EVALUATION OF TSETSE SALIVARY GLAND GROWTH FACTOR-2 AND TSETSE ANTIGEN- 5 AS MARKERS OF EXPOSURE TO TSETSE BITES IN CATTLE

BY

WAMALA SAMUEL POSIAN (BVM, MAK)

2007/HD17/8985

MMB

A DISSERTATION SUBMITED TO THE COLLEGE OF VETERINARY MEDICINE, ANIMAL RESOURCES AND BIOSECURITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTERS OF SCIENCE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY, MAKERERE UNIVERSITY

APRIL, 2013

DECLARATION

I, **WAMALA Samuel Posian,** declare to the best of my knowledge that this is my original research report and it has never been submitted for the award of any degree in any university or any other institution of higher learning.

…………………………………………   ………………………………….………..

Signature   Date

This research work has been done and submitted under the supervision and approval of;

Dr. Enock Matovu (Assoc. Prof.),

College of Veterinary Medicine, Animal Resources and

Biosecurity,

Makerere University, Kampala.

Signature …………………………………   Date ………………………………

Prof. George William Lubega,

College of Veterinary Medicine, Animal Resources and

Biosecurity,

Makerere University, Kampala.

Signature …………………………………   Date ……………………………..

Prof. Serap Askoy

Yale University, USA.

Signature …………………………………..   Date ……………………………

i

DEDICATION

I dedicate this work to my parents, Mr. and Mrs. Christopher Wamala.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

˚C   Degrees Celsius

aa   Amino acid

APCs   Antigen-presenting cells

Approx.   Approximately

$CaCl_2$ Calcium chloride

Cat   Catalogue

CDC   Centre for Disease Control

cDNA   Complementary Deoxy-ribonucleic acid

DAB   3, 3,5',5' diamino-benzidine

DNA   Deoxyribonucleic acid

ELISA   Enzyme linked immunosorbent assay

et al   And others

g   grams

H_2O_2 Hydrogen peroxide

Hr   Hour

kDa   Kilo Dalton

mM   Milli molar

NK   Natural killer

no.   Number

PARP   Procyclic acidic repetitive protein

PBS-T   Phosphate buffered saline-Tween 20

rpm: Revolutions per minute

rTag-5   Recombbinat tsetse antigen-5

rTSGF-2   Recombinant tsetse salivary gland growth factor-2

SDS   Sodium dodecyl sulphate

SDS-PAGE   Sodium dodecyl sulphate – polyacrylamide gel electrophoresis

Tag-5   Tsetse antigen-5

Th2   T- helper 2

Tryp.   Trypanosomes

TSGF-2   Tsetse salivary gland factor-2

µg/ml   Micrograms per mililitre

µl   Micro liter

v/v   Volume by volume

VSG   Variant surface glycoprotein

w/v   Weight by volume

ABSTRACT

During their blood meals, arthropod vectors including tsetse flies inject saliva into their hosts to enhance their feeding efficiency. The salivary gland secretions include a number of proteins some of which can elicit the host's immune response thereby being useful candidates for investigation as vaccine targets and/or markers of exposure to bites of these vectors which may aid disease control. This project evaluated the potential of tsetse salivary gland growth factor- 2 (TSGF-2) and tsetse antigen- 5 (Tag-5) as markers of exposure to tsetse bites in cattle.

Both rTSGF-2 (810bp falling between 375 and 1094bp of the TSGF-2 cDNA) and rTag-5 (390bp falling between 78 and 468bp of the Tag-5 cDNA) gene fragments were expressed in BL21 DE3 *E. coli* expression host to obtain the respective recombinant proteins, rTSGF-2 and rTag-5. The rTSGF-2 was purified by Ni-NTA agarose column and, the two proteins were run on SDS and trans-blotted onto a nitrocellulose membrane, which was probed with pooled plasma from cattle of different tsetse exposure status. The ELISA tests were also run to show whether rTSGF-2 could be used to distinguish between tsetse exposed and non exposed cattle as well as trypanosome positive and negative cattle.

Results revealed that rTSGF- 2 was expressed and purified in sufficient amounts of the expected size (31kDa) and it was recognized by plasma from tsetse exposed cattle. However, Western blotting and ELISA could neither distinguish between tsetse exposed from non exposed cattle (P 0.05) nor trypanosome positive from negative cattle (P 0.05). This would indicate possible cross reactions with proteins from other vectors

such as mosquitoes and ticks to which the control animals could as well have been exposed. The rTag- 5 was expressed in very low quantities which hindered its purification making some of the subsequent tests impossible. It was also recognized by plasma from the different groups of cattle as above. Therefore, both rTSGF-2 and rTag-5 are recognized by cattle plasma antibodies but they are not reliable as markers of exposure specific to tsetse bites and cannot specifically determine trypanosome infection status in cattle. Similar proteins secreted by other vectors should be evaluated for recognition by antibodies produced against rTSGF-2 and rTag-5 to check for possible cross reaction, and future studies should be based on proteins expressed from gene regions unique to *Glossina* to limit such cross reactions.

CHAPTER ONE

INTRODUCTION

1.1 Background

Tsetse flies are the biological vectors of African trypanasomiasis, a very important parasitic disease caused by organisms of the genus *Trypanosoma* (Kuzoe, 1993; Barrett *et al*., 2003, Matovu *et al*., 2003). The disease affects both humans and animals, with *Trypanosoma brucei brucei*, *T. congolense* and *T. vivax* as the major causes of animal African trypanosomiasis while *Trypanosoma brucei rhodesiense* and *T. brucei gambiense* cause human African trypanosomiasis. Control of this disease is very challenging as vaccine development is still a failure due to rapid antigenic variation exhibited by the causative agents, while chemotherapy remains largely unsatisfactory (Barrett, *et al*., 2003 and Croft *et al*., 2005). Besides this, without treatment trypanosomiasis is highly fatal (Matovu *et al*., 2003; Kennedy *et al*., 2006) and economically very important in the animal sector.

Proper management of animal African trypanosomiasis requires early identification of high risk areas through monitoring tsetse fly population and early detection of infection. *Glossina* populations are currently monitored using entomological techniques with limitations that these cannot be applied to a large area in the field. Geographical information systems are also being used to map out favorable habitats for tsetse fly infestation (Rogers and Williams*, 1993*).

1

Glossina saliva contains over 250 different proteins that are likely to be associated with blood feeding (Oliveira *et al.,* 2009) with properties that promote tsetse feeding, such as anti clotting factors (Caljon *et al*., 2006), counteraction of host responses like vasoconstriction, platelet aggregation, inflammatory reactions, and coagulation reactions (Alves-Silva *et al*., 2010; Ribeiro and Francischetti, 2003). In addition, some salivary proteins can induce a specific immune response (Poinsignon *et al*., 2007, Caljon *et al* 2006) while others have been shown to modify the vertebrate host's immune response in a manner that favors trypanosome establishment (Van Den Abbeele *et al*., 2010), a parameter that could be exploited in the diagnosis of African trypanosomiasis.

Studies on different vectors including *Triatoma (*Nascimento *et al*., 2001*), Aedes* (Palosuo 1997, sand fly (Barral *et al., 2000*), *Anopheles (*Remoue *et al*., 2006*), Ixodes damini* (Lane *et al*., 1999) and glossina (Caljon *et al* 2006) suggested that the evaluation of vertebrate host specific antibody response to vector saliva and/or to recombinant salivary protein could be used to determine exposure of individuals to vector bites and thus could be an indicator of risk to pathogens transmission (Remoue *et al*., 2006 and 2007).

Such salivary proteins include putative endonucleases including tsetse salivary gland proteins 1 and 2 (Li   *et al*., 2001), putative adenosine deaminases including tsetse salivary gland growth factors 1 and 2 (TSGF-1 and TSGF-2 respectively) as demonstrated by Li and Aksoy (2000), and tsetse Antigen 5 (Tag-5) as documented by Caljon *et al*., (2009), among others. The roles played by some of these salivary agents in the life cycle and survival of trypanosomes as well as their immunological properties is

not well understood, thus providing a discovery platform for the identification of novel immunological markers of vector exposure (Alves-Silva *et al*., 2010). In this study tsetse salivary gland growth factors 2 and tsetse antigen 5 were investigated along that line.

1.2 Problem statement

Tsetse flies are the biological vectors of African trypanosomes, the causative agents of African trypanosomiasis. The disease is very important in both animals and humans, especially in the Tropics and Sub tropics where it causes mortality and great economic losses. Control of this disease is very difficult due to the absence of an appropriate vaccine, and treatment is also ineffective (Brun *et al*., 2001) because of drug resistance to the few available drugs, which are also very expensive to the local farmers (Matovu *et al*., 2003). Unfortunately, tsetse fly infestation is extending beyond the known "tsetse fly belt", putting more animal and human populations at risk. Disease control and prevention would therefore depend on rapid identification of high risk areas for immediate actions however, lack of a reliable marker for tsetse exposure in cattle has hindered the early identification of areas that would require immediate tsetse control thereby deterring efforts to control the disease.

1.3 Justification and significance

In order to improve vector borne diseases' control, many efforts are being devoted to developing new tools for identification of geographic areas with high risk of parasite transmissions. Evaluation for cattle antibody response to tsetse salivary proteins could be an epidemiological marker of exposure to tsetse bites, and therefore indicate the risk of pathogen transmission. It helps in identification of highly exposed areas toward which

vector control should be directed and also to assess the efficiency of entomological control measures. Response to total saliva has been evaluated with possible cross reactions to other vectors (Somda, 2010). Maximal specificity could be achieved through identification of individual immunogenic proteins specific to the tsetse fly vectors. This project investigated the potential of rTSGF-2 and rTag-5 as reliable markers of exposure to tsetse fly bites in cattle. Identification of reliable markers of exposure is very useful in prioritizing areas for immediate tsetse control. This is one of the avenues to control African trypanosomiasis by interfering with disease transmission.

