1	1		0	0	1	
R009		1	1		1	1		0
R014	1	1	1		1	1	35	0
R020		1	1		1		349	0
R022		2	1		1		1002	0
R025		2	1		1		35	0
R039		1	1		1		57	0
R040		2	1		0	0	400	
R041		2	1		1		9	
R049		1	1		1		19	
R052		0	2	1	1		14	
R058		9	9		1		159	
R060		2	1		0	1	15	
R063		1	1		0	1	26	
R065	0	1	1	1	1	1	10	
R066		1	1		1		55	
R067		1	1		1		10	
R069		1	1		1		10	
R070		1	1		1	1	50	
R071		1	1		1			
R072		0	1		0	1	10	

Table A10: Bioclinical information of non-infected patients. Table shows results from the CATT test (1, positive, 0, negative) and the highest blood titration tried which still gave a negative result (expressed as fraction). Results from microscope examination of the patient's blood for trypanosomes (1, positive; 0, negative, blank, procedure was not carried out), and microscope examination after CTC and mAECT (centrifugation techniques) and lymph node puncture. Cerebrospinal fluid was also examined for trypanosomes (1, positive; 0, negative).

	CATT		BLOOD			LYMPH NODE	
ID	***CATT***	***CATT 1/4***	***WET***	***CTC***	***MAECT***	***Puncture***	Thin Blood Film
CR01			0	0	0		
CR02			0	0	0		
CR04			0	0	0		
CR06			0	0	0		
CR08			0	0	0		
CR09			0	0	0		
CR20	0		0	0	0	0	
CR40	0		0	0	0	0	0
CR41	0		0	0	0	0	0
CR43	0		0	0	0	0	0
CR48	0	0	0	0	0	0	0
CR49	0	0	0	0	0	0	0
CR50	0		0	0	0	0	0
CR52	0		0	0	0	0	0
CR59	0	0	0	0	0	0	0
CR61	0	0	0	0	0	0	0
CR62	0	0	0	0	0	0	0
CR63	0	0	0	0	0	0	0
CR64	0	0	0	0	0	0	0
CR68			0	0			

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