Vrije Universiteit Brussel
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Universiteit Antwerpen

Interuniversity Program Molecular Biology (IPMB)

Development of recombinant TgsGP antigen for serodiagnosis of *Trypanosoma brucei gambiense.*

Thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science in Molecular Biology (Human Health)

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Academic year 2014-2015

ACKNOWLEDGEMENT

I can't be more thankful to the people of VLIR-UOS who immensely contributed to my life and study in Vrije Universiteit Brussel (VUB).

I will like to acknowledge my parents, siblings, course mates and teachers for their timeless contributions to my life. I will like to say a big thank you Mom and Dad for seeing me through another academic journey.

I am happy to express my thanks to all my labmates, Dr. Nick Van Reet, Birhanu Hadush Aberra, Erika D'Haenens and Nicolas Bebronne for their constant support during my lab work.

I gratefully extend my sincere gratitude to my Promoter, Prof. Philippe Büscher; co-promoter, Prof. Stijn Deborggraeve; and supervisor Mrs. Tessa de Block for being such wonderful friends and mentors. I say a big thank you for the enthusiastic and encouraging support you gave. Thank you very much for the corrections you gave. Thank you for your continuous demand for excellent results because this work is a product of it.

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LIST OF ABBREVIATIONS

Ab	Antibody
AUC	Area under the receiver operator characteristics curve
AP	Alkaline phosphatase
APOA1	Apolipoprotein A1
APOL1	Apolipoprotein L1
CATT	Card Agglutination Test for Trypanosomiasis
cPCR	Colony PCR
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FIND	Foundation for innovative New Diagnostics
HAT	Human African Trypanosomiasis
HCT	Haematocrit centrifugation
HPR	Haptoglobin-related protein
IgA	Immunoglobin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
kDa	Kilodalton
LAMP	Loop-mediated isothermal amplification
M	Marker
mAECT	Mini anion exchange centrifugation technique
mg	Milligrams
mM	Millimole
ml	Milliliter
NASBA	Nucleic acid sequence based amplification
NCBI	National Center for Biotechnology Information

NECT	Nifurtimox-eflornithine combination therapy
OD	Optical density
PCR	Polymerase chain reaction
RDT	Rapid Diagnostic test
RIME	Repetitive insertion mobile element
RNA	Ribonucleic acid
rTgsGP-qpSP	Recombinant TgsGP without signal peptide
rpm	Revolutions per minute
SD	Standard Diagnostics
SL	Spliced-Leader
SP	Signal Peptide
SRA	Serum resistance associated
TgsGP	*T.b. gambiense* specific glycoprotein
TgsGP-SP	TgsGP with signal peptide
TgsGP-qpSP	TgsGP without signal peptide
TbHpHbR:	T. *brucei* haptoglobin-haemoglobin receptor
TLFs	Trypanosome lytic factors
VSG	Variant Surface Glycoprotein
WHO	World Health Organisation

ABSTRACT

The majority of human African trypanosomiasis (HAT) cases are caused by *Trypanosoma brucei gambiense (T.b. gambiense)*. This parasite is transmitted by tsese flies and is endemic in rural settings of West and Central Africa. The parasite can infect man because it can resist lysis by the human serum through expression of the *T.b. gambiense* specific glycoprotein (TgsGP).

Different diagnostic tools have been developed for *T.b. gambiense* HAT. Among the serological tests, detecting antibodies against *T.b. gambiense*, the CATT/*T.b. gambiense* is used for mass screening in endemic areas. Recently, a rapid diagnostic test in dipstick format has been developed for *T.b. gambiense HAT*. However, both tests are based on native LiTat 1.3 and/or LiTat 1.5 antigens. However, not all *T.b. gambiense* strains do express these immunodominant VSGs leading to lower sensitivities in certain areas. In my thesis work, I have developed an in vitro system for the recombinant expression of the *T.b. gambiense* specific TgsGP protein and evaluated its diagnostic potential in ELISA with sera from HAT patients and controls. Advances in developing recombinant antigens will lead to increased performance and affordability of serodiagnostic tests for *T.b. gambiense* HAT.

The recombinant TgsGP (rTgsGP) was successfully expressed in Origami *E. coli* cells and purified in a two-steps approach, affinity chromatography followed by size-exclusion chromatography. Purified rTgsGP antigen was first tested in western blot strips with 7 HAT patientsô sera and 5 controls. The diagnostic accuracy of the rTgsGP antigen was evaluated by ELISA with sera from 100 *T.b. gambiense* patients, 50 *T.b. gambiense*-endemic negative controls, 78 *T.b. rhodesiense* patients and 50 *T.b. rhodesiense*-endemic negative controls. Based on the ELISA OD values, the area under the receiver operator characteristics curve (AUC) was calculated at 0.86. This indicates a good diagnostic accuracy. However, native as well as recombinant LiTat 1.3 and 1.5 antigens showed higher AUC values (>0.95) in previous studies, and may thus be better antigens for the diagnosis of *T.b. gambiense* HAT.

CHAPTER I. INTRODUCTION

I.1 OVERVIEW

Human African trypanosomiasis (HAT), also called sleeping sickness, is a protozoal disease encountered on the African continent. The disease is transmitted from humans to humans by an insect, the tsetse fly, and occurs in 27 countries of the sub-saharan part of Africa. The disease occurs in two forms and people living in rural settings are more exposed to the disease. The causative agents of human African trypanosomiasis are *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense.* The two subsepecies are indistinguishable in their morphology. However the disease presents different epidemiological and clinical characteristics depending on whether it is caused by *T.b. gambiense* or *T.b. rhodesiense* (World Health Organization 2013). The geographical distribution of the HAT causative agents was initially defined in relation to the rift valley; *T.b. gambiense* is confined to the west of the rift valley, whereas *T.b. rhodesiense* is in east of the valley.

I.2 TAXONOMICAL CLASSIFICATION AND MORPHOLOGY

Human trypanosomes are unicellular parasites taxonomically classified under the domain *Eukarya*; kingdom *Protista* (unicellular eukaryotes); phylum *Sarcomastigophora* (characterised by pseudopodia and/or flagella); subphylum *Mastigophora* (characterised by flagella); class *Zoomastigophora* (characterised by absence of chloroplasts); order *Kinetoplastida* (with an extracellular DNA called kinetoplast); family *Trypanomastidae*; genus *Trypanosoma*; species *brucei* and subspecies *gambiense* and *rhodesiense* (http://parasite.org.au/para-site/text/brucei-text.html).

Trypanosomes, as hemoflagellates, share typical cell components with eukaryotes; like a nucleus, a cytoskeleton, a mitochondrion, an endoplasmic reticulum and a Golgi apparatus. They possess a kinetoplast at the basis of their flagellum; kinetoplast being an extensive organelle to mitochondrion which contains a self-replicating DNA.
Human African trypanosomes are characterised by two morphological forms: trypomastigote and epimastigote (see figure 1).

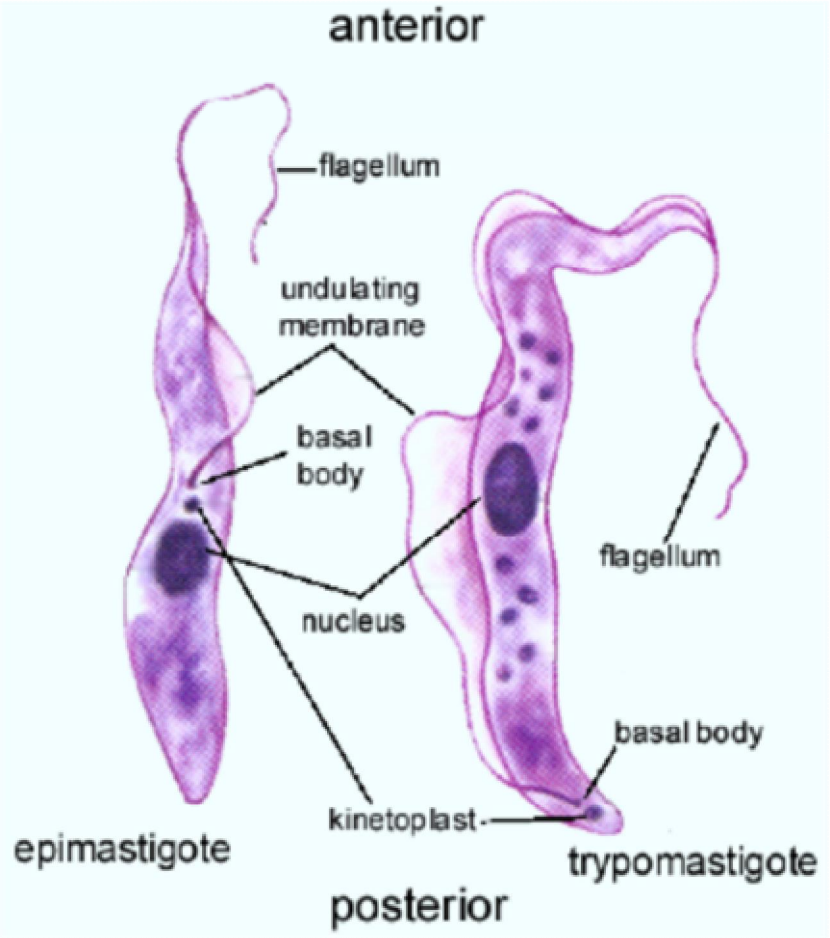

Figure 1. Epimastigote and trypomastigote forms of trypanosomes
Source: http://quizlet.com/23048252/lecture-12-blood-and-tissue-protozoa-flash-cards/

The trypomastigote is marked by a post nuclear kinetoplast that is localised at the most posterior end of the parasite. Its flagellum undulates along the whole length of the cell body. The body length varies from 12 to 40 µm. The epimastigote is the main form of the parasite found in the salivary glands of the vector tsetse fly. The epimastigote is characterised by an anterior kinetoplast to the nucleus with a flagellum undulating about a half of the parasite body length. Both trypomastigotes and epimastigotes multiply by binary fission as a mode of division (http://quizlet.com/23048252/lecture-12-blood-and-tissue-protozoa-flash-cards/).

I.3 LIFE CYCLE

The occurrence of HAT depends on the interaction of the main three factors: reservoir, vector and causative agent.

First, there are two reservoirs of HAT, human and animal for *T.b. gambiense* and *T.b. rhodesiense* respectively. Second, tsetse flies play a role of vector in both the two forms of HAT. Third, the two causative agents which are *T.b. rhodesiense* and *T.b. gambiense*.

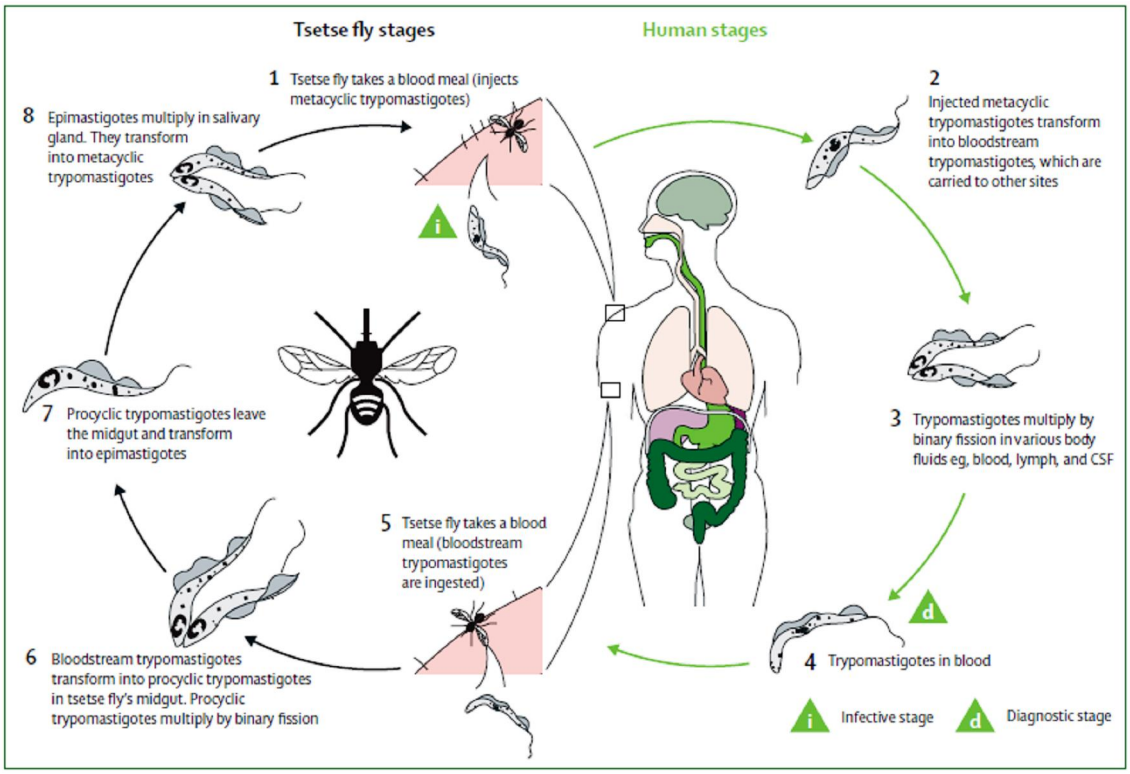

Figure 2. Life cycle of Trypanosoma brucei subspecies causing African trypanosomiasis
Source: (Kennedy, 2013)

The lifecycle starts when an infective, tsetse fly, motivated of taking the blood meal injects the metacyclic trypomastigotes forms of trypanosome into the skin of a person. Once in the skin, the parasitic form, by entering first the lymphatic system, will proceed into the bloodstream system. The stage 2 is characterised by the transformation of metacyclic trypomastigotes into bloodstream trypomastigotes, the latter form reaches the other sites of the body such as lymph and spinal fluid; and keeps replicating by binary fission in stage 3.

The vector itself, tsetse fly, gets infected in stage 4 and 5 with blood intake from an infected human. In the flyôs body, the parasitic form will transform into metacyclic trypomastigotes and multiply by binary fission like in stage 3; they will later leave the midgut to transform into epimastigotes, stage 7. The resulting epimastigotes, by migration, reach the vectorôs salivary glands and multiply by binary fission as well, stage 8. In general, the whole life cycle takes around 3 weeks.

T.b. gambiense and *T.b. rhodesiense* are the only 2 subspecies infectious to humans among 3 subspecies of *T. brucei*. Another subspecies (*T.b.brucei*) is non pathogenic to humans but rather to domestic and wild animals; and it has played an experimental role as a model in research of HAT. Being extracellular parasites, trypanosomes are microscopically distinguishable with exception of the two HAT subspecies. The latter can only be distinguished with the help of advanced molecular techniques with consideration of molecular markers. *T.b. rhodesiense* is characterised by a molecular marker known as serum resistance associated (SRA) gene compared to the *T.b. gambiense* specific glycoprotein (TgsGP) as its counterpart marker in *T.b. gambiense* (Berberof, Perez-Morga, & Pays 2001;Radwanska et al. 2002a).