In addition, both proteins have been shown to promote trypanosome establishment in the vertebrate host by modulating the host's immune response in a manner that favors survival of the parasites (Van Den Abbeele *et al*., 2010). This shows a close correlation between trypanosome infection and the immune response of the host. Therefore, by measuring the host's immune response against these proteins, one could as well determine trypanosome infection status of the host thereby enhancing disease diagnosis.

1.4.0 Objectives

1.4.1 General objective

To investigate the potential of the 31kDa fragment of tsetse salivary gland factor- 2 (rTSGF-2) and the 15kDa fragment of tsetse antigen- 5(rTag-5) as reliable markers of exposure to tsetse bites in cattle.

1.4.2 Specific objectives

- To determine whether rTSGF-2 and rTag-5 can be recognized by plasma antibodies from tsetse exposed cattle.

- To determine whether rTSGF-2 and rTag-5 can be used to distinguish between tsetse exposed and non exposed cattle

- To determine whether rTSGF-2 and rTag-5 can be used to distinguish between trypanosome infected and non infected cattle.

1.5 Research questions

- Can plasma antibodies from tsetse exposed cattle recognize rTSGF-2 and rTag-5?

- Can rTSGF-2 and rTag-5 be used to distinguish between tsetse exposed and non exposed cattle?

- Can rTSGF-2 and rTag-5 be used to distinguish between trypanosome infected and non infected cattle?

CHAPTER TWO

LITERATURE REVIEW

2.1 Animal African trypanomiasis

African trypanasomiasis is a parasitic disease of both humans and animals, caused by protozoan organisms of the genus *Trypanosoma* (Kuzoe, 1993; Barrett *et al*., 2003, Matovu *et al*., 2003) with tsetse flies as the biological vectors. *Trypanosoma. brucei brucei*, *T. congolense*, *T. vivax* and *T. simiae* are the major causes of animal African trypanosomiasis (AAT) while *T. brucei gambiense* and *T. brucei rhodesiense* cause human African trypanosomiasis (HAT).

2.2 Life Cycle of *Trypanosoma brucei*

Trypanosoma brucei undergo a series of developmental changes in both the mammalian and the insect host, the tsetse fly vector (Figure 1). When an infected tsetse fly (genus *Glossina*) feeds on the mammalian host, it injects the metacyclic trypomastigotes into the skin tissue together with saliva. The parasites enter the lymphatic system from where they enter the bloodstream and develop into long slender blood stream form trypomastigotes which are covered by the variant surface glycoprotein (Biebinger *et al.,* 1996). The trypomastigotes are carried via the blood to other sites throughout the body, reaching other body fluids including the spinal fluid, and continue to replicate by binary fission, some developing into intermediate and subsequently stumpy forms.
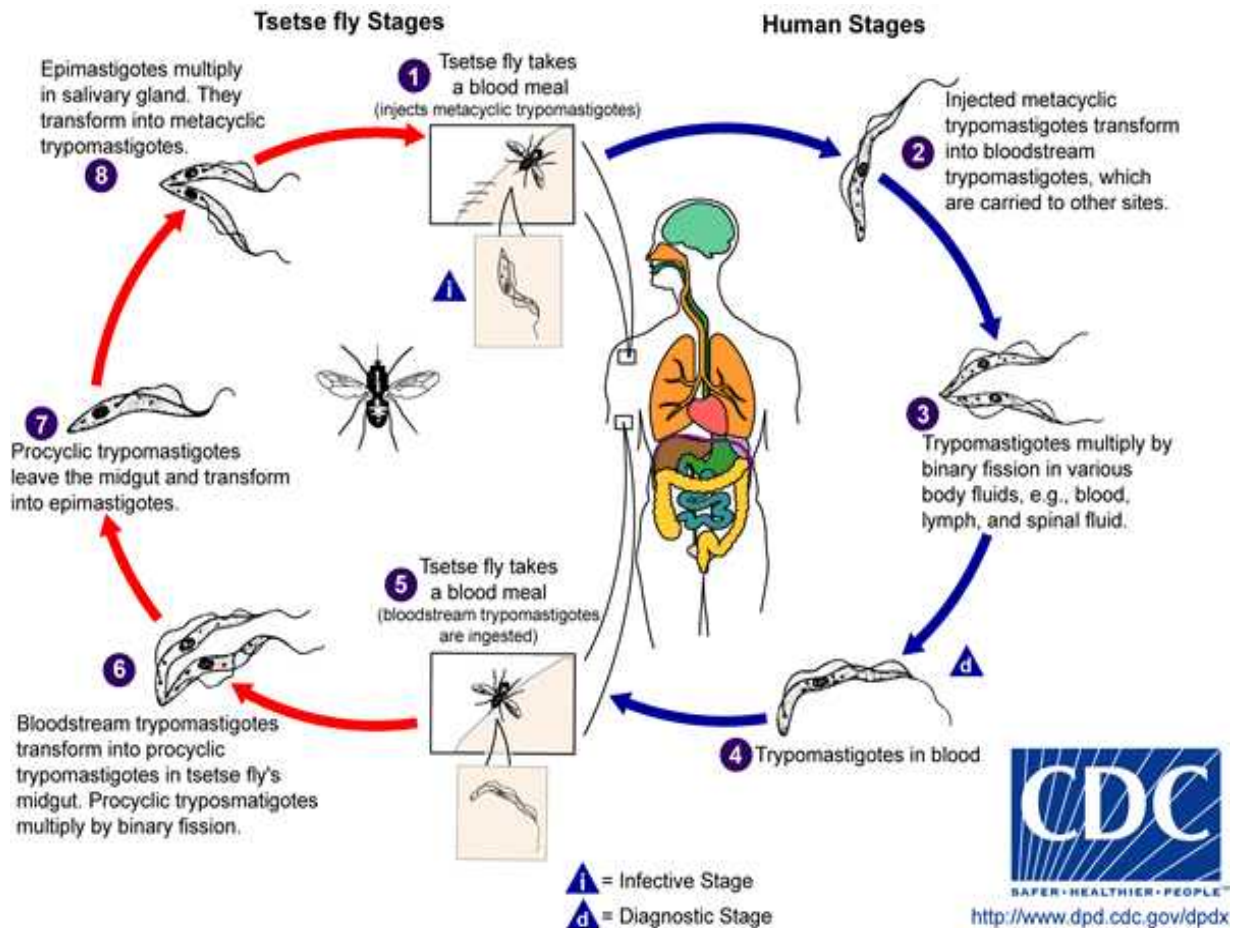
Figure 1: Life cycle of *Trypanosoma brucei*. Note: 1-8 indicates the different developmental stages, their activity and location. (Image from Da Silva and Moser; Center for Disease Control, Public Health Image Library).

Tsetse flies become infected with the trypomastigote stage when taking a blood meal from an infected mammalian host. In the fly's mid-gut, trypomastigotes transform into the 'procyclic' form and while in the tsetse fly, VSG gene transcription ceases; the VSG coat is shed and within a few hours replaced by the procyclic acidic repetitive protein (PARP) coat also known as procyclin (Biebinger *et al.,* 1996). The parasites then migrate from the mid-gut across the ectoperitrophic space and then through the proventriculus

into the foregut, where they develop into elongated and asymmetrically dividing epimastigotes (Abbeele *et al*., 1999). These epimastigotes complete the journey through the proboscis and hypopharynx to reach the lumen of the salivary gland, where the final stage of development occurs. The epimastigotes in the fly's salivary glands continue to multiply by binary fission and as they advance to the foregut and proboscis they exhibit increased motility compared to those found in the mid-gut (Abbeele *et al*., 1999). In the salivary glands, cell division is completed generating epimastigotes which attach themselves to the salivary gland epithelium through intricate membrane and cytoskeletal connections that are established between the parasite flagellum and the epithelial cell membrane. The attached epimastigotes differentiate into variant surface glycoprotein (VSG)-coated metacyclic trypomastigotes that detach from the epithelium and are now able to survive in the mammalian bloodstream. The phase of the life cycle in the fly takes approximately 3 weeks (CDC, 2006), and the entire life cycle of African trypanosomes is extracellular.

2.3 Control and prevention of animal African trypanosomiasis

Various control and prevention options are available for African animal trypanosomiasis including reduction of vectors and administration of trypanocidals, together with the use of trypanotolerant breeds (Service, 2006).

2.3.1 Chemotherapy and chemoprophylaxis

Chemotherapy and chemoprophylaxis are directed against the parasites. They involve use of drugs for treatment and prevention of the disease respectively. Drugs have been used for decades but there is a concern of development of resistance (Van Den Bossche *et al*.,

2006a) thus a need to develop new drugs. Diminazene aceturate and Isometamidium chloride are being used against the three livestock infective trypanosomes. Other drugs such as homidium bromide, quaternary ammonium trypanocides and antricides have been adandoned due to drug resistance and toxicity (www.fao.org/docrep/006/X0413E)

Use of a vaccine would be the most effective way of controlling animal trypanosomiasis but it has been hindered by rapid antigenic variation of the parasites (Vickerman and Preston, 1976). Several attempts have been made to develop trypanosomiasis vaccines but have not yet been successful. These have been based on different parasite components such as the cytoskeleton including tubulin and microtubule associated proteins (Lubega *et al*., 2002, Rasooly and Balaban, 2004 and Balaban *et al*., 1995), membrane proteins (Pays, 1997) and invariant surface glycoprotein (Nolan *et al*., 1997).