Trypanosomes necessitate a vector for their infection to be successful. Vectors involved in the lifecycle are tsetse flies belonging to the genus *Glossina*. A number of subspecies of tsetse fly has been reported to be confined to different geographical niches. The subgenus *palpalis* and *fuscipes* contain the main vectors of HAT. For instance, *Glossina palpalis gambiensis* (*G.p. gambiensis*) are responsible of transmitting *T.b. gambiense* and *Glossina fuscipes* (*G.fuscipes*) is responsible *T.b. rhodesiense* (Robays et al. 2004b). Human activities such as deforestation tend to eliminate tsetse flies, as they all mostly live in forests and vegetations. Tsetse flies are more adapted in the tropics where the HAT is endemic. This is because they optimally live at a temperature of 16-38ºC and a relative humidity of 50-80% (World Health Organization 2013).

The rate of HAT transmission is always proportional to the rate of trypanosomes inoculated in the host by tsetse fly. Thus, the transmission is influenced by important factors such as density of vectors (tsetse fly), tsetse flyôs lifespan and susceptibility to infection, tsetseôs preference towards different source of blood meal, the frequency of human-tsetse fly contact and the percentage of infectious flies (Aksoy, Gibson, & Lehane 2003;Roditi & Lehane 2008).

1.4 ANTIGENIC VARIATION

The Variant Surface Glycoprotein (VSG) covers the trypanosome against any form of human immunity such as antibodies (IgG and IgM) or the complement. The hostôs antibodies are able to attack and kill the parasite by disrupting its covering membrane.

Therefore, in this case, the number of parasites is expected to decrease. On the other hand, parasites have got their way of escaping to the immune system by rearranging and renewing their membrane coat and this leads to the non-recognition by specific antibodies (McCulloch 2004;Pays, Vanhamme, & Perez-Morga 2004).

T.b. gambiense and *T.b. rhodesiense* are among the pathogenic agents that developed mechanisms for their membrane surface proteins. To evade the humanôs immune system, the pathogen changes the composition of its VSG in the bloodstream. The parasite switches from one VSG type to another. Therefore the mechanism is very important for the *Trypanosoma* species to escape the host immune response. The molecular investigation of the antigenic variation has shown that it reacts from recombination and telomeric VSG expression (McCulloch 2004).

The VSG genes get activated in the tsetse-fly salivary gland and get inactivated upon the parasitesô return to the flyôs mid-gut. Activation of the VSG genes results into production of an important coat protecting the parasite through its life cycle. The VSG coat, covering almost the entire parasiteôs body, represents more or less 20% of the expressed proteins in the parasite; and elicits production of antiserum at different VSG levels.

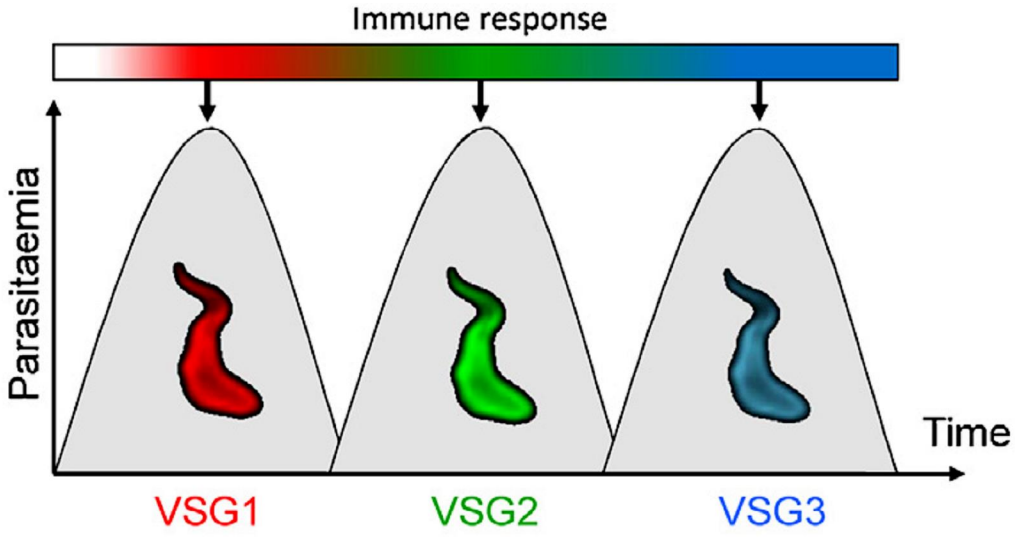

Figure 3. Antigenic variation against the host's immune system
Source:(McCulloch 2004)

The figure above shows that the switching of VSG leads to an antigenic variation, respective to the immune response at different VSG spectrum.

Consequently, as the level of antibodies increases, a number of trypanosomes will be eliminated while those with a VSG coat to which no antibodies are yet present will escape.

I.5 EPIDEMIOLOGY

As stated above in the introductory overview, HAT occurs in two major forms: *gambiense* HAT and *rhodesiense* HAT. *T.b. gambiense* causes a more chronic disease and exhibits an anthroponotic life cycle. Humans are the main reservoirs (Brun & Blum 2012;Simo et al. 2014).

I.5.1 Geographical distribution

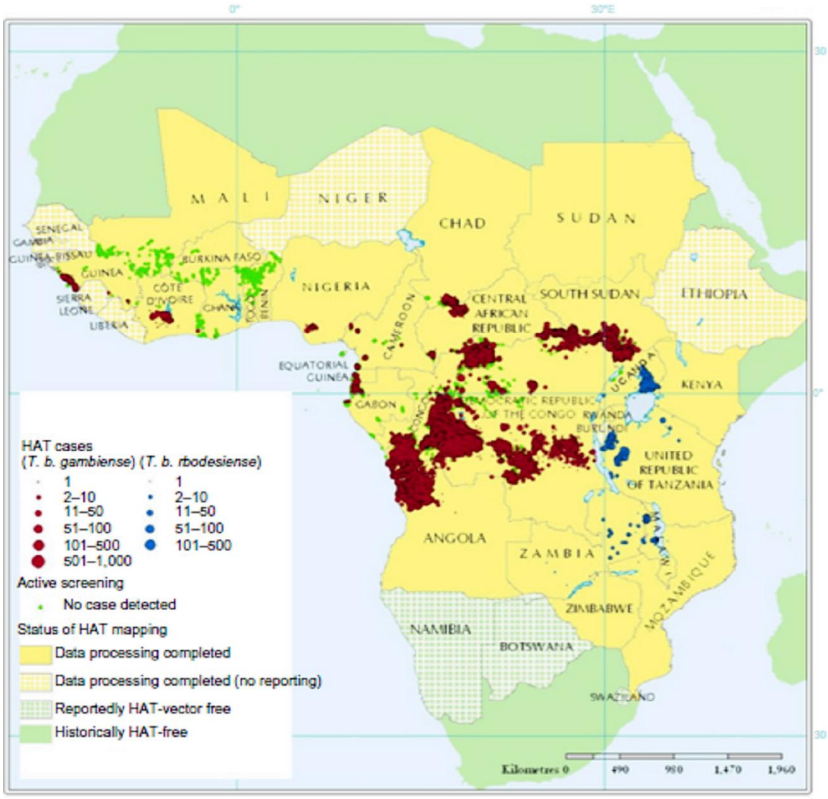

Figure 4. Geographic distribution of HAT cases reported from 2000–2009
Source: (Simarro et al. 2010)

In total, 300 *gambiense* HAT foci have been reported. They are all distributed among 24 sub-Saharan Africa countries including Nigeria, Senegal, Sierra Leone, South Sudan, Togo, Uganda, Gambia, Ghana, Guinea, Guinea Bissau, Liberia, Mali, Niger, Democratic republic of the Congo, Equatorial Guinea, Gabon, Côte d'Ivoire, Congo, Central African Republic, Chad, Cameroon, Angola, Benin and Burkina Faso**.** The Democratic Republic of the Congo (DRC) is ranked at the first place among countries presenting high number of cases of HAT followed by Chad (Brun & Blum 2012;Simarro et al. 2012).

I.5.2 Social and economic consequences

Patients suffering from HAT fail to declare their health status, because it is a stigmatising disease; therefore, they feel discriminated among the population (Mpanya et al. 2012;Robays et al. 2004a).

Since the disease is long lasting and affecting the productive age group, it harms the economy of the infected population. The indirect related cost to diagnosis and treatment constitute important expenses to the patient (Lutumba et al. 2007).

I.5.3 Risk factors

The main factors of contracting *gambiense* HAT are always associated with the intensity and frequency of risk of contact of tsetse fly with human beings. These risks include mainly the human activities and the entire environment which might be favourable to the prosperity of tsetse flies.

Tsetse flies are found widely distributed in humid areas, savannah, rivers and streams. Different human activities such as fetching firewood and water, fishing, farming, mining of diamond and gold, etc, are developed along those areas and constitute major means and/or sources of transmission (Kohagne et al. 2011;Moore et al. 1999;Robays, Ebeja, Lutumba, Miaka mia, Kande Betu, V, De, Makabuza, Deguerry, Van der Stuyft, & Boelaert 2004b). As a matter of fact, *gambiense* HAT is mostly reported in rural areas.

I.5.4 Disease control

Several control measures and efforts have been put in place to lower the burden of the disease and showed a success. This is due to the supply of different supporting forms including access to diagnosis, treatment and training in the endemic countries mentioned above. For some regions, *gambiense* HAT can be considered as it has already attained the level of control defined as ñeliminated as a public health problemò (Simarro, Cecchi, Franco, Paone, Diarra, Ruiz-Postigo, Fevre, Mattioli, & Jannin 2012).

Gambiense HAT has been a neglected disease for several years ago. However, the World Health Organisation (WHO) sets its elimination by year 2020. Several interventions have been put in place towards its eradication and control. We can state the use of the Card Agglutination Test for Trypanosomiasis (CATT) and treatment of patients in due time. The control measures put in place are quite promising as there is a remarkable decrease in HAT incidence: from 10000 new cases (in 2009) to 7216 new cases (in 2012) (World Health Organization 2013). At the same, new diagnostic tools are being assayed. However, the road to HAT elimination presents some challenges. First, some of the developed diagnostic rapid tests require trained personnel, cold chain and/or electrical power which are not all available in most HAT endemic areas. Second, a remarkable decrease in HAT prevalence would lead to pharmaceutical companies and laboratoriesô decrease in effort and investment to tackle the disease.

I.6 RESISTANCE TO HUMAN IMMUNITY

Human is protected from infection of most animal infective trypanosomes, thanks to blood protein complexes (also called trypanosome lytic factors) as part of the human innate immune system. Most of these proteins are of apolipoprotein L1 types and are able to destroy trypanosomes. Trypanosome lytic factors (TLFs) are of two types: TLF1 and TLF2. TLF1 is considered as the most highly dense lipoprotein particles, also named as fraction 3 or HDL3; these are characterised by a lipid core together with a hydrophilic layer of phospholipids and other several apolipoproteins (notably apolipoprotein A1). Contrarily, TLF2 is a complex poor in lipids but containing IgM with apolipoprotein A1 (APOA1) together with some other proteins (Hajduk et al. 1989;Raper et al. 1999).

The two TLFs share the same haptoglobin-related protein (HPR) and apolipoprotein L1 (APOL1). HPR and APOL1 show some complementarity in their functions where HPR is involved in targeting and interacting TLF1 to the parasite and APOL1 acts as a lysing toxin.

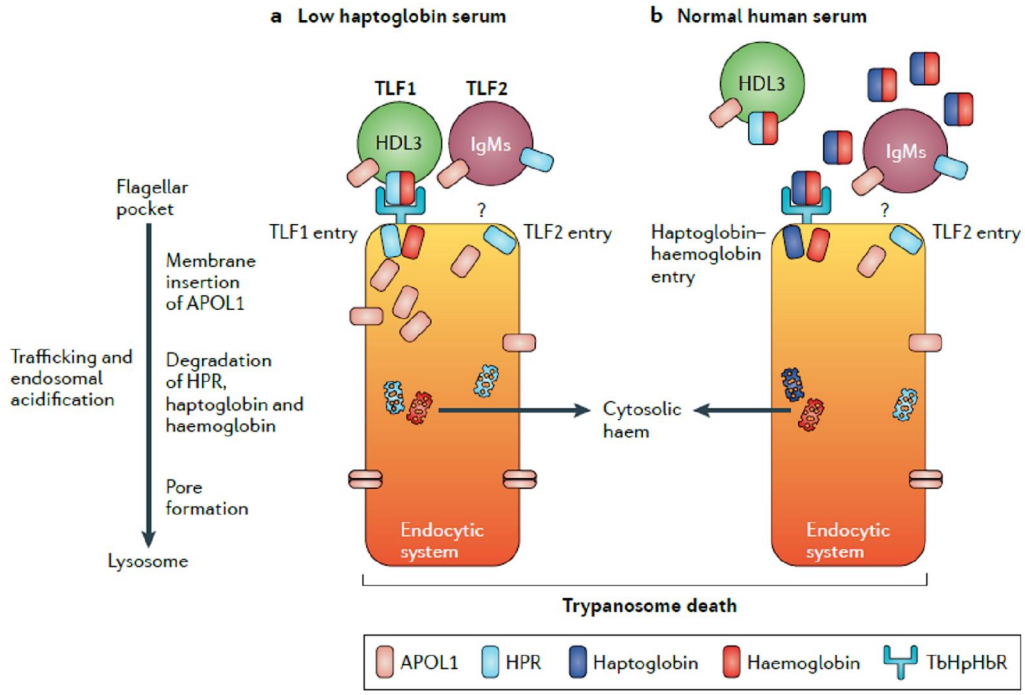

Figure 5. Illustration of the uptake and trafficking of trypanolytic factors (TLF1 and TLF2) in *Trypanosoma brucei brucei*.
Source: (Pays et al. 2014).

As it can be seen on the figure 5, TLF1 binds to T. *brucei* haptoglobin-haemoglobin receptor (TbHpHbR). HPR is also present in TLF2 but it is not known whether it can also bind to TbHpHbR. HPR results from a haptoglobin through tandem gene duplication. The association of HPR with the haemoglobin allows the uptake of TLF1 into the parasite. The latter complex (TLF1-HPR-haemoglobin) binds to TbHpHbR (Vanhollebeke et al. 2007). The question mark on the figure means that the uptake mechanism of TLF2 is not known yet. Apolipoprotein L1 gets inserted in the endosomal membrane as a way of escaping from cysteine protease. Unless it gets inserted into the endosomal membranes to create pores, APOL1 will be degraded by cathepsin L. Due to its insertion in the endosomal membranes, APOL1 creates pores inducing the cell lysis. The definite cell deathôs mechanism is unknown (Shiflett et al. 2007).

Part a of the figure 5 shows the special case in Western countries of Africa, whereas part b shows the situation in normal human serum. The haptoglobin-haemoglobin down-regulates the APOL1 uptake in TLF1 pathway; whereas for TLF2, its uptake enhances the APOL1 entry to kill the trypanosome parasite. The colour change (yellowish to brownish) in the two boxes shows the level of acidification in the endocytic pathway. TLF1 and TLF2 interact with trypanosomes through two different mechanisms. TLF1 acts through the HPR (protein surrounding TLF1) and TbHpHbR (embedded in the flagellar pocket of trypanosome) interaction (Vanhollebeke et al. 2008). TLF2 interacts with the parasite through the same mechanism but it may also present other ways of uptake (Bullard et al. 2012).