2.3.2 Trypanotolerant breeds

For long it has been noted that certain breeds of cattle in West and Central Africa, mainly the N'Dama and West African shorthorn, along with their sheep (Djallonke) and goat (Dwarf West African) counterparts, possess the ability to survive and be productive in tsetse-infested areas where other breeds rapidly succumb to trypanosomiasis (ILCA., 1979). Susceptibility studies have shown the N'Dama to be the most resistant breed followed by the West African Short-horned cattle. The large and more recently introduced Zebu is the most susceptible (Murray *et al*., 1979).

However, widespread use of trypanotolerant cattle breeds is challenged by the limited number of resistant animals available (Adeniji, 1993), and the possibility of animals

acting as reservoirs of trypanosomes. In addition, the cost of transport over great distances, along with the acclimatization period required for adaptation to different environmental conditions requires a long-term investment before any significant development and economic returns can be achieved. Also, the degree of trypanotolerance is influenced by various factors including the level of tsetse challenge. When tsetse challenge is high in N'Dama cattle, stunting, wasting, abortion, extreme lethargy and death may occur as a result of trypanosomiasis. Trypanotolerance is therefore a relative rather than an absolute trait, which is severely affected by heavy challenge, malnutrition, stress, breed, age, season, and concurrent disease (Kalu, 1995; Feldmann and Hendrichs, 2001).

2.4 Blood sucking in arthropods

Blood sucking arthropods depend on their host's blood for nutrient acquisition. They must deal with the hosts' defense against blood loss, based on blood clotting, platelet aggregation and vasoconstriction, as well as defense mechanisms triggered by inflammation and immune reactions. These defense responses may cause death to the insect or, at the least, interrupt blood meal acquisition (Ribeiro and Francischetti, 2003) and also greatly affect the pathogenic organisms vectored by these organisms. The saliva of hematophagous arthropods can counteract these barriers by using a complex mixture of pharmacologically active components, which are injected into the host skin during the probing and ingestion phases of feeding (Ribeiro and Francischetti, 2003; Ribeiro, 1995). Accordingly, at the site of skin penetration, the hosts' response is pharmacologically modified by these salivary agents, which may inadvertently lead to enhanced transmission of pathogens (Oliveira *et al*., 2009) or otherwise.

10

2.5 The tsetse fly

Tsetse flies are insects belonging to genus *Glossina*, which are widely distributed in the tropics and the subtropics where they put large animal and human populations at risk of getting infected with African trypanosomiasis, the disease they transmit biologically (Kettle, 1995).

2.5.1 Morphology of tsetse flies

Tsetse adults are relatively large flies with large heads and distinctly separated eyes. The tsetse thorax is quite large, while the abdomen is wide rather than elongated and shorter than the wings. They have a distinct *proboscis* which is a long thin structure attached to the bottom of the head and pointing forward. When at rest, tsetse flies fold their wings completely one on top of the other creating an appearance of a closed pair of scissors. The discal cell of the wing has a characteristic hatchet shape resembling a butcher's cleaver or a hatchet. The antenna has an arista with hairs which are themselves branched (Kettle, 1995).

2.5.2 Life cycle of tsetse flies

Tsetse flies have an unusual life cycle where the female flies only fertilize one egg at a time and retain each egg within their uterus to have the offspring develop internally during the first larval stages, a strategy called adenotrophic viviparity. During this time, the female feeds the developing offspring with a milky substance secreted by a modified gland in the uterus. In the third larval stage, the tsetse larvae finally leave the uterus and crawl into the ground to form a hard outer shell called the puparial case, in which it completes its morphological transformation into an adult fly. This life stage has a variable

duration, generally twenty to thirty days, and the larva must rely on stored resources during this time. The importance of the richness of blood to this development can be seen since all tsetse development before it emerges from the puparial case as a full adult occurs without feeding, based only on nutritional resources provided by the female parent. The female must get enough energy for her needs, for the needs of her developing offspring, and to store the resources which her offspring will require until it emerges as an adult (Kettle, 1995).

2.5.3 Control of tsetse flies

There are many techniques available to control tsetse populations, with earlier, crude methods recently replaced by methods that are cheaper, more directed, and ecologically friendly.

2.5.3.1 Slaughter of wild animals

One early technique involved slaughtering all the wild animals tsetse fed on. For example, the island of Principe of the west coast of Africa was entirely cleared of feral pigs in the 1930s, which led to elimination of the fly. While the fly eventually re-invaded in the 1950s, the new population of tsetse was free from the disease (Fatic, 2011).

2.5.3.2 Bush clearing

Tsetse tend to rest on the trunks of trees so removing bush and woody vegetation make the area inhospitable to the flies. Preventing re-growth of woody vegetation requires continuous clearing efforts, which is only practical where large human populations are present. The clearing of woody vegetation has come to be seen as an environmental

problem more than a benefit. Bush clearing eliminates breeding sites of the vector and is still useful especially when used in conjunction with other methods (Uilenberg, 1998).

2.5.3.3 Insecticide campaigns

Insecticides have been used to control tsetse flies starting initially during the early part of the twentieth century in localized efforts using the inorganic metal based pesticides, expanding after the Second World War into massive aerial and ground based campaigns with organochlorine pesticides such as DDT applied as aerosol sprays at Ultra-Low Volume rates (Leak, 1996). Later, more targeted techniques used pour-on formulations in which advanced organic pesticides like synthetic pyrethroids with low toxicity to the cattle and highly toxic to *Glossina* were applied directly to the backs of cattle (Zebra, 1988).

2.5.3.4 Trapping

Tsetse populations can be monitored and effectively controlled using simple, inexpensive traps. These often use blue cloth, since this color attracts the flies. Early traps mimicked the form of cattle but this seems unnecessary and recent traps are simple sheets or have a biconical form. The traps can kill by channeling the flies into a collection chamber or by exposing the flies to insecticide sprayed on the cloth in case of insecticide impregnated tsetse traps or targets (Vale *et al*., 1988a). Tsetse flies are also attracted to large dark colors like the hides of cow and buffaloes. The use of chemicals as attractants to lure tsetse to the traps has been studied extensively in the late 20th century, but this has mostly been of interest to scientists rather than as an economically reasonable solution. Attractants studied have been those tsetse might use to find food, like carbon dioxide,

13

octenol, and acetone; which are given off in animals' breath and distributed downwind in an odor plume. Synthetic versions of these chemicals can create artificial odor plumes. A cheaper approach is to place cattle urine in a half gourd near the trap. Non insecticide impregnated traps are also commonly used (Kuzoe, 2005; Brightwel *et al*., 1991).

2.5.3.5 The sterile insect technique (SIT)

The release of irradiated males has been used to reduce tsetse populations. This technique involves the rearing of large numbers of tsetse, separation of the males, irradiation of these flies with large doses of gamma rays to make them sterile and then release into to the wild. Since females only mate once in their lifetime, any mating with a sterile male prevents that female from giving birth to any offspring. The sterile insect technique has been used in Zanzibar Islands. Like other eradication efforts, early indications are that the fly numbers have been devastated, with the fly possibly extirpated (locally eradicated) from the island. However, this approach is very costly, technically demanding and requires isolated areas of tsetse infestation (Leak, 1996).

Additionally, using the parasite refractory strains is another method to control the tsetse that means providing the blood meal containing the trypanocide before releasing the sterilised males. One can also consider using the cytoplasmic incompatibility strategy to control the population of tsetse. With the development of genetic engineering, the releasing of engineered parasite refractory counterparts is another strategy to control the population of tsetse (Kettle, 1995).

2.5.4 Tsetse fly as vectors of trypanosomes

Tsetse flies are the biological vectors of the different pathogenic African trypanosome species, which has to go through a complex developmental cycle in the alimentary tract and salivary glands of the fly for successful transmission to occur (Van Den Abbeele *et al*., 1999). *Trypanosoma brucei* undergo the final developmental phase in the salivary glands. Once this trypanosome population has been established in the salivary glands, it is continuously maintained at high density throughout the remaining life span of the tsetse fly by their repetitive division. Successful establishment of trypanosomes in tsetse mid-gut as well as their migration to salivary gland depends on their ability to adapt rapidly to new environmental conditions and to traverse the physical barriers (Simo *et al*., 2010).

2.5.4.1 Tsetse fly saliva

Tsetse salivary gland secretion is essential for the hematophagous behavior of the tsetse fly by counteracting host responses such as vasoconstriction, platelet aggregation and coagulation reactions involving serine proteases such as thrombin (Ribeiro and Francischetti 2003). Trypanosomes have shown ability to promote their transmission and facilitate infection onset upon their inoculation in the host skin through manipulation of the tsetse feeding behavior by modifying the composition of the saliva (Van Den Abbeele *et al*., 2010). Several compounds have been implicated in facilitating blood feeding. The saliva of *Glossins morsitan morsitans* contains over 250 proteins. These proteins are likely to be associated with blood feeding. It includes proteins of known functions and new polypeptide families some of which are unique to *Glossina*. However, the functions of most of these proteins are not known (Alves-Silva *et al*., 2010).

2.5.4.2 Tsetse salivary gland growth factor 2 (TSGF-2)

Tsetse salivary gland growth factors 2 (TSGF-2) is a putative adenosine deaminase with a cDNA that encodes for an open reading frame of 506 amino acid residues (approx. 58.2 kDa) and it is similar to TSGF-1, a closely related protein (Li and Aksoy, 2000). The TSGF-2 and TSGF-1 encoding genes are preferentially expressed in the salivary glands of male and female adult flies. In addition to salivary glands, both transcripts can be detected from the gut tissue. Only transcripts specific for TSGF-2 are detected in ovary and testes of adults and puparia. None of the genes is expressed during the larval developmental stages. The N-terminal region of TSGF-2 contains a hydrophobic sequence with secretory signal peptide characteristics. The deduced protein sequence of the cDNA displays extensive similarities to two other proteins: insect derived growth factor (IDGF) characterized from *Sarcophaga peregrina* with growth-factor activity, and atrial gland specific antigen characterized from *Apylasia californica*. In addition to growth factor similarity, all four related proteins share the evolutionarily conserved amino acid residues associated with the enzymatic deamination of adenosine. While both genes are present and expressed in *G. m. morsitans* and *G. p. palpalis*, only TSGF-1 is present in *G. austeni* (Li and Aksoy, 2000).

2.5.4.3 Tsetse Antigen- 5

The full-length transcript encoded by Tsetse Antigen 5 (Tag-5) cDNA is 926 bp without the poly (A) tail. It has an open reading frame of 259 amino acids, encoding for a protein of 28. 9 kDa (Li *et al*., 2001). The putative product of Tag-5 is similar to Agr and Agr2 cDNAs from Drosophila and LuLoAG5 from Lutzomyia. The N terminus of Tag-5 contains a hydrophobic region with signal peptide characteristics indicating that it may be

secretory in nature. Transcripts specific for Tag-5 gene can be detected in all developmental stages of tsetse. The Tag-5 transcripts can be detected from proventriculus and midgut tissues of the fly in addition to salivary glands. The salivary glands of adult males are found to express higher levels of Tag-5 in comparison to females. The expression of these cDNAs in different tsetse species (*G. m. morsitans*, *G. austeni* and *G. fuscipes*) shows wide variations (Li *et al*., 2001).

Tsetse antigen- 5 is a homolog of Antigen 5 sting venom allergen. Antigen 5-related proteins are represented as functional allergens also in stinging insects (Caljon *et al*., 2009). Antigen-5 related proteins belong to an ubiquitous group of secreted proteins within the CAP family (cysteine-rich secretory protein). Most of these animal proteins have no known function; in the few instances to the contrary, their function diverges from proteolytic activity in *Conus* (Milne *et al*., 2003) through smooth muscle-relaxing activity in snake venoms (Yamazaki and Morita (2004), to salivary neurotoxin in the venomous lizard *Heloderma horridum* (Nobile *et al*., 1996). Members of the antigen 5 family are expressed in the salivary glands of all blood feeding insects studied so far, including sandflies and *Culicoides* (Calvo *et al*., 2007, Anderson *et al*., 2006; Campbell *et al*., 2005). In mosquito salivary glands, several genes of this family are expressed, including those which are salivary gland specific (Arca *et al*., 2007). Tsetse antigen 5 has been reported to be immunogenic (Caljon *et al*., 2009), and antigen 5 of the *Stomoxys calcitrans* bind immunoglobulins with specificity (Ameri *et al*., 2008), perhaps having an anti-complement activity. Exceptionally, a tabanid salivary protein of this family is a

potent platelet aggregation inhibitor (Xu *et al*., 2009) that would enhance blood flow during feeding.

2.5.4.4 Tsetse salivary gland proteins 1 and 2

Tsetse salivary gland proteins 1 and 2 are putative endonucleases. The cDNAs tsetse salivary gland proteins 1 and 2 encode for mature proteins of 45. 6 kDa (399 amino acids) and 43.9 kDa (389 amino acids), respectively, and their putative products exhibit over 42% identity to one another (Li *et al*., 2001 and Alves-Silva, 2010).

2.5.4.5 Serine proteases

As noted above, many tsetse salivary proteins are in the category of serine proteases, which are trypsin like proteases and play a role in digestion. They are relatively small enzymes of about 220 amino acids in length and lack substrate specificity. Larger enzymes function in more specific ways, for example, in the activation of proteolytic cascades of immunity pathways such as activating the prophenoloxidase enzyme that produces pathogen melanization (Kanost *et al*., 2004)

2.5.4.6 Phosphatases and collagenases

These are basically lysosomal enzymes, but are highlighted here because fleas have co-opted this gene family to be abundantly expressed in their salivary glands. The GM-6557 codes for a collagenase-related metalloprotease, which could play a role in blood feeding, if secreted.

2.5.4.7 Anti-thrombin peptide

This is an anti-clotting agent found in *G. m. morsitans* saliva which was previously identified as an anti-thrombin but later molecularly characterized as a small 53 amino acids unique peptide (Cappello *et al*., 1998). Only one type of this peptide is found in the salivary gland cDNA library, despite the abundance of this product in the salivary glands of adult *Glossina*.

2.5.4.8 Serpins

Serpins are serine protease inhibitors with ubiquitous tissue expression indicating that they may function primarily in the control of proteolytic cascades such as in prophenoloxidase activation (Kanost, 1999). Notwithstanding this, a serpin family member contributes to the anti-clotting activity of *Aedes aegypti* saliva, which specifically blocks Factor Xa of the blood coagulation cascade (Stark and James 1998).

2.5.4.9 Prostaglandin E2 synthase

There are two types of prostaglandin E2 synthase from the salivary gland library, together with three from the fat body library. This enzyme may produce an endogenous mediator of salivary gland function, or may indicate that tsetse saliva may contain prostaglandin E_2, which is a potent skin vasodilator (Wallengren and Håkanson, 1992). Prostaglandin E_2 and other lipid mediators are found in the saliva of ticks (Ribeiro *et al*., 1992).

2.6 Immunity to tsetse saliva

Focusing on the early stage of trypanosome infection in the mammalian host, the effect of tsetse fly salivary components on parasitemia onset and on the involved host antiparasite immune effectors has been poorly investigated. Studies with other blood-sucking

arthropods, such as ticks and sand flies, have demonstrated that salivary proteins are potent modulators of host innate and adaptive immune responses. A broad repertoire of immune modulatory activities has been described. These modulatory activities include the predominant induction of a Th2 response with an overall inhibition of proinflammatory and Th1 cytokines (Kovar, *et al*., 2001) suppression of the effector functions of antigen-presenting cells (APCs) and modulation of T-cell and B-cell responses, as well as the inhibition of granulocyte infiltration and NK-mediated cytotoxicity (Kubes *et al*., 2002). Generally, the immunological effect in several vector-host interaction models is an anti-inflammatory action of the salivary components and the occurrence of a Th2-associated cytokine response in exposed hosts (Kovar *et al*., 2001).

2.7 Biomarkers of exposure to vector bites

Biomarkers of exposure to vector bites are biological molecules which can be used to show exposure of a given host to bites of the respective vector. Various studies have been carried out on different hosts to determine their exposure to vector bites with varying degrees of success (Remoue *et al*., 2006, Caljon *et al* 2006 and Nascimento *et al*., 2001). Of particular interest is the study by Somda (2010) who showed that measuring the immune response to *Glossina* saliva could be useful in detection of cattle exposure to tsetse bites. However, total saliva produced a lot of non specific reactions, suggesting that the best approach would be determination of host's antibody response to individual salivary protein thus the current study (Somda, 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study investigated samples collected from both tsetse endemic and non endemic areas. For tsetse endemic, Serere district was chosen, a district located in North- Eastern Uganda. This district was chosen because tsetse flies, human and animal African trypanosomiasis are prevalent in the area, with several species of *Genus Trypanosoma* including *T. brucei*, *T. congolence* and *T. vivax* (Ocaido *et al*., 2005; Enyaru *et al*., 2006) and *Glossina spp* (Ocaido *et al*., 2005) prevalent. For non-tsetse endemic areas, samples were collected from animals at the College of Veterinary Medicine, Animal Resources and Biosecurity, Kampala district. Laboratory analysis was conducted at the Molecular Biology Laboratory, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University.

3.2 Study design

The study was cross sectional in nature, involving collection of cattle blood and plasma samples from tsetse endemic and non endemic areas of Uganda. This was followed by various laboratory procedures including gene expression, protein purification and quantification, SDS and Western blotting; and ELISA tests.

3.3 Sample collection

Blood samples were collected from 80 heads of cattle from Serere district and 24 heads of cattle from Kampala, including calves. This was done by venipuncture of the jugular

21

vein to obtain about 5mls of blood per animal, into an EDTA anti-coagulated vacutainer. The blood samples were used for microscopic examination by hematocrit centrifugation technique (Woo *et al.*, 1969), to check for the presence of trypanosomes and about 4mls of the remaining portion centrifuged for collection of plasma that was frozen in liquid nitrogen and transported to the laboratory for storage at -80^0C. Plasma from both microscopically trypanosome positive and negative animals from the tsetse infested areas were used. Plasma samples were also obtained from blood of non-tsetse exposed cattle, collected from Kampala district. Negative cattle control serum was obtained from animals that had not been exposed to bites of arthropod vectors and it was imported from Sigma-Aldrich.

3.4 Propagation of rTSGF 2 and rTag 5 genes

Two sets of recombinant pET 28a expression vector (appendix VII), one containing the Tsetse salivary gland growth factor-2 gene fragment and the other Tsetse antigen- 5 gene fragment were provided by Prof. Serap Aksoy, Yale University, School of Medicine, USA. The recombinant plasmids were scaled up by transforming *E. coli* BL- 25 DE3 expression cells made competent by Calcium chloride method (Sambrook *et al.*, 1989) as shown in section 3.4.1 below. The scaling up maintained the availability of recombinant plasmids in sufficient amounts, kept as aliquots of 50µl at -20^0C.