The uptake of APOL1 through TbHpHbR is depending on the ratio HPR-haemoglobin/haptoglobin-haemoglobin. Normally, serum contains a higher concentration of haptoglobin than HPR (Muranjan, Nussenzweig, & Tomlinson 1998) (Raper, Nussenzweig, & Tomlinson 1996). Thus, in vivo, TbHpHbR is more saturated of haptoglobin-haemoglobin than HPR-haemoglobin as haptoglobin usually has a higher affinity to TbHpHbR than HPR. Therefore, the lysis of trypanosomes results only from the TLF2 pathway. TLF1 pathway may only occur in vivo by diluting the serum or removing the haptoglobin (Vanhollebeke, Nielsen, Watanabe, Truc, Vanhamme, Nakajima, Moestrup, & Pays 2007).

According to its structure, APOL1 is composed of three important domains; a multihelical anion pore-forming domain; a double-helical membrane addressing domain and a long carboxy-terminal amphipatic Ŭ-helix involved in the control of APOL1 activity (see figure 6 below).

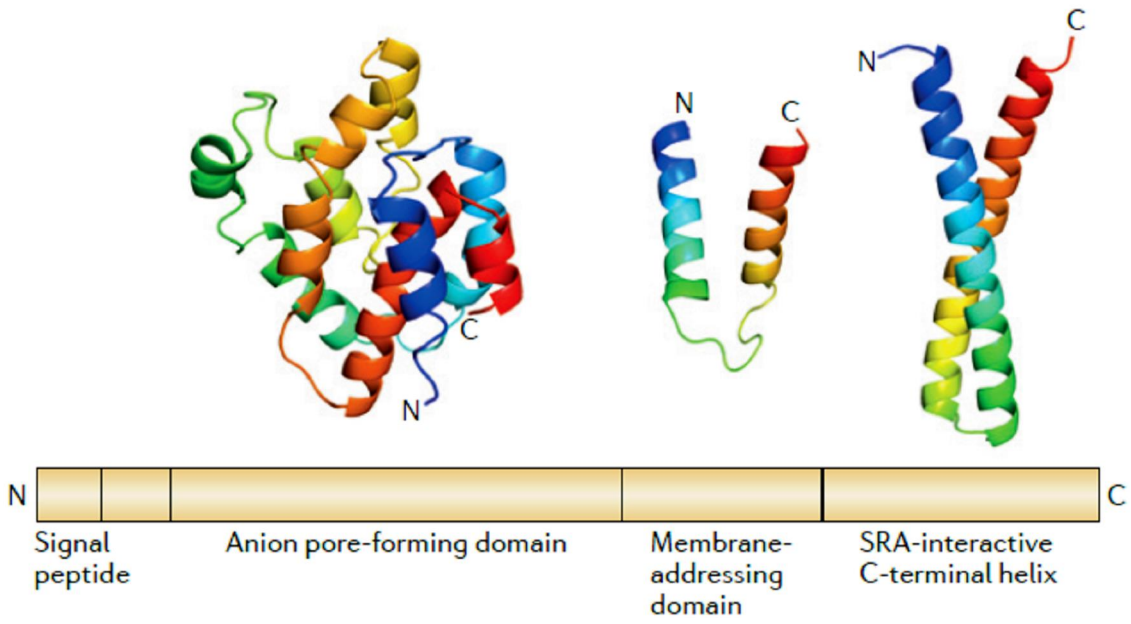

Figure 6. Structure of APOL1.
Source : (Pays, Vanhollebeke, Uzureau, Lecordier, & Perez-Morga 2014).

Trypanosoma brucei rhodesience and *Trypanosoma brucei gambiense* have acquired different means to overcome the human defence system making them infectious to human (Bullard, Kieft, Capewell, Veitch, Macleod, & Hajduk 2012). The mechanism of human serum resistance are conferred by truncated VSG: serum resistance associated (SRA) protein in *T.b. rhodesiense* and *T.b. gambiense* specific glycoprotein (TgsGP) in *T.b. gambiense* (Capewell et al. 2013;De & Hamers 1994).

The SRA inhibits the APOL1 pore forming in the endosomal membrane by strongly binding to it. The binding is ensured by a coiled-coil interaction to the C-terminal helix of APOL1 by the N-terminal amphipatic helix A of SRA (Vanhamme et al. 2003;Lecordier et al. 2009). The helix A of SRA has a stable binding when it interacts with helix of APOL1 than its counterpart helix B in the SRA.

Contrary to SRA, TgsGP does not interact directly with APOL1. APOL1 inhibition by TgsGP results from the hydrophobic ß-sheet found between helix A and helix B (see figure 7) which causes the stiffening of the endosomal membranes when TgsGP is inserted in this membrane (Uzureau et al. 2013). This probably could slow down the insertion of APOL1 into membranes, resulting into degradation of APOL1 by endosomal proteases.

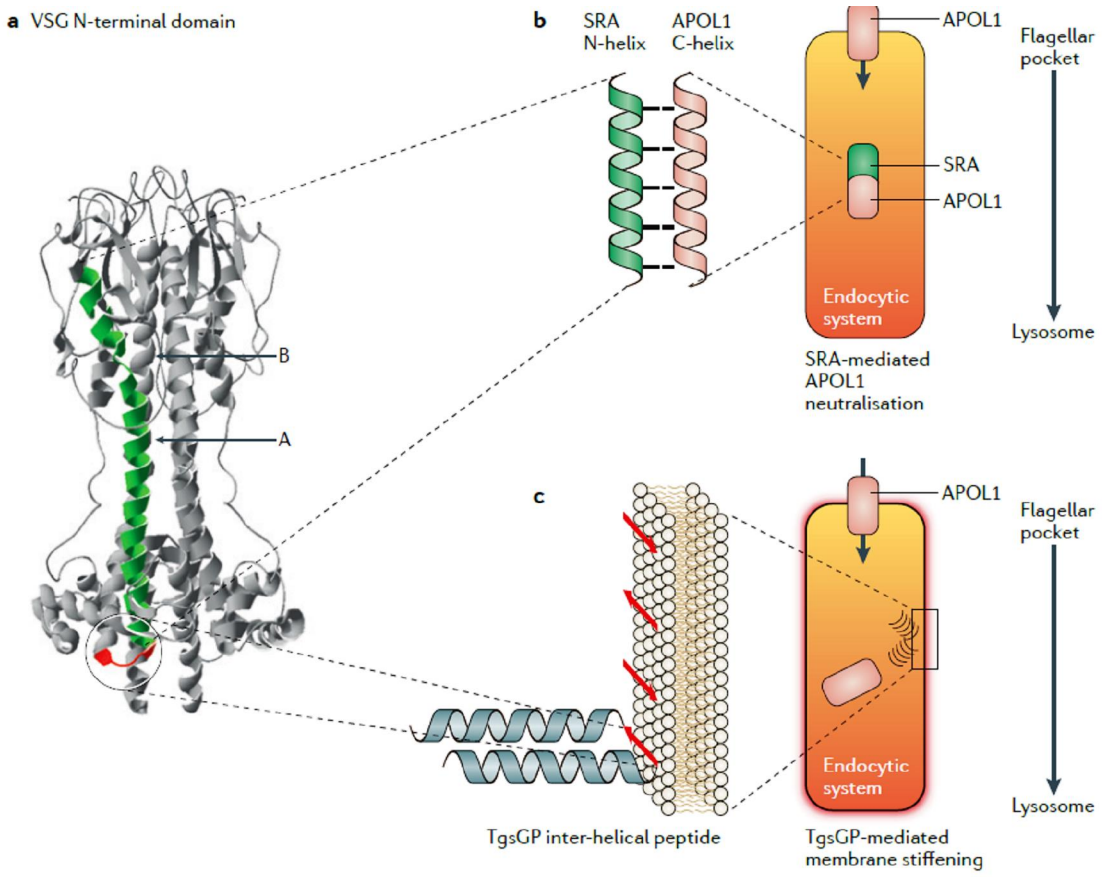

Figure 7. VSG-derived adaptive proteins in human-infective *Trypanosoma brucei* subspecies. Source: (Pays, Vanhollebeke, Uzureau, Lecordier, & Perez-Morga 2014).

The figure above represents the endocytic compartments and the colour gradient through the endosomal acidification between the flagellar pocket and the lysosomes. Part a of the figure illustrates amino-terminal domain of the VSG, highlighting the amphipatic helices A in green and B in grey, with an inter-helical peptide in red forming a hairpin structure. Part b shows the neutralisation of APOL1 by a coiled-coil interaction with SRA. Part c shows how the inter-helical peptide of TgsGP protects *T.b. gambiense* from APOL1 toxicity by lipid stiffening through antiparallel peptide organisation.

I.7 DIAGNOSIS of *T.b. gambiense*

The following steps are taken into account while diagnosing HAT: clinical examination; serological test; parasitological examination (in case the previous serological test is positive) for confirmation; and stage determination (between haemolytic and meningoencephalitic stages) through the examination of the cerebrospinal fluid (CSF).

The latter step is very important for treatment. Among the reported weaknesses of the diagnosis steps mentioned above, we can first mention the symptoms of HAT shared with other tropical diseases such as malaria, enteric fever or tuberculosis; which sometimes hinder the first step with clinical diagnosis. Second, the switching and changing of the parasiteôs VSG remains an obstacle for serological test such as CATT. In addition, serological tests fail due to difficulties to differentiate active form from cured form of infection, this because antibodies can prolongedly remain in blood after treatment. Most of the serological tests need some specific requirements such as power and well-trained personnel. Thus, some seem not to be adequate for remote and endemic areas for HAT.

I.7.1 Clinical stages and clinical diagnosis

Human African trypanosomiasis presents two clinical stages. The first stage, shorter for systems. It is also called the hemo-lymphatic stage (World Health Organization 2013). Whereas, the second stage is characterised by the trypanosome crossing the blood brain barrier and invading the central nervous system. It is also called the meningo-encephalitic stage. The latter stage results in a serious neurological damage (World Health Organization 2013). The neurological phase of HAT can be diagnosed and confirmed by the presence of parasites and a high count of white-blood cells in patientsô cerebrospinal fluid (Miezan et al. 2000). If untreated, HAT can results into coma, severe organ failure and death (Jamonneau et al. 2012).

In terms of clinical presentations, *gambiense* HAT has an average duration of chronic course of 3 years. Signs and symptoms vary among individuals and are associated with the two stages of the disease mentioned above. The first stage is mostly accompanied with headache, fever, trypanosomal chancre, dermatologic problems, anaemia, asthenia, lymphadenopathies, weakness, cardiac disorders, hepatosplenomegaly and musculoskeletal pains. The second stage is associated with sleeping disturbances and some psychiatric signs (World Health Organization 2013). The clinical distinction of the two stages happens unclear due to the fact that sometimes symptoms in two stages overlap.

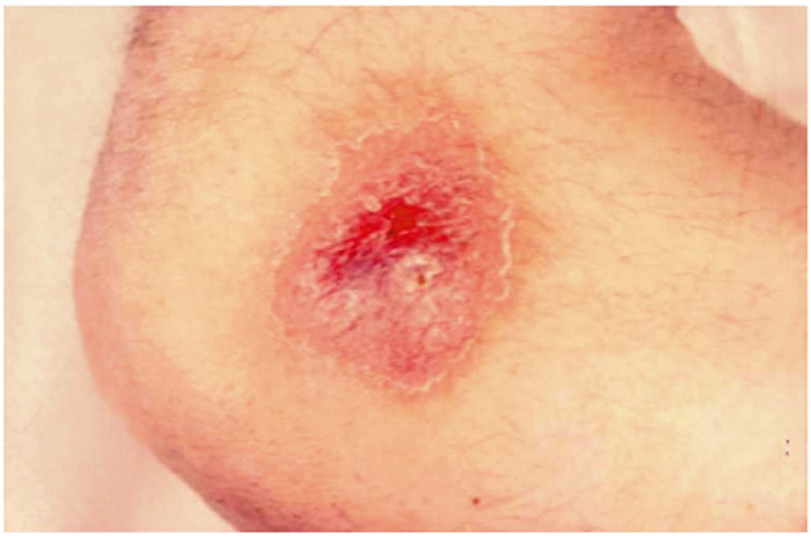

Figure 8. Trypanosomal chancre (*T.b. gambiense*)
Source: (Lejon et al. 2003).

I.7.2 Laboratory diagnosis

Laboratory diagnosis of HAT is based on the accurate identification of blood parasites in patientsô blood, lymph and cerebral spinal fluid (CSF) sample. It is mainly based on parasitological detection tools, molecular and serological methods. For example, the Card Agglutination Test for Trypanosomiasis (CATT) is used as a mode of screening those positive for HAT from the negative ones in endemic areas. CATT detects the presence of produced antibodies against *T.b. gambiense* in patientsô blood.

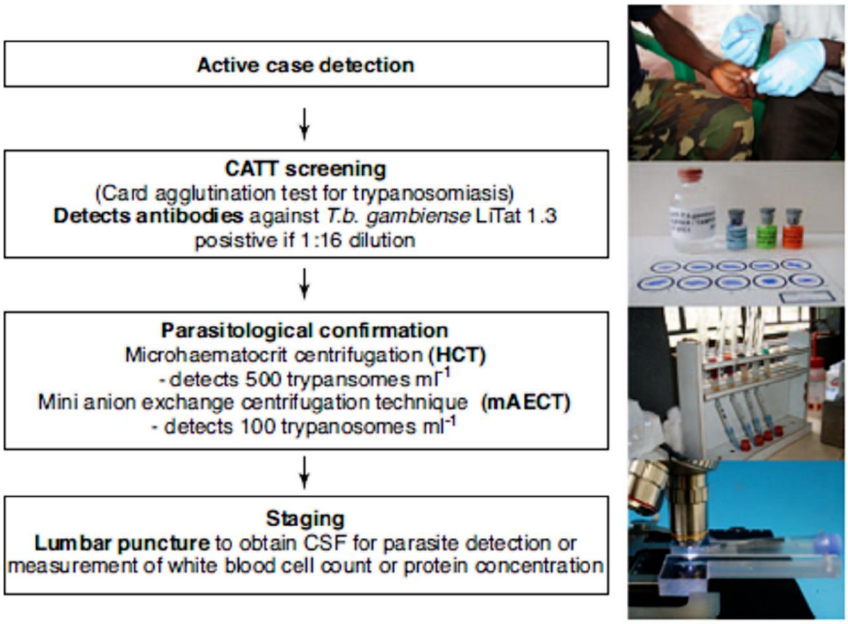

Figure 9. Staging of *T.b. gambiense*

Source: (Wastling & Welburn 2011).

I.7.2.1 Serological diagnosis

Specific immunoglobulins IgM and IgG are usually produced in high concentration following the *T.b. gambiense* infection. They are directed against the parasiteôs VSG antigen. Therefore, there are a number of techniques put in place for the serology based diagnosis of HAT; we can mention the CATT/*T.b. gambiense*, agglutination test, enzyme-linked immunosorbent assay (ELISA), Western blot, immunotrypanolysis and immunofluorescence test (IFAT) (Rebeski et al. 2001). The most efficient serological diagnosis tool is the CATT in *gambiense* HAT based on the *T.b. gambiense*ôs LiTat 1.3 antigen (Magnus, Vervoort, & Van Meirvenne 1978).

CATT is a simple and quick type of agglutination test for detecting specific antibodies to *T.b. gambiense* in the patientôs blood, serum or plasma. The antigen used for CATT test is produced from the bloodstream trypanosome forms of *T.b. gambiense* LiTat 1.3 variable antigen. The extracted trypanosomes will then be subject to fixation, staining with coomassie blue and then freeze-dried. The test kits contain the reagent, control sera, a 12V DC-card rotator and a 220V AC power source or a 12V car battery in case of field survey.

The test consists in mixing one drop of the reagent with one drop of the blood sample and shake of the mixture on the card rotator for 5 minutes. The test result (either positive or negative agglutination) can be macroscopically read on the card.

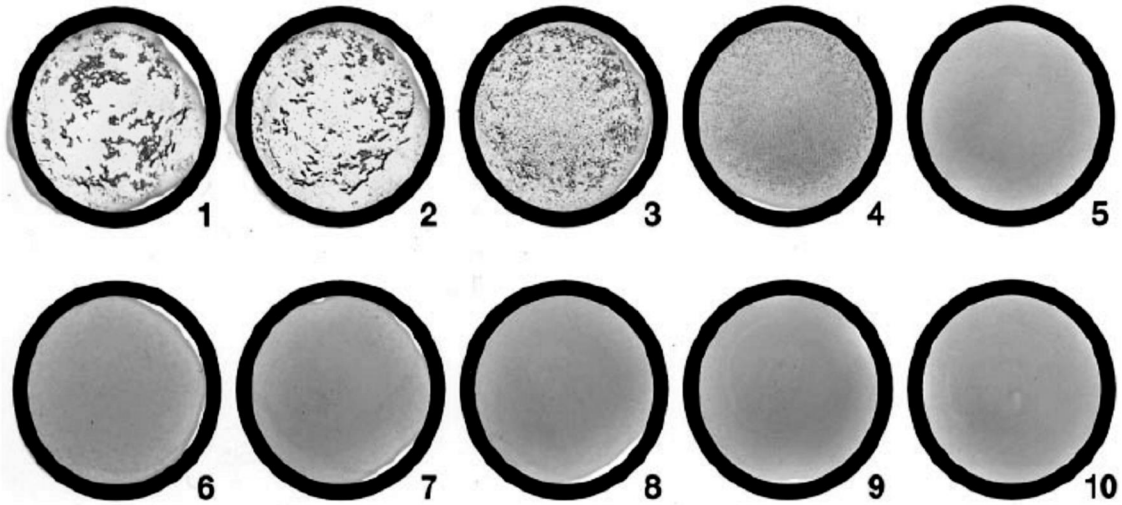

Figure 10. An example of CATT test results carried out on 10 times diluted serum sample. Source: (Chappuis et al. 2005a)

From the figure above, we observe that samples 1 to 3 are strongly positive, whereas sample 4 is weakly positive. Sample 5 to 10 are strongly negative.

The test has a high sensitivity 85% to 98% and a specificity of 95% on undiluted whole blood. However, it can result into false-negatives for patients infected with *T.b. gambiense* strains deprived of LiTat 1.3 gene. Therefore, in the latter case, an alternative serological test is needed. In case of low blood dilution <1:4 for instance, agglutination can be inhibited. This is called prozone and it is due to complement factors affecting the test sensitivity.

The Latex/*T.b. gambiense* technique uses a combination of three purified antigens LiTat 1.3, 1.5 and 1.6 coupled to latex particles. Latex test shares some similar steps with CATT such as the use of a rotator. Contrary to the CATT, latex presents a high specificity of 96% with similar sensitivity to CATT (Büscher et al. 1999).

The immunofluorescence assay has been used in some endemic countries such as Equatorial Guinea and has been successful with a high sensitivity and specificity. They use a standardised antigen and a serum or filter paper eluate as sample (Chappuis et al. 2005b).

For the ELISA, samples can be serum, filter paper eluate or cerebrospinal fluid (CSF). The type of ELISA used in *T.b. gambiense* diagnosis relies on VSG conferring an immunogenic reaction to antibodies in patientôs blood sample (Holland et al. 2002).

The immune trypanolysis test is the reference serological test used for *T.b. gambiense* serodiagnosis (Jamonneau et al. 2010). This test is based on the complement-fixation principle and requires live trypanosomes, patient blood sample and guinea pig serum; and it is advantageous due to its high specificity and the fact that the sample can be on filter paper (Van Meirvenne, Magnus, & Büscher 1995).

Most of the currently used serological tests can at least detect antibodies after 3 or 4 weeks of infection. This becomes again an obstacle as antibodies can still persist in a patient up to 3 years after treatment; this leads to false positive cases. Therefore, results of any antibody detection test should always be supplemented by analysis of an historical background of the patient. In addition, the microscopy should also be carried out on the same sample to confirm the presence of the parasitic agent.

Following the use of CATT which led to a decreasing prevalence of trypanosomiasis, rapid diagnostic tests have been developed to be used in primary health centres and in rural areas marked by poor conditions for the efficient use of CATT.

One rapid diagnostic test (RDT) for *gambiense* HAT, SD BIOLINE HAT, was developed and deployed in the field by the foundation for Innovative New Diagnostics (FIND) and standard Diagnostics (SD). It uses native VSG LiTat 1.3 and LiTat 1.5 to detect antibodies (IgG, IgM and IgA) to *T.b. gambiense*. It is tested in either serum, plasma or whole blood and gives results in 15 minutes (http://www.standardia.com/en/home/product/rapid/infectious-disease/HAT.html).

Two other rapid diagnostic tests for *gambiense* HAT have been developed and evaluated so far for *T.b. gambiense*: the HAT-Sero-K-SeT and the HAT Sero-Strip. On one side, the HAT Sero-strip is a dipstick, whereas the HAT-Sero-K-SeT is in a lateral-flow device. The two tests can be performed on blood (30 µl) or plasma (15 µl) and they rely on variant surface glycoproteins of *T.brucei gambiense* variable antigen types LiTat 1.3 and LiTat 1.5 that they contain. The results can be provided in 15 minutes (Büscher, Gilleman, & Lejon 2013).

Test	Specimen	HAT	Control	Sensitivity (95% CI)	Specificity (95% CI)
		number		*percent*	
Trypanolysis LiTat 1.3	Plasma	198	99	98.5 (96.3–100)	100 (100–100)
Trypanolysis LiTat 1.5	Plasma	198	99	98.5 (96.3–100)	100 (100–100)
HAT Sero-Strip	Plasma	198	99	98.5 (96.3–100)	96.0 (91.0–100)†
HAT Sero-Strip	Blood	198	99	97.5 (94.7–100)	98.0 (94.4–100)
HAT Sero-*K*-SeT	Blood	99	99	93.9 (87.9–99.9)†	99.0 (96.5–100)

* CI denotes confidence interval.
† The result was significantly lower than that for immune trypanolysis (P<0.05 by the chi-square test).

Table 1. Sensitivity and Specificity of Tests for Human African Trypanosomiasis (HAT), according to reactions with plasma and reconstituted samples.
Source: (Büscher, Gilleman, & Lejon 2013).

Comparing immune trypanolysis to the HAT Sero-Strip, the latter shows a better sensitivity and a slightly lower specificity in plasma (p=0.005). In the case of reconstituted blood, the two test didnôt show any difference (P>0.05 for both comparisons); for the HAT Sero-K-SeT, the situation was a bit different: sensitivity was lower (P=0.01), but the specificity was not significantly different (P=0.32) compared to the immune trypanolysis. Therefore, the HAT Sero-K-Set and the HAT Sero-Strip are valuable tools in the control of trypanosomiasis.

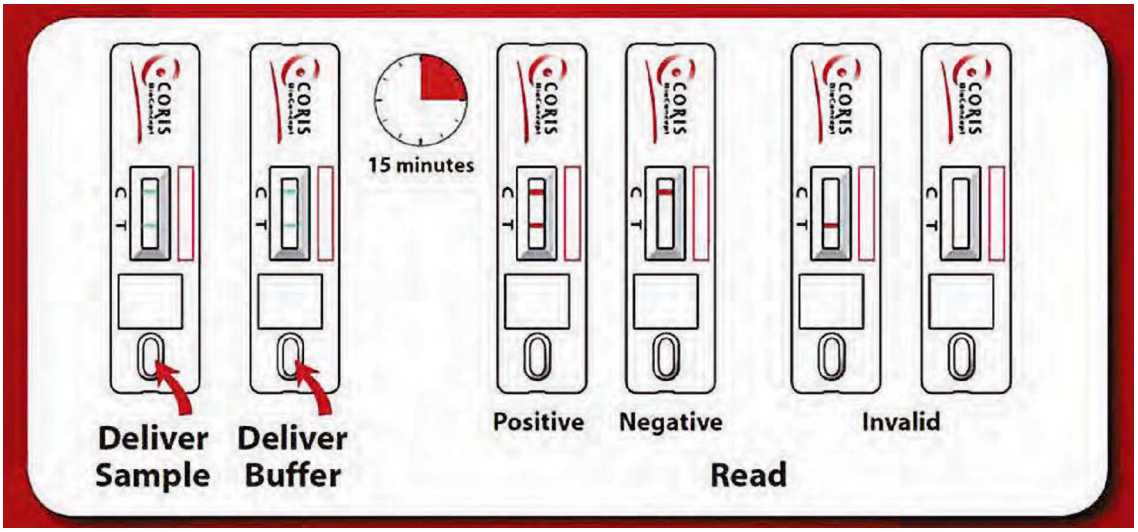

Figure 11. HAT Sero K-SeT.
Source: http://www.corisbio.com/Products/Human-Field/Human-African-Trypanosomiasis.php

At the same, The HAT Sero-K-SeT shares another similarity with both CATT and immune trypanolysis of using a native antigen VSG; CATT uses LiTat 1.3 whereas HAT Sero-K-SeT uses LiTat 1.5 in addition to LiTat 1.3.

HAT Sero-K-SeT is well adapted in health facilities with a low number of suspect cases and with no enough power (electricity) supply (Büscher et al. 2014).

The next generation serodiagnostics for *T.b. gambiense* use recombinant antigens and mimotopes. Recombinant expression of *T.b. gambiense* VSGs LiTat 1.3 and LiTat 1.5 was proven successful in *Pichia pastoris*; and the expressed antigens have shown a diagnostic potential in ELISA tested on *gambiense* HAT samples (Rogé et al. 2014). On the other side, short synthetic peptides mimicking epitopes of *T.b. gambiense's* antigens have been developed. Synthetic biotinylated peptide for VSG LiTat1.3 and VSG LiTat1.3 have been investigated and have shown a potential to be incorporated in serodiagnostic tests for *T.b. gambiense* (Van Nieuwenhove et al. 2013).

I.7.2.2 Parasitological detection

Parasite detection can only be done by microscopy. Samples used in this case are usually blood, lymph and cerebrospinal fluid. This common diagnostic method presents some inconveniences like the difficulty to distinguish between *T.b. rhodesiense* and *T.b. gambiense*. Microscopy is also less sensitive with a detection limit of 10,000 trypanosomes per ml. However, it has been evidenced that the sensitivity can be improved by concentrating the parasite (Magnus, Vervoort, & Van Meirvenne 1978). Parasite concentration in a sample is accomplished by haematocrit centrifugation (HCT). A better microscopic examination requires a preliminary separation of parasites from red blood cells by a mini anion exchange centrifugation technique (mAECT). It allows an unusual low detection down to <30 trypanosomes per ml (Lumsden et al. 1979).

I.7.2.3 Molecular diagnosis

In *gambiense* HAT, PCR is considered as not only a specific but also sensitive diagnostic method. Both nested and single round PCRs have been put in place to detect at least 10 trypanosomes per ml (Radwanska et al. 2002b). In large scale surveys, blood sample are the first collected and stored on Whatman FTA cards (Cox et al. 2010).

Isothermal amplification methods have been developed following limitations in remote areas such as a need for a thermocycler and electric power.

There are two isothermal amplification techniques: Loop-mediated isothermal amplification (LAMP) and Nucleic acid sequence based amplification (NASBA).

LAMP assays have been developed for an isothermal amplification of specific sequences in PFRA gene and repetitive insertion mobile element (RIME) (Kuboki et al. 2003;Njiru et al. 2008), it uses a specific strand-displacement polymerase and requires an incubation period of one hour at 58-65ºC with a termination at 80ęC. The RIME LAMP is characterised by a detection limit of 0.001 parasites per reaction, which is approximately 10,000 fold lower than in the case of PFRA gene LAMP. The same low detection limit was reached using the TgsGP (Njiru et al. 2011). A high sensitivity of more than 90% of the assay was reported (Matovu et al. 2010).

A similar isothermal technique detecting the RNA has been developed and applied to diagnosis of *T.b. gambiense*. The nucleic acid sequence-based amplification (NASBA) assay relies on the overall activity of reverse transcriptase, T7 RNA polymerase and RNase to amplify target RNA sequence without using thermocycler. Two forms of NASBA targeting the 18s ribosomal RNA have been developed: The real-time NABSA assay and a lateral flow (Mugasa et al. 2009). The detection limits of the NASBA assays fall in the same range as the RIME LAMP. One of the limitations of NASBA assays is that there is always a possibility of RNA degradation by nucleases and hydrolysis.

A novel RNA based diagnostic test was recently developed; this is the spliced-Leader (SL) RNA assay. A lower detection limit of 100 parasitesô SL-RNA per millilitre of blood was observed; with a sensitivity and specificity of 92% and 96% respectively. The advanced advantage of the technique is that it detects the parasiteôs mRNA as the best marker for a living parasite (Gonzalez-Andrade et al. 2014).

According to Mugasa et al. (2012), most of the currently available molecular diagnostic tests have sensitivities falling between 82% and 100%, whereas their respective specificities fall between 59% and 100% (Mugasa et al. 2012). There is no single molecular test that promising at the clinical facilities in endemic areas. The main reason is that most of the endemic areas for HAT are in remote areas where diagnosis means are scarce.

I.8 TREATMENT of *T.b. gambiense*

The treatment of *gambiense* HAT presents some problems as the current drugs are toxic to the patients. As a matter of fact, it should always follow the assessment of the disease stages: lymph, blood and/or CSF HAT stage. HAT can be classified among the latter stages based on the examination of CSF. The disease will be classified under stage 1 if the CSF examination results into a white cell count of less or equal to 5 and trypanosome count of zero per µl (Weisser & Hall 2009). For stage 2, the examination results in a white blood cell and/or trypanosomes count of more or equal to 20 per µl.

For patients diagnosed with a first stage infection, they get an intramuscular pentamidine, for a daily administration in 7 to 10 days. In case of second-stage of disease, melarsoprol can be of use for treatment. On the other hand, patients diagnosed with second-stage of disease can also be treated with eflornithine and is safer than melarsoprol; but it has to be administered as intravenous infusions, four times a day in 14 days. However, the treatment for the two stages of disease presents side effects of encephalopathy which may also lead to fatality rate of 2 to 10% in case of melarsoprol and less than 1% in case of pentamidine (Chappuis, Loutan, Simarro, Lejon, & Büscher 2005a).