3.4.1 Preparation of *E. coli* competent cells

Competent cells were prepared by adding 1ml. of overnight *E. coli* culture to 100mls Luria Bertani (LB) media. This was grown at 37°C while shaking until OD_{600} reached 0.6 to 1, which took about 3 to 4 hours. The culture was cooled to 4^0C and centrifuged at

4000 rpm for 10 minutes at 4˚C to harvest the cells. The cell pellet was re-suspended in 10mls of 0.1M CaCl$_2$ for every 50mls of culture and incubated on ice for 30 minutes. The cells were recovered by centrifugation at 4000rpm for 10 minutes at 4˚C, as above. The pellet was dissolved in 2mls of 0.1M CaCl$_2$, aliquoted in 100µl volumes and kept at 4˚C for a maximum of 2 days before addition of 20% glycerol for longer periods of storage at -80˚C.

3.4.2 Transformation of competent *E. coli* cells

The aliquoted (competent) cells from above were thawed slowly on ice and 100µl of the cells were pipetted into a clean eppendorf tube. About 1µg of plasmid DNA (from the stock kept at -20ºC) was added, mixed by gentle tapping on the tube and kept on ice for 30 minutes. The mixture was heat shocked by incubation at 42˚C for 40 seconds in a water bath. The content was then incubated on ice for 2 minutes, and then 800µls of LB was added, mixed and incubated at 37˚C for 1 hour in an orbital shaker at 150rpm. About 100µls of this culture was plated onto a selective LB agar plate containing 25µg/ml kanamycin. This procedure was repeated for the second set of plasmid DNA (for rTag-5) and the plates were incubated at 37˚C overnight while inverted.

3.4.3 Selection of colonies capable of expressing the recombinant plasmids

Four colonies for rTSGF-2 and 4 colonies for rTag-5 were picked from the LB agar plates (see section 3.4.2 above) and grown in 20mls of LB broth containing 25µg/ml of kanamycin to an OD$_{600}$ of 0.6. In each case, 10mls of the culture were induced using 1mM IPTG to express the respective recombinant proteins and the cultures were incubated at 30ºC for 3 hours. Cells were harvested by centrifugation at 8000rpm and

analyzed for recombinant protein expression as described in sections 3.6 and 3.7 below. The remaining 10mls of the non induced culture were kept at 4°C.

3.5 Expression and time course analysis of recombinant rTSGF-2 and rTag- 5 genes

This was achieved by inoculating 100μl of the non induced culture into 100ml of LB broth containing 25μg/ml kanamycin which was then grown for 3 hours with shaking. After this time the OD_{600} was about 0.6. A pre-induction sample of 2 mls was collected at this optical density before induction with 1mM IPTG per milliliter of culture, followed by 2mls hourly aliquots taken from the culture for 5 hours. This was done to determine optimum time of expression prior to harvesting of the cells.

The aliquots were centrifuged at 8000 rpm for 5 minutes after which the supernatant was removed. The pellets were mixed with a reducing sample buffer (appendix VII), boiled for 4 minutes and then stored at -20°C. The rTag-5 expression was carried out at varying temperatures (25°C, 30°C 33°C and 37°C) using different expression hosts including BL 21 DE3 and Rosetta. Also, the culture volume in this respect was increased to 1litre, in attempts to optimize rTag-5 expression.

3.6 Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis analysis

This was carried out to check for expression and confirm that the expressed proteins are the desired ones. The samples collected and prepared (refer to section 3.5 above) were used. The samples were picked from -20^0C, thawed, boiled again and ran on a 15% SDS-PAGE (separating) gel for both rTSGF-2 and rTag-5 and, 5% SDS-PAGE (stacking gel) using a BioRad Mini-Protean III Electrophoresis cell and an electrophoresis buffer

(250mM Glycine, 0.1% SDS, and 25mM Tris) according to the method of Laemmli, (1973).

3.7 Purification of recombinant proteins

About 500ml of LB broth containing 25μg/ml kanamycin were inoculated with 1ml of the non induced overnight culture of the transformed bacteria containing rTSGF-2 recombinant plasmids and grown up to OD_{600} of 0.6. Expression was induced as described in section 3.5 above. The rTSGF-2 was purified by Nickel- Nitriloacetic acid (Ni-NTA) agarose column (QIAexpressionist™ protocol 17) by initial preparation of a cleared *E. coli* lysates (QIAexpressionist™ protocol 10) under denaturing conditions according to manufacturer's instructions (appendix III). An aliquot of the purified protein was subjected to SDS-PAGE using a 12% gel and Western blotting using anti-histidine antibodies (as described in 3.9 below) was carried out to detect the identity of purified protein.

3.8 Electrophoretic transfer to nitrocellulose membrane and Western blotting

This was done to determine expression of the desired protein by probing with anti-histidine antibodies and to determine their recognition by bovine plasma that was obtained from blood collected from tsetse exposed cattle. To confirm expression, SDS-PAGE was performed as described earlier (section 3.6); the gels were equilibrated by soaking in the transfer buffer for 30 minutes and assembled with the blot membrane and pads. The proteins were blotted onto a nitrocellulose membrane (Amersham Biosciences) using a Miniprotein III Transblot unit (BioRad) making sure no air was trapped in between the blotting papers and the pads or between the gel and the membrane to ensure complete transfer. Transfer was carried out at 250 mA and 100 volts constant for 1 hour

using a transfer buffer (appendix VIII). The nitrocellulose membrane was rinsed in PBS-T. Non specific binding sites were blocked by incubating the membrane in 0.1% BSA overnight at 4^0C. The membrane was washed with PBS-T for 3 minutes and probed with mouse anti Histidine IgG from Qiagen® at a dilution of 1:2000. This was followed by washing with PBS-T as above and then probed with anti mouse IgG (Sigma-Aldrich) conjugated to horse raddish peroxidase as secondary antibody, at a dilution of 1: 5000 and incubated for 1hr and washed with PBS-T. For Histidine tag detection, 3, 3, 5', 5' diamino benzidine (DAB) solubilized in PBS and methanol to which 0.004 % H_2O_2 had been added was used to visualize any positive reactions.

3.9 Quantification of the purified rTSGF- 2 protein

The purified rTSGF- 2 protein was quantified using the Bradford's assay. Bovine Serum Albumin (1mg/ml) was diluted with 1XPBS to make standards of varying concentrations 50, 100, 150, 200 and 250µg/ml. To a 96 well-micro plate, 10µl of each standard and of the purified protein was loaded in duplicate. To each well with a sample, 200µl of Biorad® Bradford's dye which had been diluted with distilled water in a ratio of 1:4 was added. The samples and dye were mixed thoroughly and incubated in darkness at room temperature for 10 minutes and the optical densities of the samples were read by a micro plate reader (Biorad) at 495nm. Using Microsoft Excel spreadsheets, a graph of optical density against concentration of the standards was plotted and using the equation of the line of best fit, the concentration of purified protein was approximated.

3.10 Analysis for the recognition of rTSGF-2 and rTag-5 by cattle plasma antibodies

Un-purified and purified rTSGF-2, and un-purified rTag-5 were run on SDS-PAGE and transferred to nitrocellulose membrane as shown in section 3.8 above. The membrane was blocked with 0.1% BSA overnight at 4^0C. It was washed by agitating it in PBS-T for 5 minutes at room temperature. This was followed by addition of 15mls of plasma (at a dilution of 1: 200 in PBS) from trypanosome positive tsetse exposed cattle, microscopically trypanosome negative tsetse exposed cattle, cattle from non tsetse infested areas including calves and cattle negative control serum imported from SIGMA, and incubated for 1hr at room temperature, then washed with PBS-T 4 times for 5 minutes each. Horse raddish peroxidase conjugated anti- bovine IgG (secondary antibody) at a dilution of 1:2000 in PBS was added and incubated for 1 hr at room temperature and the membrane then washed three times with PBS-T for 5 minutes each wash. This was followed by addition of 9mls of 1X PBS containing 0.006g of DAB and 50µl of 30% hydrogen peroxide. The results were considered as positive if a signal of the expected molecular size was detected.

3.11 The rTSGF-2 indirect ELISA tests

Indirect ELISA tests were carried out with rTSGF-2 antigen to distinguish tsetse exposed from non exposed cattle, trypanosome infected from non infected cattle as well as adults and calves from non tsetse endemic areas. Purified rTSGF-2 at a concentration of 204.4ug/ml was diluted to a concentration of 1µg/ml with coating (bicarbonate) buffer and 100µl of this solution was pipetted into the different wells of the ELISA plate to coat overnight at 4^0C. The solution was discarded and the wells were washed with 300µl of

PBS-T and 200µl of blocking buffer were added and left to stand for one hour at room temperature to block non specific binding. The wells were again washed five times while shaking at five minutes interval with 300µl of PBS-T and cattle plasma samples at a dilution of 1: 500 were added as follows; to thirty six (36) wells were added 12 plasma samples (in triplicates) from tsetse exposed cattle, 36 wells received 12 plasma samples (also in triplicates) from cattle from non tsetse endemic areas and 6 wells received negative control serum samples from Sigma. Three wells were left as the blanks. The plate was incubated for 1 hour at room temperature after which it was washed five times while shaking at five minute intervals with 300µl of PBS-T. The secondary antibody (goat anti-bovine conjugated with horse raddish peroxidase) was then added and incubated for 1 hour at room temperature. The plate was washed 7 times with PBS-T, dried on tissue paper and 100µl of the substrate solution (TMB substrate reagent cat. No. 555214, BD OptEIA^{TM}) was added and kept in the dark for about 30 minutes. The reaction was stopped by adding 50ul of 2M sulphuirc acid before reading the optical densities at a wavelength of 490nm using the Gen 5 ELISA reader (Biotech from Northstar Scientific Ltd). The above procedure was repeated with samples from different animals of same categories as above to make a total of 24 for the non exposed group, of which 17 were adults and 7 calves; and 48 samples for tsetse exposed, 24 of which were from trypanosome infected cattle and 24 from trypanosome negative cattle.