In 2009, the nifurtimox-eflornithine combination therapy (NECT) was introduced to replace the eflornithine monotherapy (Alirol et al. 2013;Eperon et al. 2014). Referring to the data provided by different clinical trials, the combination therapy has shown a clear safety and efficacy in endemic areas. As a matter of fact, it has been accepted as an essential medicine by WHO; and it is the currently recommended therapy for the second stage of *gambiense* HAT (Alirol, Schrumpf, Amici, Riedel, de, Quere, & Chappuis 2013;Steinmann et al. 2015). It is true that NECT has played an important role in treating patients. However, there have been reported difficulties in deploying and using the same combination therapy in rural endemic areas (Eperon, Balasegaram, Potet, Mowbray, Valverde, & Chappuis 2014). Therefore, new oral compounds: flexinidazole and oxaborole SCYX7158 have been developed and are in clinical trials. The two drugs are now being evaluated through different clinical trials and are expected to be submitted for approval in 2016 and 2017 respectively for fexinidazole and Oxaborole SCYX-7158 (Eperon, Balasegaram, Potet, Mowbray, Valverde, & Chappuis 2014;Steinmann, Stone, Sutherland, Tanner, & Tediosi 2015).

I.9 RESEARCH PROBLEM AND AIM

As it has been discussed under the section of diagnosis above, it is clear that different diagnostic tools have been developed so far; with microscopy remaining a primary diagnostic tool for parasite detection. However, despite the fact that different molecular diagnostic tools have been largely investigated and developed, they are more of use in research settings than in clinical practice; because they are expensive and require sophisticated equipment and many skills.

There are accurate serological tests available for the diagnosis of *T.b. gambiense* HAT, leading to a remarkable decrease in *gambiense* HAT prevalence. However, these tests may be hindered by the antigenic variation of the parasite. Indeed, not all T.b. gambiense strains have the immunodominant LiTat 1.3 and LiTat 1.5 VSGs. Therefore, for eliminating the HAT by 2020 as set by WHO, it is necessary to develop additional serological tests based on other antigens. In addition, the current RDTs are based on native antigens and the production of such antigens is expensive, time consuming and laborious. In this work, we have developed a system for the recombinant expression of the *T.b. gambiense* specific TgsGP protein and evaluated its diagnostic potential. This will result into a *T.b. gambiense* specific test that is appropriate in the case of small numbers of suspects reporting at the health centres and in endemic areas where there is usually not enough electricity supply.

I.10 OBJECTIVES

I.10.1 General objective

The general objective of our study is to develop a recombinant TgsGP as a diagnostic antigen for serodiagnosis of *gambiense* HAT.

I.10.2 Specific objectives

-To recombinantly express TgsGP in *E. coli.*

-To purify the recombinant TgsGP by affinity chromatography and size-exclusion.

-To determine and evaluate the diagnostic potential of the recombinant TgsGP in ELISA.

CHAPTER II. MATERIALS AND METHODS

1. TgsGp gene sequence

The complete nucleotide sequence of the TgsGP gene (Gibson, Nemetschke, & Ndung'u 2010) was retrieved from NCBI (with accession number FN555993). The sequence was translated into an amino acid sequence with the online translation tool òtranslateò (http://web.expasy.org/translate/). In the TgsGP protein sequence, sequences for glycosylphosphatidylinositol (GPI) anchor and signal peptide (SP) were predicted using PredGPI (http://gpcr.biocomp.unibo.it/predgpi/pred.htm) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) online tools respectively, and were cut out. The resulting TgsGP protein sequence was then submitted to the GenScript company (GenScript, USA). GenScript made the gene sequence codon optimised for *E. coli* and provided a pET22b(+) vector containing the optimized TgsGP gene.

```
Optimized      6   GAAGATTCTGCGGGCGAAAATGGCGGCACCTATGCTGCTCTGTGTACCCTGCTGACGGAA
Original       6   GAGGACAGTGCAGGCGAAAATGGCGGCACGTACGCAGCCCTCTGTACACTGCTAACAGAA

Optimized     66   GCTCTGGGCGAAGTGGACCAAGCTCAACCGACCAAAGGTTGGGAACAGGCTTATGCGAGC
Original      66   GCACTTGGTGAAGTTGACCAAGCGCAGCCCACTAAAGGATGGGAACAGGCATATGCTTCT

Optimized    126   ATTCTGGAAGCCAACATGTCTGCGGCCGGTCCGGATTGGCGTAATCAGTTTGTCAGCTCT
Original     126   ATTCTTGAAGCCAACATGTCGGCAGCGGGACCGGATTGGAGAAACCAGTTCGTCAGCAGC

Optimized    186   AAAGGCGTGAAACAAGAATGGGAACCGACCGCCAAACATAAAGCCGTTGCAGAAGCTTGG
Original     186   AAAGGTGTTAAGCAGGAATGGGAGCCAACAGCGAAGCACAAAGCCGTAGCGGAGGCATGG

Optimized    246   GCGCGCTCATATGCGGGTTGGATTAACACCGCTCTGGTCCTGTATGCGGGCGGTTCGGAT
Original     246   GCGCGTAGCTACGCGGGATGGATCAACACAGCACTCGTCCTTTATGCAGGCGGCAGCGAC

Optimized    306   GACCGTAAACGCGCGATCTCAAAATTCGACTCGATGGGCGATGCCACCCGTAAACTGGCA
Original     306   GACAGGAAACGAGCGATCAGCAAATTTGACAGCATGGGAGATGCAACTCGCAAGCTAGCA

Optimized    366   CAGCGCAAACTGGAAGCTATCCTGGCGAAAGTTCAACCGCTGCGTAGTAAACTGTCCGCC
Original     366   CAGCGGAAGCTGGAAGCCATTTTGGCAAAAGTGCAGCCGCTGAGAAGCAAGCTCAGTGCA

Optimized    426   CTGAAAGCAGTGGTTGAAGCTGGCACCGGTAAAGCGGTGACGGACCTGCTGAAAGCAGCT
Original     426   CTTAAAGCGGTAGTAGAAGCGGGAACGGGCAAGGCTGTCACGGACTTGCTCAAGGCGGCG

Optimized    486   CTGTATGGCGGTATTGATGGCGGTTCAGATTTTGAAGACGCGACCAAAGATAAAGACGGT
Original     486   CTCTACGGCGGTATCGACGGCGGCAGTGACTTCGAGGACGCGACAAAGGACAAGGACGGC

Optimized    546   GAACGTGTTCGCGGCATCTGCAAAGCGGCCGGTAAAGTTAAAGGCAACCAGACCCTGGCC
Original     546   GAACGTGTGCGAGGCATCTGCAAGGCCGCCGGAAAAGTGAAGGGTAACCAGACACTGGCA

Optimized    606   GATGTCCTGCTGTGCGTCTGTGTGACGGCAGTGTCCTACGGCGATGACGGTAATAAGAAA
Original     606   GATGTGCTGCTTTGCGTATGCGTAACCGCAGTAAGCTACGGCGACGACGGCAACAAAAAA

Optimized    666   ATTTGCGCCAAACTGAGTGGCAAACGTGGTGCAAAACAATGGGATCTGTCCGACCGCGGT
Original     666   ATTTGTGCCAAACTGAGCGGTAAGCGCGGAGCGAAGCAGTGGGACCTTAGCGACAGGGGC

Optimized    726   GATGTGGCAGCTGTTTTTGGTGAACTGCGTCAGGGCTGTAATAGCAAACAAGAACACAAA
Original     726   GATGTCGCTGCTGTGTTCGGAGAGCTCAGACAGGGCTGTAATAGCAAGCAAGAGCACAAA

Optimized    786   ACCACCGCTGGCGGTATCCGTGCCGCACTGGCCACCATTCGCTCTAAATTCCAGATCGAT
Original     786   ACCACAGCAGGCGGGATCAGGGCGGCCCTGGCGACGATAAGGAGCAAATTCCAAATTGAC

Optimized    846   GGCGACAACGGTTATCTGGGCCGCTACGATACGGATGGTAATTGTACCGGTACCGCACCG
Original     846   GGGGACAACGGCTATCTAGGAAGGTACGACACCGACGGCAACTGCACAGGAACGGCGCCA

Optimized    906   GGCGGTGTTTGTGTCAAATATGCGGGCTACGGTACCAACACGGGCAATGGTTGGCATGAT
Original     906   GGCGGTGTCTGCGTTAAATATGCCGGCTACGGCACCAACACTGGGAACGGTTGGCACGAT

Optimized    966   ATTCAGTGGGTGCGTCATGCCACCGCAGCTGCCGCAGCAATTGAAGCGGGTGCACGCGCT
Original     966   ATTCAATGGGTCAGGCATGCAACGGCCGCAGCTGCAGCAATTGAGGCAGGAGCCCGAGCG

Optimized   1026   GCCAGCACGATGGCCGCACTGGAACCGCTGCTGGAAGCTGCCGCCGTGGAAGCCTGGGAA
Original    1026   GCAAGCACGATGGCAGCGCTGGAGCCCCTGCTAGAAGCAGCGGCGGTTGAAGCTTGGGAA

Optimized   1086   GTTGCCAATACGACCGCCTCTAAT
Original    1086   GTGGCAAACACCACAGCAAGCAAT
```

Figure 12. Original and optimised fragment of TgsGP gene sequence; with the red color showing the optimized region.

2. Recombinant expression in *E. coli*

2.1 *E. coli* cells

E. coli Rosetta and *E. coli* Origami cells have been used to recombinantly express TgsGP (rTgsGP). These two *E. coli* strains present different but important characteristics in recombinant gene expression. Rosetta strain is known to enhance expression of rare eukaryotic codons such as AUA, AGG, AGA, CUA, CCC and GGA in *E. coli*; whereas Origami strain enhances disulfide bond formation as it has mutations in the glutathione reductase and thioredoxin reductase.

2.2 Expression vectors

For recombinant expression experiments we used the pET-22b(+) vector (Novagen, USA) (Figure 12). This vector presents advantages for the expression of recombinant proteins such as multiple cloning sites and a strong promotor, T7 lac, which allows regulation of expression recombinant proteins by induction with isopropyl ß-D-1-thiogalactopyranoside (IPTG). In addition, pET-22b(+) has a signal sequence (pelB leader) for secretion of expressed protein to the periplasm; a C-terminal His-Tag sequence for affinity purification; f1 origin of replication and confers ampicillin resistance to host cells.

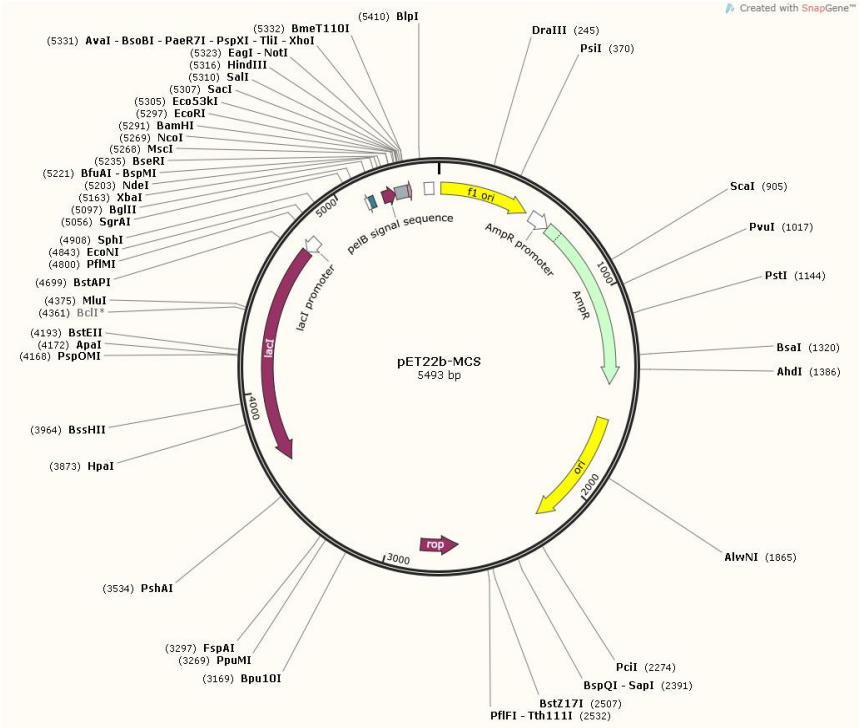

Figure 13. The pET-22b(+) vector; generated with SnapGene Viewer 1.1.3

The vector pET-22b(+) illustrated above presents important parts such as multiple cloning sites, strong promoter T7 lac, pelB leader (signal sequence), a C-terminal His-Tag sequence, F1 origin of replication and ampicillin resistance gene.

2.3 TgsGP constructs engineering

The TgsGP gene was cloned under two forms: rTgsGP with signal peptide (rTgsGP-SP) and rTgsGP without signal peptide (rTgsGP-qSP). The two TgsGP constructs are presented in Figures 14 and 15. The rTgsGP-qSP was derived from the rTgsGP-SP construct provided by GenScript (USA) using the restriction enzymes NdeI (CA/TATG) and Xhol (C/TCGAG) and the In-Fusion HD cloning kit (Clontech, USA) (Figure 16). The empty pET-22b(+) was subjected to double digestion with NdeI and Xhol by cutting out the pelB leader (figure 15). For the In-Fusion PCR, TgsGP specific primers (forward primer: 5ô-AAGGAGATATACATATGGAAGATTCTGCGGGCGA-3ôand reverse primer: 5ô-GGTGGTGGTGCTCGAGATTA-3ôwith 15bp extensions homologous to pET-22b(+) vector ends were designed to amplify the TgsGP gene. Two µl cloning enhancer was added to 5 µl PCR products and incubated for 20 minutes at 80ęC.

The In-Fusion cloning reaction was done by mixing 2 µl In-Fusion enzyme premix, 1.5 µl linearised pET-22b(+), 1 µl TgsGP and 5.5 µl H_20 in the same eppendorf tube. The resulting cloning reaction mixture was incubated at 50ęC for 15 minutes. In principle, the linear pET-22b(+) will integrate at the site of 15bp overlap with a TgsGP amplicon by homologous recombination. This resulted in a recombinant TgsGP without signal peptide (rTgsGP-ęSP).

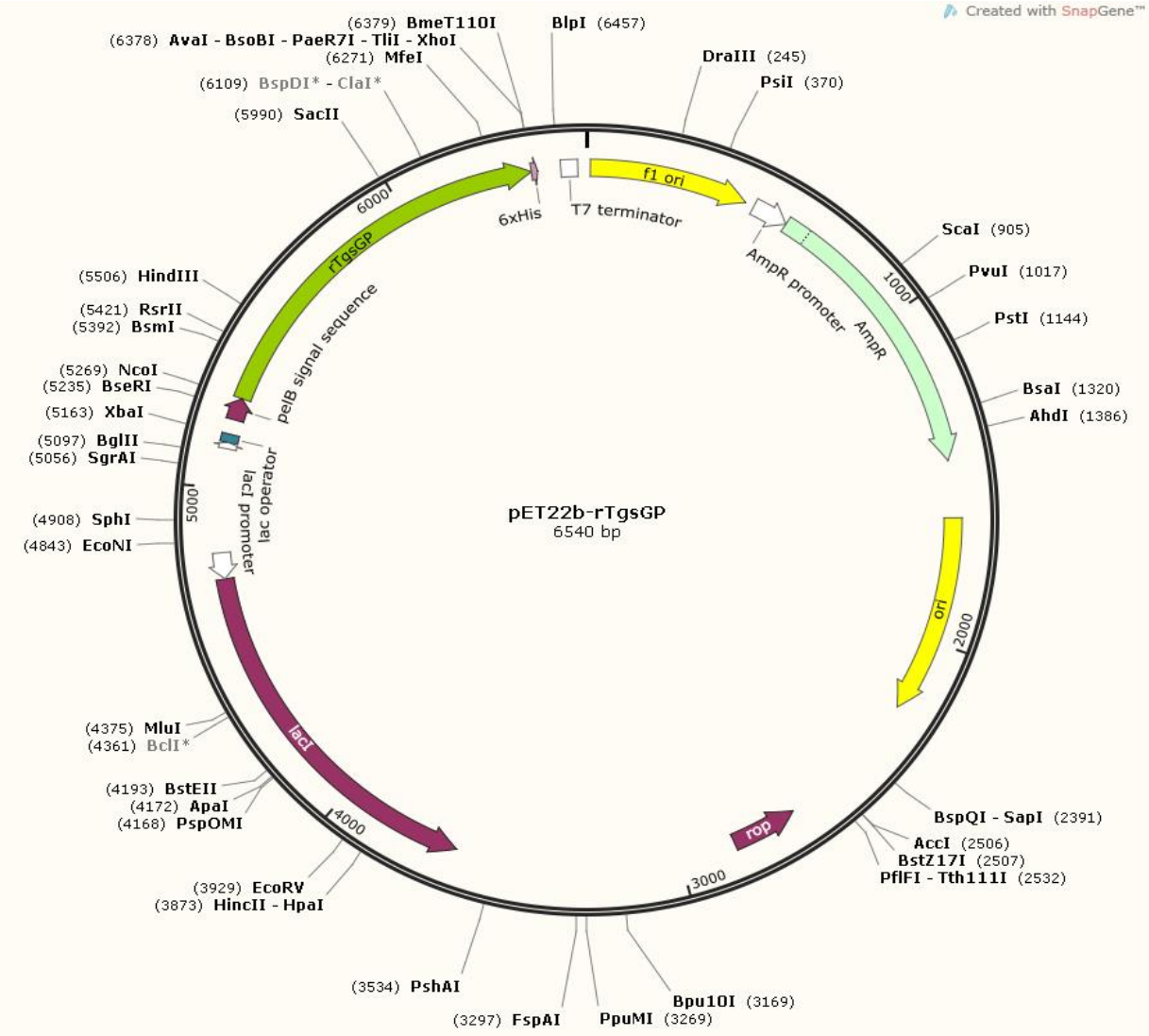

Figure 14. The TgsGP gene cloned in pET-22b(+) vector with signal peptide (rTgsGP-SP); generated with SnapGene Viewer 1.1.3

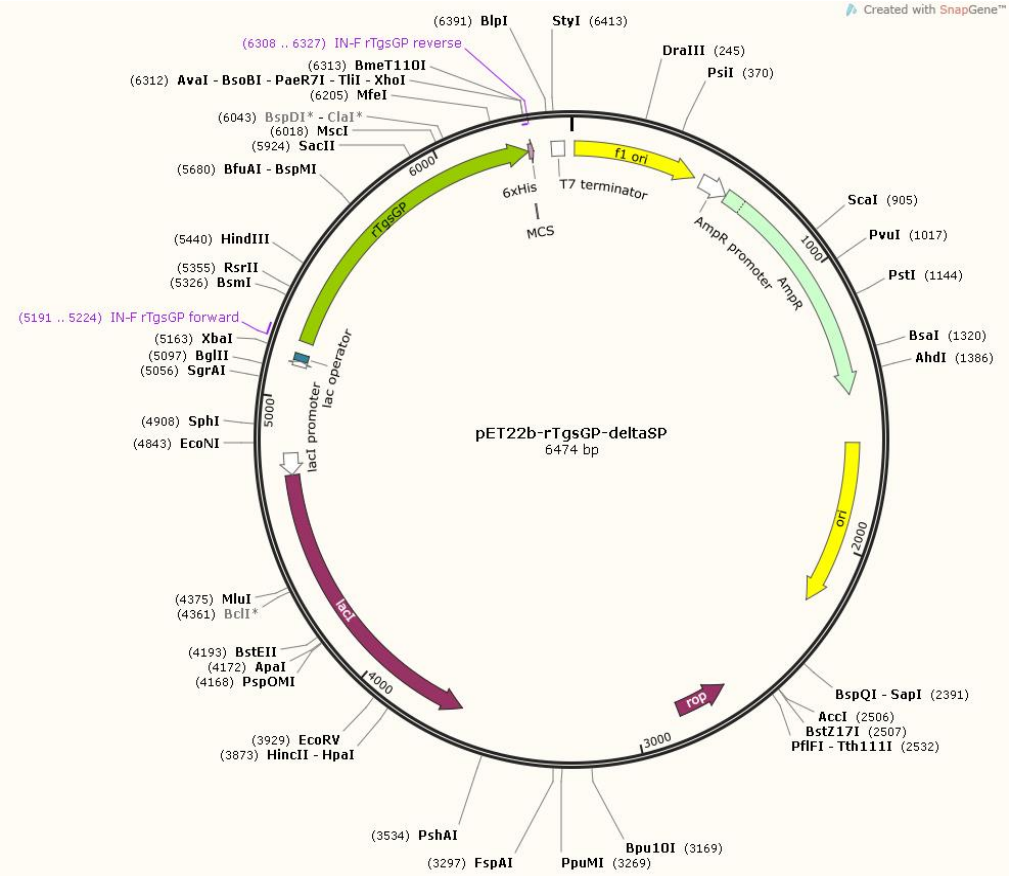

Figure 15. The TgsGP gene cloned in pET-22b(+) vector without signal peptide (rTgsGP-ΔSP); generated with SnapGene Viewer 1.1.3

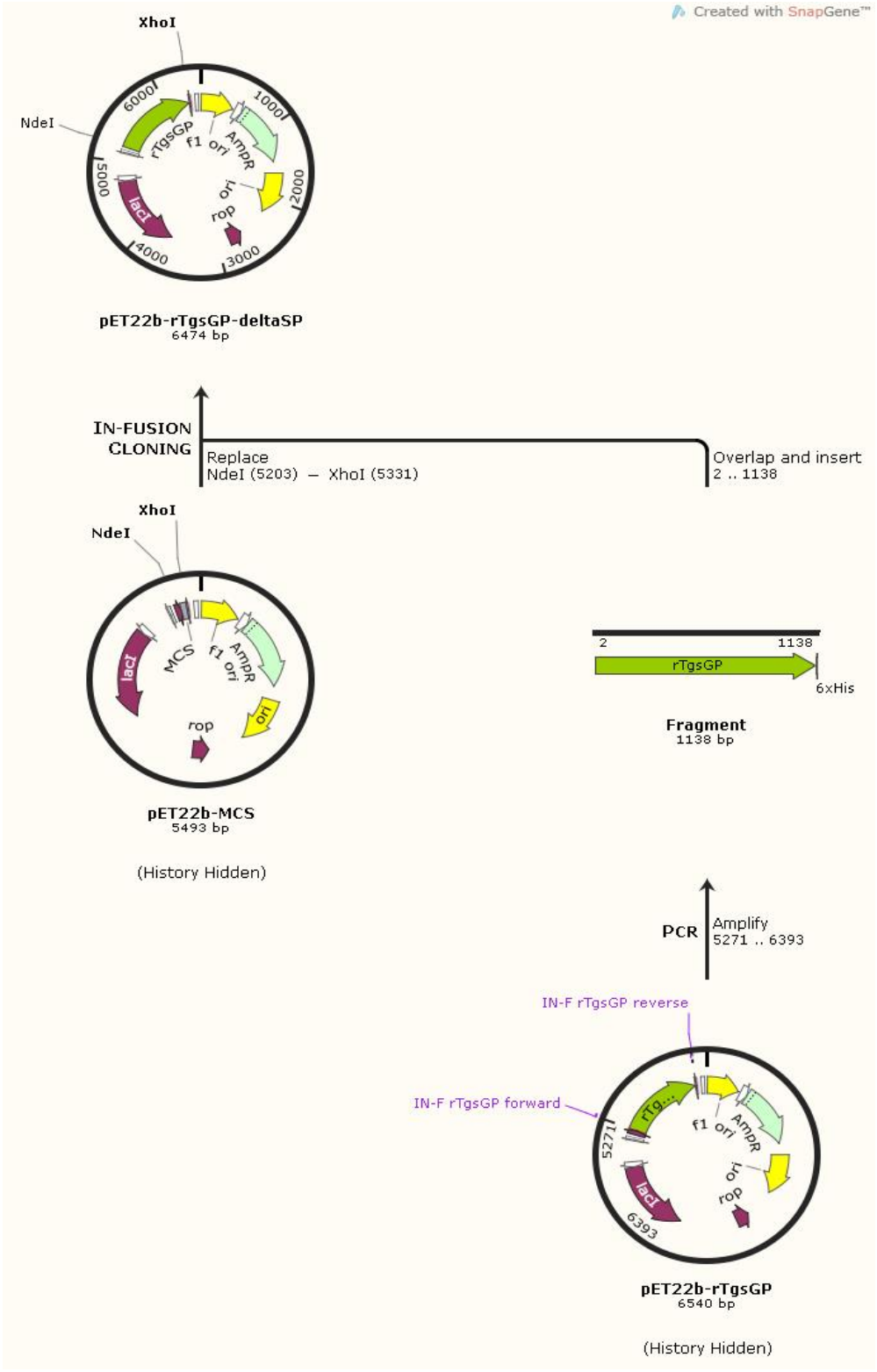

Figure 16. The TgsGP-ΔSP construct engineering through In-fusion PCR; generated with SnapGene Viewer 1.1.3

2.4 Transformation of *E. coli* cells

The TgsGP gene constructs were transformed in stellar competent *E. coli* cells grown on selective Luria Bertani (LB) medium with appropriate antibiotics following the transformation protocol (Novagen, USA). To identify transformed cell colonies with the right insert (either TgsGP-SP or TgsGP-qpSP), a colony PCR (cPCR) was run directly from colonies grown on selective LB agar plates supplemented with a 100 µg/ml carbenicillin. The insertion of a known TgsGP size (1115 bp) in a pET-22b(+) vector by using specific primers that bind on either side of the insertion was checked on agarose gel with ethidium bromide under UV exposure.

2.5 Protein expression

Successfully transformed cells containing the vector pET-22(b+)-TgsGP were grown in 5 ml LB medium with 100 µg/ml carbenicillin; and the culture was incubated with shaking at 37°C/250 rpm overnight. The next morning, the overnight culture was transferred to a fresh 500 ml LB medium containing carbenicillin antibiotic and incubated back with rotation at 200 rpm to reach an optical density (OD_{600}) of 0.6 required for induction. When OD of 0.6 was reached, a 50 µl aliquot was collected as a non-induced control sample in gel analysis; and 1M IPTG (with a 1:1000 dilution factor) was added to the rest of the culture to induce the expression of rTgsGP. The culture was then incubated overnight at 37°C, 200 rpm whereafter, a 50 µl aliquot was collected for gel analysis.

2.6 Denaturation and solubilisation of inclusion bodies

The rest of induced culture was centrifuged at 4°C, 3,500 g for 15 minutes to harvest the *E. coli* cells pellet. The cell pellets were resuspended in 8 different buffers: (A) sodium carbonate-sodium bicarbonate pH 9.9 + 6M GuHCl, (B) TrisHCl pH 6.8 + 500 mM NaCl, (C) TrisHCl pH 6.8 + 1 mg/ml lysozyme + Tween 20, (D) TrisHCl pH 6.8 + 1 mg/ml lysozyme + 1.5M NaCl, (E) TrisHCl pH 6.8 + 1 mg/ml lysozyme, (F) sodium carbonate-sodium bicarbonate buffer pH 9.9 + 1 mg/ml lysozyme + Tween 20, (G) sodium carbonate-sodium bicarbonate buffer pH 9.9 + 1ml/mg lysozyme + 1M NaCl, and (H) sodium carbonate-sodium bicarbonate buffer pH 9.9 + 1 mg/ml lysozyme.

All buffers included protease inhibitor (Thermo scientific) resuspended pellets were subjected to 3 freeze-thaw cycles, incubated on ice, and subsequently disrupted using a sonicator (Sonics and materials inc. Danbury, USA) at 60% amplitude, 1 minute on/off in 6 cycles. The homogenate was dispensed in 1.5 ml eppendorfs and centrifuged for 30 minutes at 13,000g at 5°C to remove cell debris. A 100 µl aliquot was taken from the supernatant and kept into a clean eppendorf on ice to serve as a soluble sample in gel analysis; and the rest of supernatant was discarded. The pellets were resuspended in the same 8 buffers mentioned above; and considered as an insoluble sample for gel analysis.

3. SDS-PAGE

SDS-PAGE was carried out through its three main standard steps: pouring the separation and concentration gels, loading samples and running the gel. SDS-PAGE was used to analyse both expression and purity of rTgsGP.

A 12 % acrylamide separation gel [SDS (10 %), H_2O, Tris-HCl 1.5 M (pH 8.8), protogel (National Diagnostics), ammoniumpersulphate (10 %), TEMED (BIO-RAD] was prepared and poured (4.50 ml) between the assembled glass plates. It was left to polymerise for 45 minutes before a 4% acrylamide concentration gel [SDS (10 %), H_2O, Tris-HCl 1 M (pH 6.8), Protogel, ammoniumpersulphate (10 %), TEMED (BIO-RAD) could be poured (2.0 ml) at the top; and combs were immediately dipped into to create wells and the gel was allowed to polymerise for 30 minutes. The two glass plates holding gels were then placed vertically into the electrophoresis chamber, Mini-PROTEAN® Tetra Cell (Bio-Rad) containing 10x diluted SDS-PAGE electrode buffer [0.25 M Tris base (pH 8.3), 1.92 M glycine, 1 % SDS].
A 5 µl sample was added to 5 µl SDS-PAGE reducing sample buffer [0.125 M Tris-HCl (pH 6.8), 4 % SDS, 20 % sucrose, 0.04 % bromophenol blue and 0.2 M ß-mercaptoethanol] and heated at 105°C for 5 minutes and then spinned down for 5 minutes, 13,000g. 10 µl of the resulting sample solution were loaded into each well including the molecular weight standards in one outside well. In case of ladders, 5 µl of either prestained SDS-PAGE Standards (6.9-210 kDa, Bio-Rad) or Precision Plus ProteinÊ DualColor Standards (10-250 kDa, Bio-Rad) were loaded. The gel was run at 200 V for 50 minutes (PowerPac^{Ê} HC Power Supply (Bio-Rad); and after electrophoresis, gels were used for either Coomassie brilliant blue staining or Western blot.