3.12 Blast search

To detect presence of similar proteins to TSGF-2 (rTSGF-2), BLAST searches were performed against TSGF-2 (Gene Bank accession number: AAD52851.1) and rTSGF-2 protein sequence via NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignments were then carried out using the ClustalW2 alignment tool to establish the extent of similarity between rTSGF-2 fragment and some of the similar proteins, with special interest to similar proteins produced by arthropod vectors feeding on cattle.

3.13 Data analysis and presentation

Each sample was run in triplicate and its average optical density was obtained. The actual value of each sample was calculated by subtracting the average of the blanks from the samples OD. The cut off was then determined as the average of the negative controls plus two (2) times the standard deviation. Any value above the cut off was considered positive while values below the cut off were considered negative.

The results were analyzed using GraphPad prism 5 soft ware to determine the relationship between variables (using the Chi-square test to calculate P- values). Graphs were also drawn using the same program. Visual observation of gels and blots for presence or absence of band signals was also employed. Gel and blot pictures were taken for record and presentation of the results.

CHAPTER FOUR

RESULTS

4.1 Transformation results

Recombinant plasmids (containing rTSGF-2 gene and rTag-5 gene fragments) were used to transform competent BL21 DE3 *E. coli* expression hosts. The genes were expressed to obtain the rTSGF-2 and rTag-5 recombinant proteins for SDS-PAGE analysis, purification and analysis of their recognition by plasma from tsetse exposed cattle. Several colonies were obtained following transformation reactions and all the eight colonies selected for analysis (four colonies for rTSGF-2 and four colonies for rTag-5) expressed the protein of interest showing they had been correctly transformed.

4.2 Expression of rTSGF-2 and rTag-5 proteins

4.2.1 Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

This was carried out to show the expression of rTSGF-2 and rTag-5 proteins, their respective optimum expression time, and their detection by anti- His antibodies since the two proteins were His- tagged. The results revealed that both rTSGF-2 and rTag-5 were successfully expressed though in very low concentrations for rTag-5 as shown in Figure 2 and Figure 3 respectively below;
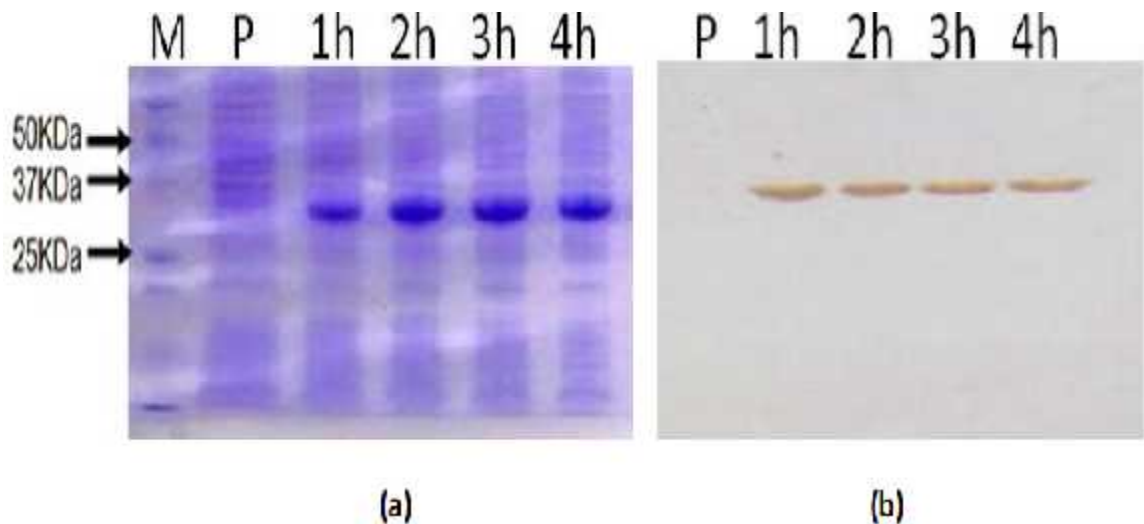
Fig. 2: Expression of rTSGF-2 in BL21 DE3 cells. M: Precision plus Protein standard, P: Pre-induction, Lanes 1, 2, 3 and 4 are samples collected at 1, 2, 3, and 4 hours post induction.

From the figure, Panel (a) is a Coomasie blue stained gel. It shows a time course analysis of rTSGF-2 in BL21 DE3 *E. coli* cells using a 15% SDS PAGE from pre-induction to 4 hours post induction on SDS-PAGE gel stained with Coomasie blue. The presence of a 31kDa protein band (corresponding to the protein under study) in the induced samples which were missing in the pre-induction sample. (b) A Western blot of the same gel in (a), showing the detection of the rTSGF-2, a 6x His- tagged protein as detected by Qiagen PentaHis® antibodies. The signals shown on the blot confirmed that the protein of interest had been expressed. There was no obvious increase in the quantity/amount of the expressed protein after 1hr as observed from the Coomasie blue stained gel.

With respect to rTag-5, the expression results were as indicated in the Figure 3 below;
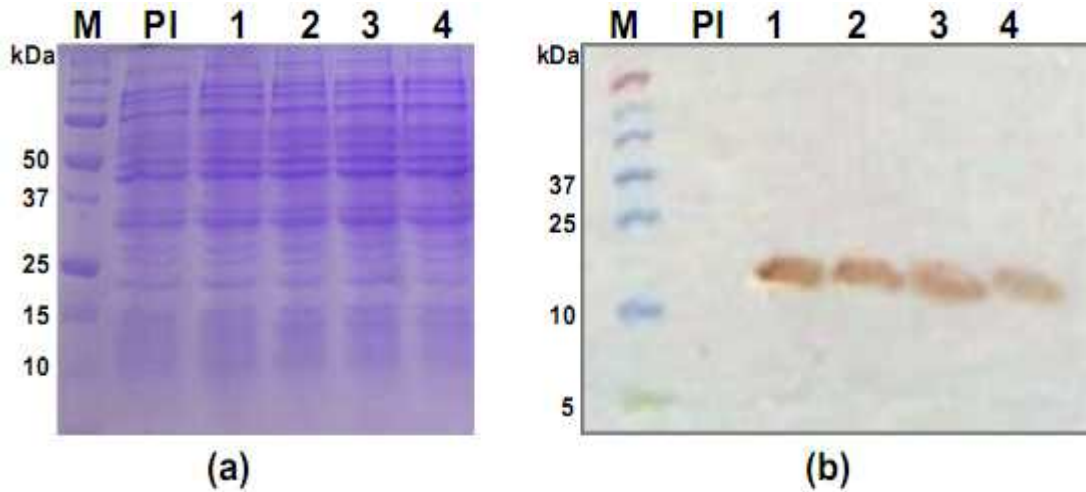
Fig. 3: Expression of rTag-5 in BL21 DE3 cells. M: Protein molecular marker, PI: Pre-induction, Lanes 1, 2, 3 and 4 are samples collected at 1, 2, 3, and 4 hours post induction. (NB: In panel 'a', M = precision plus Protein standard, un stained from BIO RAD; Panel 'b' M= PageRuler^{TM} Prestained Protein ladder from Fermentas Life Sciences)

From the figure, Panel (a) is a Coomasie blue stained gel. It shows a time course analysis of expression of the rTag-5 fragment in BL21 DE3 *E. coli* cells using a 15% SDS PAGE stained with Coomasie blue. (b) A Western blot of the same gel in (a), showing histidine detection using Qiagen PentaHis® antibodies. M: Protein molecular marker, PI: Pre-induction sample. The rTag-5 did not express in sufficient amounts in the different expression volumes (up to 1 litre), temperatures and host cell strains including *E. coli* BL21 DE3 (which showed the best results as detected by anti- his anti- bodies). However, anti- histidine Western blotting detected that a 15kDa hisitidine tagged protein (corresponding to the protein under study) was being expressed, despite failure to visualize the corresponding bands with Coomasie blue staining.

4.2.2 Purification of the rTSGF-2 and rTag-5 proteins

Recombinant TSGF-2 was purified using Ni-NTA agarose column and the purification products were run on 15% SDS-PAGE after which they were probed with anti-histidine antibodies. Tsetse salivary gland growth factor-2 was successfully purified and the pure recombinant protein was detected by the anti-histidine antibodies as evidenced by the signals on the blot as shown in the Figure 4 below. However, purification of rTag-5 protein was not attempted due to the very low expression levels (section 4.2; Fig. 3).
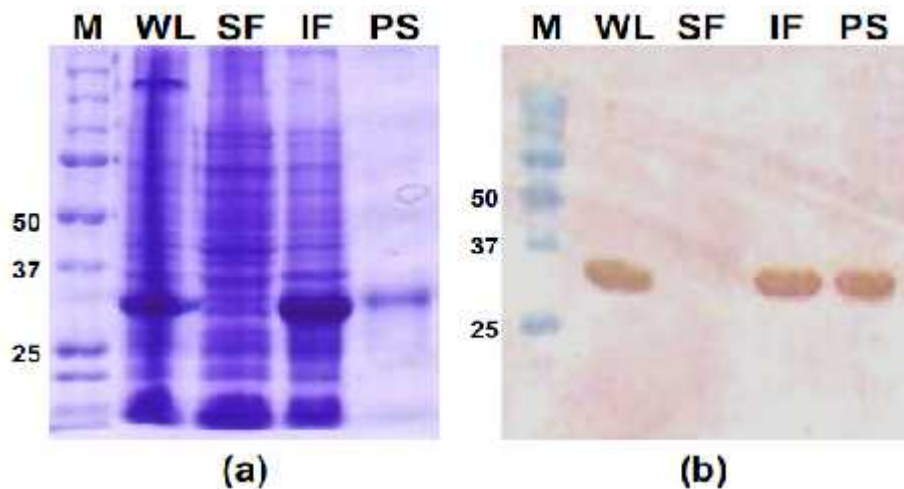
Figure 4: Purification of rTSGF-2 protein. M: marker, WL: Whole cell lysate, SF: Soluble fraction, IF: Insoluble fraction, PS: Purified sample (Elution).

From the figure, (a) Coomassie blue stained gel. (b) Western blot of the same gel, showing anti Histidine detection using Qiagen PentaHis® antibodies. The signals shown on the blot confirmed that the protein under study had been expressed and purified.

4.2.3 Quantification of the purified recombinant rTSGF-2 protein

The concentration of the produced rTSGF-2 was estimated by spectrophotometry. The optical densities of the Bovine serum albumin standards were read and plotted on a scatter graph, and the line of best fit (Y=0.0024X-0.0482) determined: it was considered the line of best fit because it produced the highest R^2 value (0.9875) which corresponded to the level of accuracy of the approximation using that line. The corresponding concentration to the read optical density of the purified protein was the protein concentration (203.4µg/ml) as shown in the in Figure 5 below;
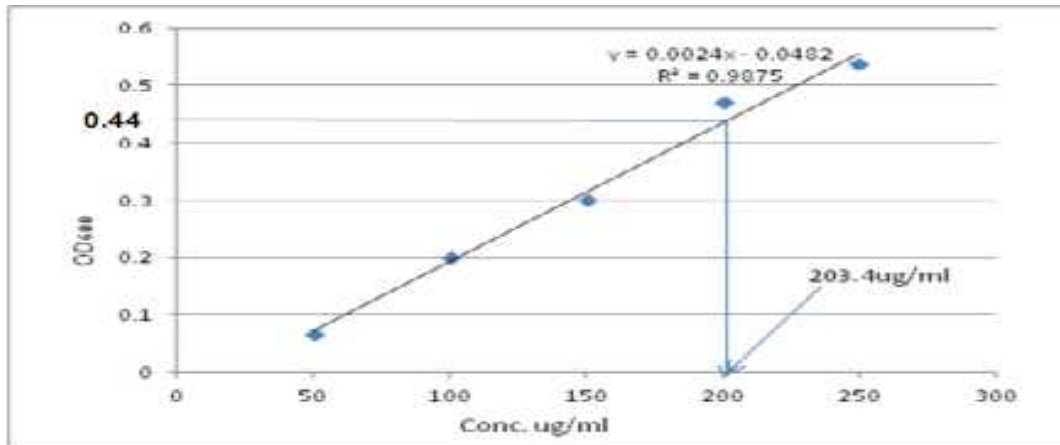
Figure 5: Standard curve (OD vs protein concentration) for determination of the concentration of purified rTSGF-2. The OD_{600}: Optical density at wavelength of 600nm, OD_{600} of test sample = 0.44nm

The protein sample optical density at 600nm (OD_{600}) was read off using spectrophotometer, which approximated to 0.44nm. Using the graphical scale, the position of 0.44nm along the OD_{600} was determined, and line parallel to the x- axis was drawn to touch the line of best fit and then a penpedicular line dropped to touch the x-axis and its corresponding value read-off; this was the approximate protein concentration, which was 203.4µg/ml.

4.3 Western blotting with tsetse exposed cattle plasma antibodies

To determine the immunoreactivity (recognition) of plasma from tsetse and/or trypanosome exposed cattle, Western blots of rTSGF-2 and rTag-5 were done and probed with plasma from tsetse exposed cattle, The *T. brucei* exposed cattle and serum from non exposed cattle, commercially obtained (SIGMA-Aldrich) were used as negative controls. The results revealed that both rTSGF-2 and rTag-5 were recognized by plasma from tsetse exposed cattle. Plasma from calves not exposed to tsetse flies also recognized the two proteins while the negative control serum did not recognize any of the proteins. The results are as shown in Figures 6 and 7 below;
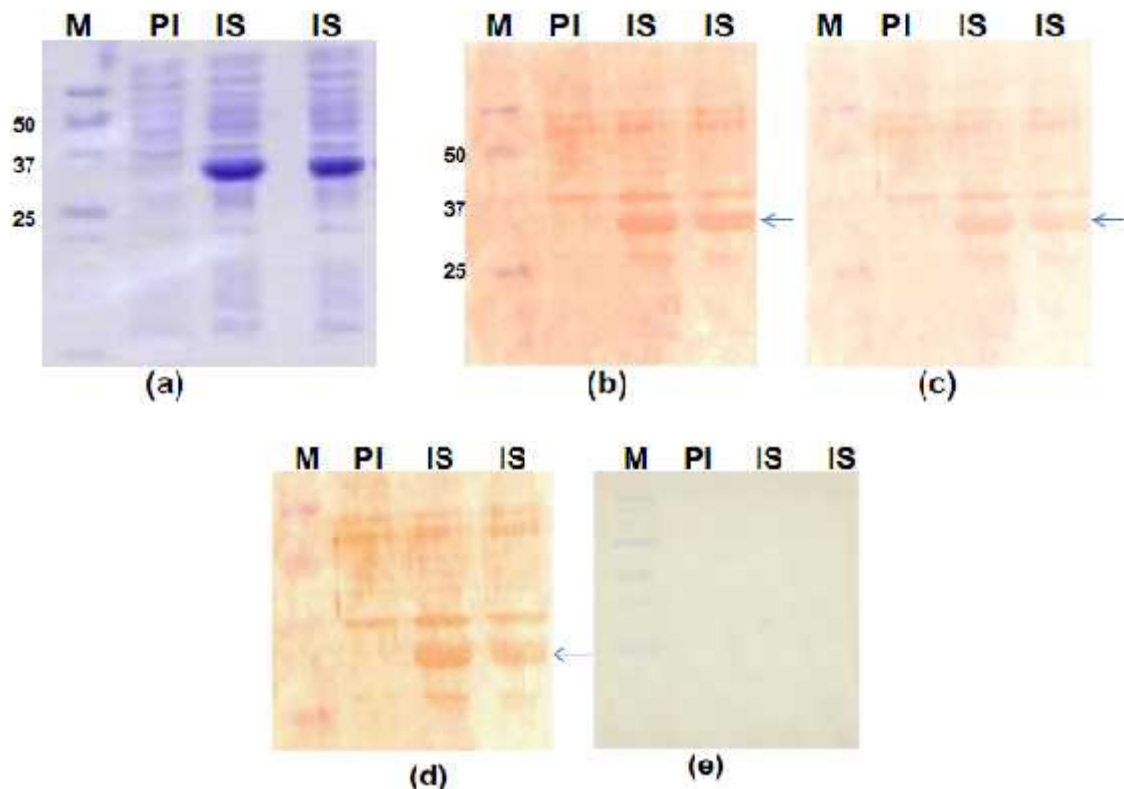
Figure 6: Recognition of rTSGF-2 by cattle plasma antibodies. M: protein marker, PI: Pre-induction sample IS: induced non purified rTSGF-2 sample. Arrows point at rTSGF-2 signals (31kDa).

From the Figure, (a) shows a Coomassie blue stained SDS-PAGE gel of rTSGF-2. **(b)**, **(c) (d)** and **(e)** show Western blots of the same gel (a), with pooled plasma from trypanosome positive cattle, pooled plasma from trypanosome negative cattle, pooled plasma from cattle from a non tsetse endemic area (Each pool was constituted by 5 different plasma samples), and negative control cattle serum from Sigma respectively, all as detected by DAB.

The recognition of rTag-5 by the various groups of cattle plasma is as shown in the figure 7 below;
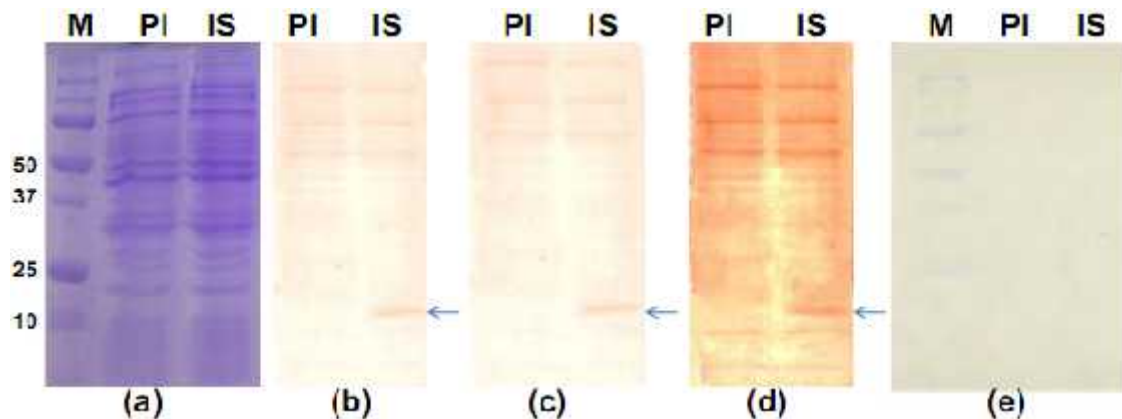
Figure 7: Recognition of rTag-5 by cattle plasma antibodies. M: Marker, PI: Pre-induction sample IS: induced non purified rTag-5 sample. Arrows point at rTag-5 signals (15kDa). From the Figure, **(a)** shows a Coomassie blue stained SDS-PAGE gel of rTag-5. **(b), (c)**

(d) and (e) show Western blots with pooled plasma from trypanosome positive cattle, pooled plasma from trypanosome negative cattle, pooled plasma from cattle from non tsetse endemic areas, and negative cattle control serum from Sigma respectively, as detected by DAB. Despite the poor expression levels of rTag-5, both trypanosome positive and negative tsetse exposed bovine pooled plasma recognized it as indicated by the Western blots in figure 7 (b, c and d) above.