For coomassie staining, SDS-PAGE gels were submerged into 0.5% Coomassie brilliant blue staining solution [50 % dH_2O + 40 % methanol (Merck) + 10 % acetic acid (VWR) + 0.025 % CBB R-250 (Bio-Rad)] and left on a shaker (Labnet Orbit 1000; Biolegio). After 1 hour of staining, staining solution was discarded and replaced by a destaining solution [85 % dH_2O + 10 % acetic acid + 5 % denaturated ethanol (Disinfectol®, Chem-Lab)] and left on shaker for 30 minutes (50 rpm). To observe more clear protein bands, ultrapure water was used to continue destaining. Gels were then equilibrated in shrinking solution [2 % glycerol (Merck), 60 % methanol (Merck), 7 % acetic acid (VWR)] for 1 hour. Gels were then covered and held by clips in a drying frame by previously saturated cellophanes (BIOzym) in 10% glycerol *(*28.75 ml 87 % glycerol + 221.25 dH_2O). Gels were left to dry at room temperature.

To prepare Western Blot strips, a preparative gel electrophoresis was done with the same materials and procedures as SDS-PAGE. The only difference was based on the specific two wells comb dipped into the gel to generate one narrow lane for standard protein marker (5µl) and a wider lane for sample (100 µl). After electrophoresis, the preparative gel was subject either to Coomassie brilliant blue staining or Western blot.

4. Western blot analysis

The electrophoresis gel was placed on top of a filter paper (previously submerged in blot buffer) lying on the top of a fiber pad saturated with 1xblot buffer [10x Blot buffer: 0.25 M Tris, 1.92 M glycine (pH 8.3)] and covered by a previously submerged nitrocellulose membrane [Amersham Hybond™-P PVDF transfer membrane (GE Healthcare Life Sciences)] into 100% methanol for 10 seconds and subsequently submerged into ultrapure water). A piece of filter paper [(Amersham Biosciences)] was placed on top of the membrane followed by a second fiber pad and a roller was gently applied on the top of the resulting sandwich to remove any possible air bubbles that could be trapped in. The sandwich was then locked in a cassette and placed vertically in the already assembled blotter tank [Criterion$^{\hat{E}}$ Blotter (Bio-Rad)] filled up with 1x blot buffer and placed on a mixer. The tank lid was placed on and its power cables plugged into the power supply [PowerPac$^{\hat{E}}$ HC Power Supply (Bio-Rad)] and let run at 100 V for 30 minutes. The observation of colored markers on the membrane was a mark of a successful protein blotting.

The immuno-detection of proteins transferred on the membrane was started by blocking the membrane overnight at 4°C with TBS-Blotto [TBS 1x + 5 % skimmed milk powder (Fluka)]; followed by series of washing (3 x 5 minutes) with TBS (0.1 M Tris-HCl (pH 7.5), 2.5 M NaCl, 0.02 % NaN_3). After washing, the membrane was incubated for 1 hour at room temperature with a serum (Mouse Anti Histidine Tag alkaline phosphatase, ABD Serotec) diluted 1:200 in TTBS (TBS 1x + 0.05 % Tween 20) per membrane; followed by two series of washings: 5 minutes wash with TTBS (3 times) followed by a 5 minutes wash with AP-buffer [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 5 mM $MgCl_2$]. Finally, the membrane was incubated with 50 ml AP-buffer, 132 µl NBT (Sigma-Aldrich)*:* 1 g in 20 ml 70 % dimethyl formamide and 66 µl BCIP (Sigma-Aldrich)*:* 0.5 g in 10 ml 100 % dimethyl formamide at room temperature for around 15 minutes (until sufficient colour was produced). To stop the reaction, the membrane was washed for 5 minutes with ultrapure water; and then the membrane was let on the bench for drying. All the membrane immunostaining steps stated above were performed on an orbital shaker (Labnet Orbit 1000: Biolegio).

To perform western blot test strips, the same western blot materials and methods were generally followed. WHO HAT sera bank and Red Cross sera bank samples were used. Each 2 ml sample, diluted 1:100, was incubated with a membrane strip for 1 hour at room temperature within a reaction tray. A 2 ml volume of the conjugate [goat anti-human Ab-AP (Sigma-Aldrich, Belgium)], diluted 1:10000 TTBS, was added to each washed strip.

5. Purification by affinity and size-exclusion chromatography

Samples for purification were prepared by denaturation and solubilisation of inclusion bodies in cells pellet with a different buffer, 50 mM phosphate buffer at pH 7.4 + 500 mM NaCl + 6 M GuHCl. This is because the latter buffer is more compatible with HisTrapÊ excel column than the previously used GuHcl based buffer (see section 2.6 above).

The cells pellet was suspended in 50 mM phosphate buffer + 500mM NaCl at pH 7.4, with one tablet of protease inhibitor/10 ml (Thermo scientific) and 1 mg/ml lysozyme (Sigma). The resulting dissolved pellet was incubated at 37°C for 30 minutes and subsequently disrupted using a sonicator at 60% amplitude, 1 minute on/off in 5 cycles.

The homogenate was dispensed in 1.5 ml eppendorfs and centrifuged for 30 minutes at 13,000g at 5°C to remove cell debris. An aliquot was taken from the supernatant and kept into a clean eppendorf on ice to serve as a soluble sample in gel analysis; and the rest of supernatant was discarded. The resulting pellet washed with Triton-X-100: the pellet was suspended in 50 mM phosphate buffer at pH 7.4 + 500 mM NaCl with 1% Triton-X-100 and proteinase inhibitor (Thermo scientific). The dissolved pellet was sonicated at 60% amplitude, 1 minute on/off in 5 cycles. Then sonicated mixture was centrifuged for 30 minutes at 13,000g, 5°C to remove cell debris. An aliquot of the first Triton wash supernatant was kept for SDS-PAGE analysis. The resulting pellet was subjected to a second round of Triton wash, sonicated (at 60% amplitude, 1 minute on/off in 5 cycles) and centrifuged (for 30 minutes at 13,000g at 5°C); and at the same time, an aliquot of the second Triton wash supernatant was kept for SDS-PAGE analysis. The resulting pellet was subjected to two rounds of extraction: the pellet was dissolved in 50 mM phosphate buffer at pH 7.4 + 500 mM NaCl + 6 M GuHCl and sonicated at 60% amplitude, 1 minute on/off in 3 cycles; the resulting homogenate was then incubated overnight at 4°C on a rotator and centrifuged for 60 minutes at 13,000g, 5°C. Supernatant was collected at each round of extraction and kept as a sample (12 ml in total) for affinity chromatography with AKTA HPLC machine.

Purification of proteins was first done with AKTA HPLC machine based on affinity chromatography followed by size-exclusion chromatography.

5.1 Automated affinity chromatography

To purify the rTgsGP from insoluble samples (pellet), an automated affinity chromatography was carried out using the AKTA HPLC system supplemented with prepacked chromatography columns with Ni Sepharose excel and flow filters, operated with UNICORN software. The AKTA HisTrapÊ excel columns were pre-equilibrated with equilibration buffer (50mM phosphate buffer pH 7.4 + 500mM NaCl + 6 M GuHCl). The extraction supernatant was loaded on the column and the flow through sample fraction was collected labelled as "unbound" (UB). Columns were subsequently washed with a wash buffer (50mM phosphate buffer pH 7.4 + 500mM NaCl + 6 M urea + 20mM imidazole) and resulting sample was collected and labelled as "wash" (W).

To elute the His-tagged TgsGP, an elution buffer (50 mM phosphate buffer pH 7.4 + 500 mM NaCl + 6 M urea+ 0.4 M imidazole) was loaded on the column and an elution fraction, labelled as "eluted" (E), was collected to be analysed on SDS-PAGE together with the previously collected samples. We harvested 2.8 ml of his-tagged proteins.

5.2 Automated size-exclusion (gel filtration) chromatography

Eluted affinity chromatography fractions were further subject to gel filtration. The same AKTA purification system was used by switching to gel filtration columns. A 2. 8 ml elution sample were first concentrated with a 0.5 ml Amicon filter, with molecular weight cut-off of 3 kDa (MERCK) through a centrifugation at 1,000g for 8 hours; which ended up with 600 µl. A 500 µl concentrated rTgsGP sample was loaded on SuperdexÊ 75 10/300 GL. Eluted fractions were collected as 200 µl aliquots and run on SDS-PAGE to check the purity.

The protein concentration in the size-exclusion eluted fractions was measured with a Nanodrop^{TM} Spectrophotometer (ISOGEN Life Science).

6. ELISA

For ELISA, 640 µl purified antigen (0.25mg/ml) were diluted in 40 ml 0.01 M phosphate buffer pH 6.5, and coated with 100 µl/well in ELISA plates (MAXISORP NUNC). On each plate, half of the wells were left empty to serve as antigen-free wells. Plates were incubated at 4^{o}c overnight. Unbound protein was discarded and the plates were subsequently blocked with 350 µl/well of PBS-Blotto and incubated for 1 hour at room temperature followed by three times wash with 350 µl PBS-Tween. Sera were first centrifuged at 5°C and 3000 rpm for 5 minutes and diluted 1:150 with PBS-Blotto. 150 µl of the diluted serum were then added to an antigen-containing and an antigen-free well. After incubation for 1 hour at room temperature, all wells were washed three times with 350 µl PBS-Tween before a second incubation was done with a conjugated antibody. A 150 µl volume of the conjugate [goat anti-human IgG (H+L)-peroxidase (Jackson ImmunoResearch) diluted 1:80000, was added to each plate well and allowed to incubate at room temperature for 1 hour; followed by a 3 times wash with 350 µl PBS-Tween. All plate wells were then incubated for 1 hour at room temperature with a 150 µl 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid: ABTS (Roche Diagnostics) per well with a dilution factor of one tablet in 100 ml PBS-Tween.

The production of green colour in a well was an indication of presence of TgsGP antigen specific antibodies in serum. Finally, the absorbance values at 414 nm in each well were read with an ELISA reader (Labsystems Multiskan RC 351).

7. Statistical analysis

For the ELISA results analysis, Sigma Plot 12.5 software was used to construct receiver operator characteristics curves (ROC) and to calculate the area under the curve (AUC).

CHAPTER III. RESULTS

We did not observe any expression of TgsGP-SP, neither in Origami, nor in Rosetta strains. Thus, from here on, we will only present results on the expression of rTgsGPï qSP.

III.1 Expression and solubility of rTgsGP-ΔSP

Figure 17 shows the presence of expressed rTgsGP-qSP in supernatant (S, lane 1) samples and pellet (P, lane 3) samples harvested from cultured cells after an overnight induction. Western blot confirmed the presence of expressed His-tagged rTgsGP-qSP (39.8 kDa) in the pellet (Figure 17B), whereas, there was no detectable His-tagged rTgsGP-qSP in the supernatant sample.

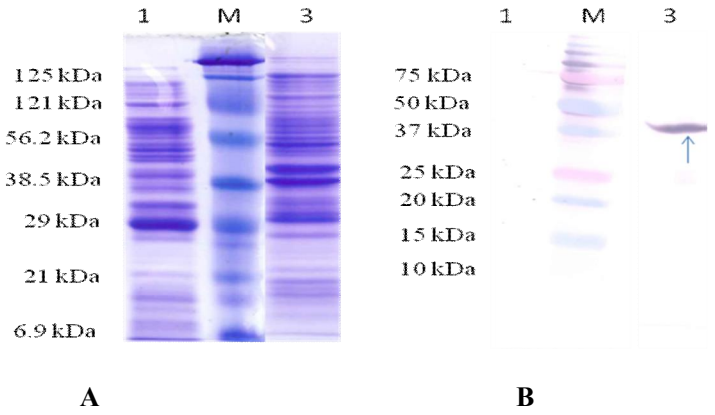

Figure 17. Solubility of rTgsGP-ΔSP in Origami cells.

Panel A. Coomassie brilliant blue stained SDS-PAGE gels.
Panel B. Western blot with anti-His tag antibody; Protein Markers (M): Prestained SDS-PAGE standard protein marker and Precision plus protein dual color standard protein marker; lane 1: oriTgsGP-qSP supernatant (S); lane 3: oriTgsGP-qSP pellet (P).

III.2 Denaturing and solubilisation of rTgsGP-ΔSP from Origami cell pellets.

According to the results shown in figure 17, our protein of interest (rTgsGP-ɸSP) is expressed as inclusion bodies (insoluble form). Therefore, we need to extract the recombinant protein from the pellet and we have tested several buffers for solubilisation (as described in section 2.6 above, under materials and methods).

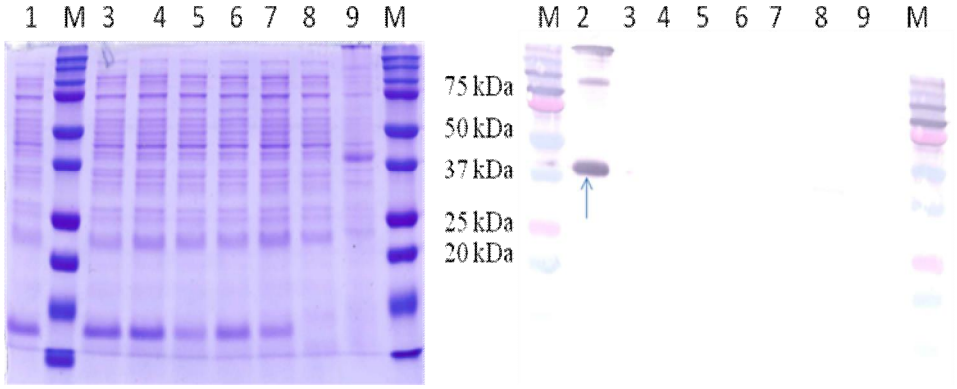

Figure 18. Solubility of rTgsGP-ΔSP in different buffer supernatant samples.

Panel A. Coomassie brilliant blue stained SDS-PAGE gels. Lane 1: H; lane M: Precision plus protein dual color standard protein marker; lane 3: G; lane 4: F; lane 5: E; lane 6: D; lane 7: C; lane 8: B; lane 9: A.
Panel B: Western blot with anti-His tag antibody. Lanes M: Precision plus protein dual color standard protein marker; lane 2: A; lane 3: B; lane 4: C; lane 5: D; lane 6: E; lane 7: F; lane 8: G; lane 9: H.

Based on the figure 18, we could determine which buffer resulted in more proteins in soluble form. We observe a strong band of rTgsGP-ɸSP (39.8 kDa) by Western blot for buffer A (panel B, lane 2), which was the guanidine based buffer: sodium carbonate-sodium bicarbonate pH 9.9 + 6M GuHCl.

III. 3 Purification of rTgsGP-ΔSP

AKTA HPLC purified samples were run and analysed in SDS-PAGE and Western blot in comparison with previously prepared samples from induction up to the extraction of rTgsGP-ɸSP protein from inclusion bodies (figure 19).

Samples were named as follows: sample after induction (II); supernatant sample (S); pellet Triton X-100 wash 1 (P1); pellet Triton X-100 wash 2 (P2); extraction supernatant (load); flow through (FT); purification wash sample (W) and purification elute sample (E).

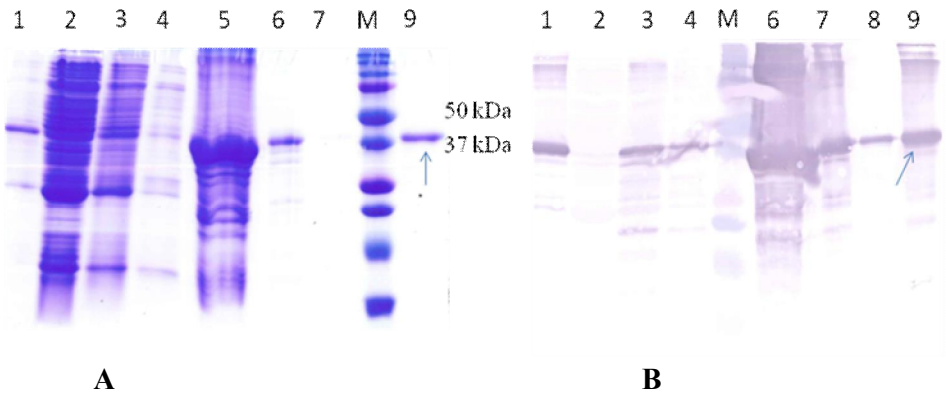

Figure 19. Purification of rTgsGP-ΔSP.

Panel A. Coomassie brilliant blue stained SDS-PAGE gel. Lane 1: II; lane 2: S; lane 3: P1; lane 4: P2; lane 5: load; lane 6: FT; lane 7: W; lane M: Precision plus protein dual color standard protein marker; lane 9: E.
Panel B: Western blot. Lane 1: II; lane 2: S; lane 3: P1; lane 4: P2; lane M: Precision plus protein dual color standard protein marker; lane 6: load; lane 7: FT; lane 8: W; lane 9: E.

Figure 19 shows that there is a 39.8 kDa rTgsGP-qpSP purified protein in lane 9 panel A and it is confirmed by Western blot (lane 9 panel B). Thus, the rTgsGP-qpSP has been successfully purified by HPLC.

III.4 Size-exclusion purification

Because the HPLC purified sample (lane 9 in figure 19) still contained contaminating proteins, we further purified the sample by size exclusion chromatography. The chromatogram of this purification (Figure 20) displays a major peak after 17.10 minutes containing our target protein rTgsGP-qpSP.

Chromatogram

Chromatogram Questions
No 1: Sample Volume and Type
500 µl TgsGP

No 2: Column
Superdex 75 10/300 GL

No 3: Eluent A
PBS Filtered/Degassed

No 4: Remarks
6M urea 500 mM NaCl 20 mM Phosphate

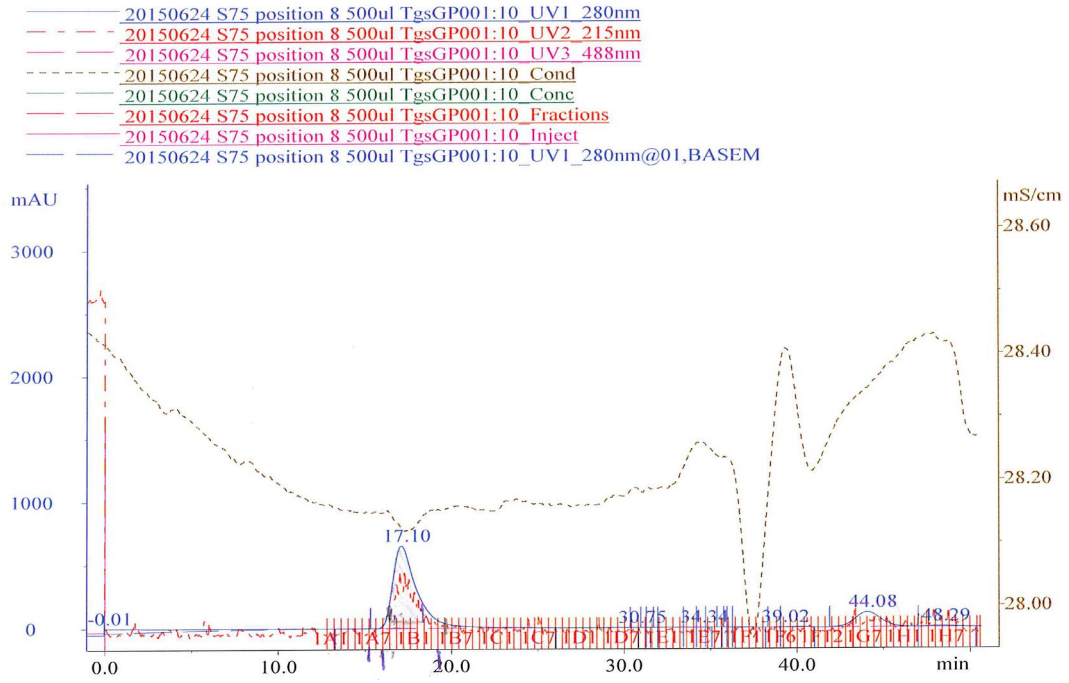

Figure 20. Chromatogram of size-exclusion purification process

The rTgsGP-qSP was purified in a total volume of 200 µl and in three eluted fractions (E1, E2 and E3). The protein concentration of the eluted rTgsGP-qSP fraction was measured with the Nanodrop™ Spectrophotometer and found to be 0.25 mg/ml. The 3 elution fractions together with the HPLC purified samples were run on SDS-PAGE. Samples were named as follows: sample before concentration (Bc); sample after concentration (load); flow through (FT); elute 1 (E1); elute 2 (E2); and elute 3 (E3).

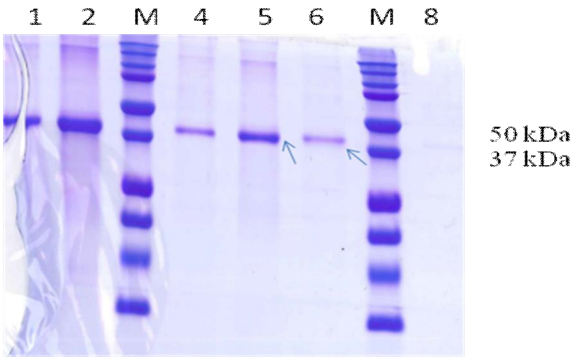

Figure 21. Coomassie stained SDS-PAGE gels of size-exclusion elute samples.

Lane 1: Bc; lane 2: load; lanes (M): lane M: Precision plus protein dual color standard protein marker; lane 4: FT; lane 5: E1; lane 6: E2; lane 8: E 3.

From figure 21, we observe purified rTgsGP-qSP in the elution fractions 1 and 2 and they are pure. This leads us to the next step of testing the purified rTgsGP with *gambiense* HAT sera to check for its diagnostic potential.

III.5 Diagnostic potential of rTgsGP-ΔSP for antibody detection in sera from patients and controls by Western blot

Serum samples from 4 *T.b. gambiense* HAT patients and 8 controls consisting of 2 T.b gambiense HAT endemic controls, 3 T.b. rhodesiense HAT patients and 3 *T.b. rhodesiense* HAT endemic controls, were tested with purified rTgsGP-qSP for antibody detection. They were named as follows: *T.b. gambiense* patientsô serum (gHAT+); *T.b. rhodesiense* patients' serum (rHAT+); *T.b. rhodesiense*-endemic negative control serum (rHAT-) and non-endemic negative control serum (NEneg).

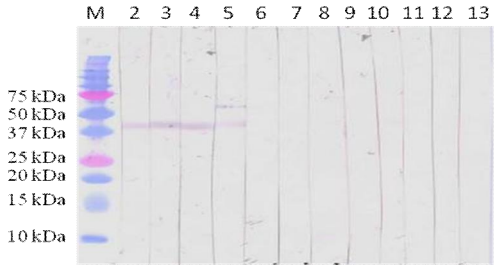

Figure 22. Western blot of rTgsGP-ΔSP immunoreaction with *T.b. gambiense* patients' sera, non-endemic control negative sera, *T.b. rhodesiense* patients' sera and *T.b rhodesiense* endemic control negative sera.

Strips 2 to 5: gHAT+ sera 1 to 4; strips 6 and 7: NEneg 1 and 2; strips 8 to 10: rHAT+ 1 to 3; strips 11 to 13: rHAT- 1 to 3.

From the figure 22, we observe a strong band of reaction (positive Abs detection) on strip 2, 3, 4 and 5 tested with *T.b. gambiense* patients sera and a weak band (false positive) on strip 10 tested with one of the *T.b. rhodesiense* patients sera. No reaction was detected in the rest of the strips.

III.6 Diagnostic potential of rTgsGP- ΔSP for antibody detection in sera from HAT patients and controls by ELISA

In a next phase, the rTgsGP-ΔSP was evaluated in ELISA assay with 100 *T.b. gambiense* patients' sera, 50 *T.b.gambiense*-endemic negative control sera, 78 *T.b.rhodesiense* patients' sera and 50 *T.b. rhodesiense*-endemic negative control sera. Test OD values were analysed with SigmaPlot software; and the resulting receiver operating characteristics (ROC) curves are presented on figure 23.

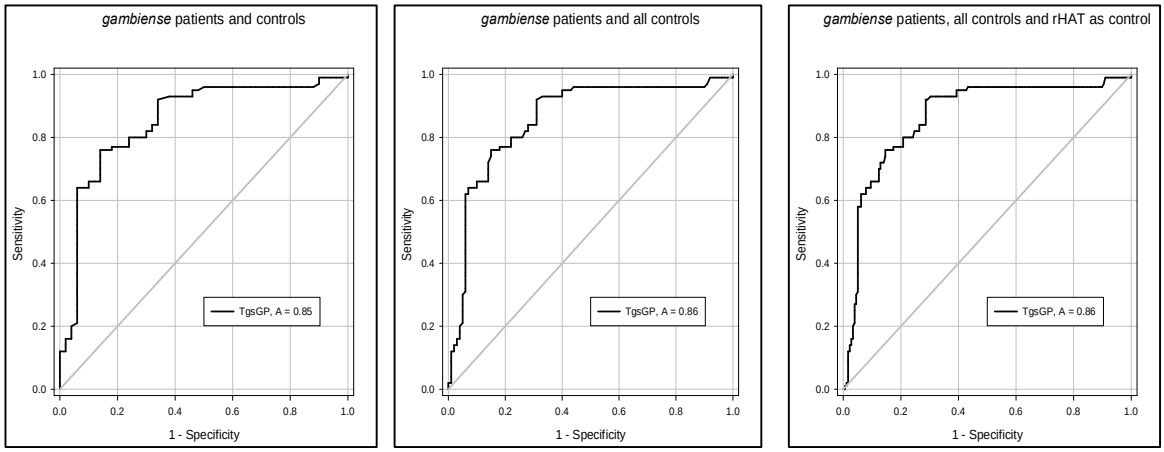

Figure 23. Receiver operator characteristic (ROC) curves and area under the curve (AUC, denoted as A in this figure) constructed from ELISA results.

Panel A: rTgsGP-ΔSP tested with *T.b. gambiense* patients' sera and *gambiense*-endemic negative control sera.
Panel B: rTgsGP-ΔSP tested with *T.b. gambiense* patients' sera and both *gambiense* and *rhodesiense* endemic negative control sera.
Panel C: rTgsGP-ΔSP tested with *T.b. gambiense* patients' sera, *gambiense* and *rhodesiense* endemic control sera and with *T.b. rhodesiense* patients' sera considered as negative controls.

As observation, the area under the curve is more or less the same in all the three scenarios presented under figure 23: A=0.85, A=0.86 and A=0.86 respectively. ROC values between 0.8 and 0.9 are considered as good (http://gim.unmc.edu/dxtests/roc3.htm).

CHAPTER IV. DISCUSSION

The rTgsGP-qpSP was successfully expressed in Origami cells which was proven by a band of 39.8 kDa on Coomassie stained gels and was confirmed in Western blot by anti-his-tag antibody. However, we didnôt succeed to express rTgsGP-SP in Origami nor Rosetta cells. The level of rTgsGP-SP expression was not high enough to be either detected on SDS-PAGE or Western blot (results not shown). This might be due to different factors. First, with their usual signal peptide which influences protein chaperon recognition, folding and translocation to the periplasm, a number of expressed proteins may get degraded or misfolded in the cytoplasm due to inefficiency or insufficiency of chaperons in the cell compartment (Keiler, Waller, & Sauer 1996). Second, due to some proteinases present in the periplasm and/or host cell envelope, secreted recombinant proteins may fall into degradation (Choi & Lee 2004).

On the other hand, thanks to the Origami strains, we could express rTgsGP-qpSP but this protein was expressed in an insoluble form, probably in inclusion bodies. A first reason may be that we used a prokaryotic expression system while *T.b. gambiense* is eukaryotic, which may have different folding factors, pH and osmolarity factors. To get the rTgsGP protein in soluble form, we tried 8 different buffers and we succeeded with only one of them, the buffer based on sodium carbonate-sodium bicarbonate pH 9.9 and 6M GuHCl. This means that only GuHcl was able to denature inclusion bodies from cell pellets and then get proteins solubilised in supernatant.

The rTgsGP-qpSP was successfully purified with HPLC affinity chromatography using Ni sepharose column (figure 19). The second step of purification, size-exclusion chromatography, availed pure rTgsGP-qpSP (figure 21); and the concentration of the purified protein was calculated as 0.25 mg/ml.

Finally, purified rTgsGP antigens were evaluated for their diagnostic potential for *T.b.gambiense* HAT. This was first done in Western blot. We observed a strong reaction band with all *T.b.gambiense* patientsô sera tested (figure 22). No reaction is observed in non-endemic patientsô sera. This confirms that the purified rTgsGPôs immunoreactivity is specific. However, we observed a faint reaction band (a false positive) with *T.b rhodesiense* patients' serum (figure 22).

The ELISA test results were not far from the ones we got on Western blot with patientsô sera. There were some false positive reactions in the *rhodesiense* HAT patients' sera. This means that our purified rTgsGP-qpSP may share some epitopes with *T.b.rhodesiense* antigens (cross-reactivity). The negative reaction with some *gambiense* HAT sera is probably due to two reasons: (i) TgsGP is a truncated VSG and routed to the lysosomes where it blocks pore-formation of the ApoL1. The TgsGP protein may thus be not enough exposed to the human serum to generate enough antibodies, (ii) another explanation may be that we used denatured and thus unfolded antigens in ELISA while patients have been exposed to the native antigen.

The area under the ROC curve with all samples was 0.86 (figure 23), which is considered as good. But it is much lower if you compare it to the native LiTat 1.3 and LiTat 1.5 antigens (AUC>0.99) and recombinant LiTat 1.3 and LiTat 1.5 antigens (AUC>0.95) (Rogé, Van Nieuwenhove, Meul, Heykers, Brouwer de, Bebronne, Guisez, & Büscher 2014;Van, Büscher, Balharbi, Humbert, Guisez, & Lejon 2013).

CHAPTER V. CONCLUSION

We have successfully expressed and purified recombinant TgsGP using Origami cells. The expression did not initially attain its solubility; therefore, we had to extract the expressed rTgsGP from the pellet in denaturing conditions. The ELISA test with purified rTgsGP showed a good diagnostic potential (AUC of 0.86) in 100 endemic gambiense HAT patients sera and 178 controls. However, the observed diagnostic potential of TgsGP was lower than the previously reported recombinant *T.b. gambiense* antigens LiTat 1.3 and LiTat 1.5.

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