4.4 Differentiation between the various groups of cattle using rTSGF-2 ELISA

The ELISA tests were performed to determine whether rTSGF-2 could be used to distinguish between tsetse exposed and non exposed cattle. Results are as indicated in Figure 8 below;
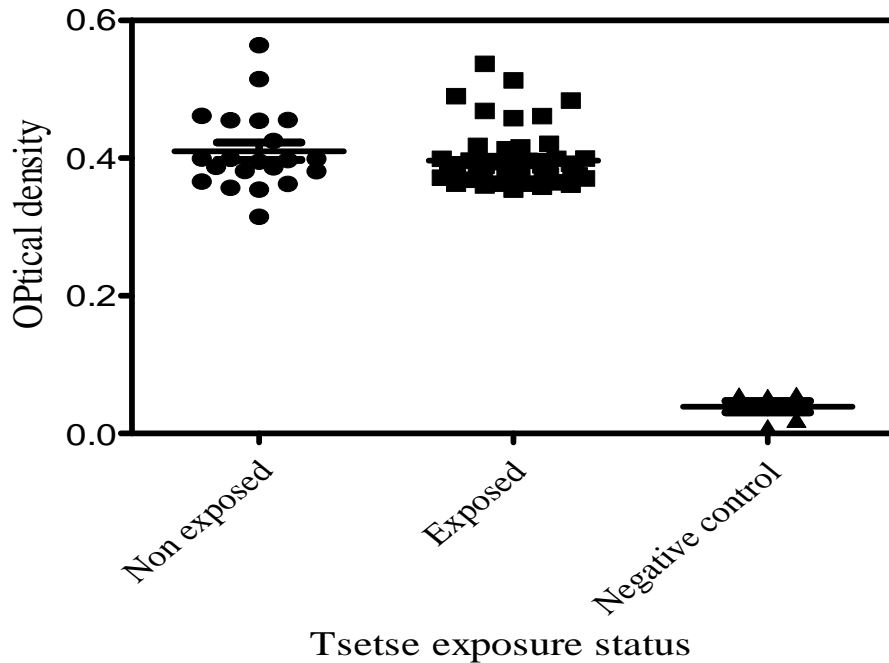
Figure 8: Differentiation between tsetse exposed and non exposed cattle using rTSGF-2 ELISA test. Non exposed: plasma from cattle not expose to tsetse bites, Exposed: Plasma from cattle exposed to tsetse bites. Negative control: Negative cattle control serum (SIGMA).

Results revealed that rTSGF-2 protein could not distinguish between tsetse exposed and non exposed cattle as there was no significant difference in OD values between the two groups (P 0.05), the average though was slightly higher in exposed cattle. However, there was a significant difference (P 0.05) on comparing these two groups with the commercially obtained negative cattle control serum (Figure 8).

Tsetse salivary gland growth factor II ELISA test was also performed to show whether it could be used to differentiate between adults and calves from non tsetse endemic areas, on assumption that calves were less likely to have been exposed to TSGF-2 and other similar proteins from biting flies and ticks. Results are indicated in figure 9 below;
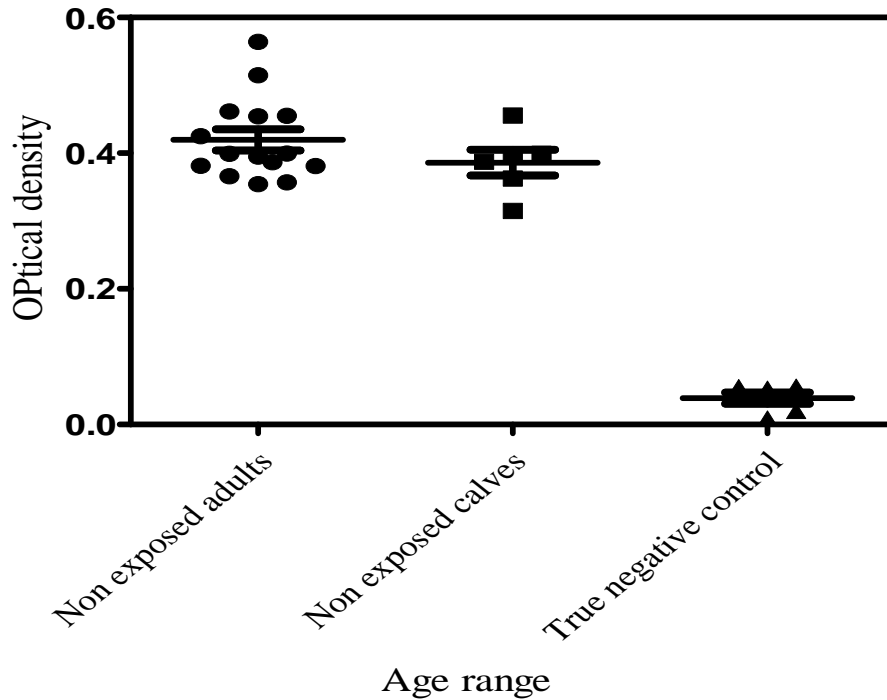
Figure 9: Differentiation between adults and calves from non tsetse endemic areas. Non exposed adults: Adult cattle not exposed to tsetse bites, Non exposed calves: calves not exposed to tsetse bites, Negative control: serum from cattle that had not been exposed to vector bites.

The results revealed no significant differences (P> 0.05) between the two groups although the average OD values of adults were slightly higher than those of calves. However, there was a significant difference (P< 0.05) between the OD valves of either of these two groups and the negative control (Figure 9).

A similar test to the ones above was performed to determine whether rTSGF-2 can be used to distinguish between trypanosome infected and non infected cattle and the results obtained are indicated in figure 10 below;
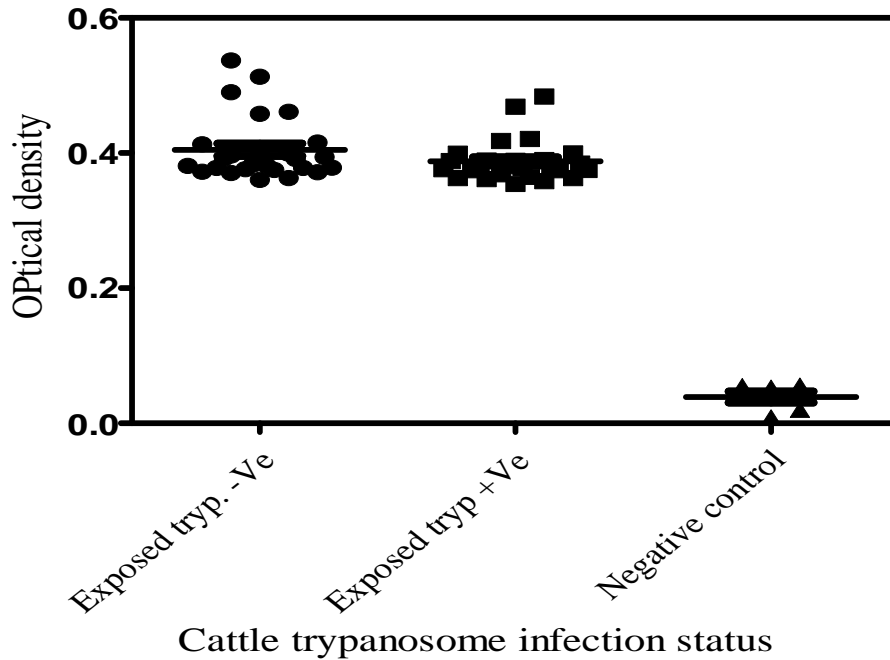
Figure 10: Differentiation between trypanosome infected and non infected cattle using rTSGF-2 ELISA test. Exposed tryp.-ve: cattle exposed to tsetse bites but negative for trypanosomes on microscopy, Exposed tryp.+ve: cattle exposed to tsetse bites and positive for trypanosomes on microscopy, Negative control: cattle not exposed to vector bites and trypanosomes.

Results revealed that rTSGF-2 protein could not distinguish between tsetse exposed trypanosome positive and tsetse exposed trypanosome negative cattle as there was no significant difference in OD values between the two groups (P 0.05), but the average for trypanosome positive was slightly lower. However, there was a significant difference (P 0.05) on comparing these two groups with the commercially obtained negative cattle control serum (Figure 10).

A cut off value was calculated to establish whether rTSGF-2 ELISA could be used to distinguish tsetse exposed from non tsetse exposed cattle. This value was obtained as the average of the negative control + 2SD (where SD= standard deviation) and it was found to be 0.09. Any OD value above the cut off would mean that the source animal of the corresponding plasma was exposed to tsetse bites (positive) while those below would be regarded non exposed (negative). However basing on this criterion, all the animals had valves above the cut off including those from non tsetse infested areas. Details of the results are shown in Appendix I. This further confirmed the statistical calculations that showed no significant differences between the OD values of the test groups.

Also, the Blast search performed revealed other arthropod parasites feeding on cattle which express significantly similar proteins to TSGF-2 as shown in table 1 below;

Table 1: Percentage similarity of the 31kDa TSGF-2 fragment with related proteins

Name	Length	Accession code & source organism	Length	Score (%)
31kDa	240	AAD52851.1; *G. m. morsitans* (TSGF-2*)*	506	100.0
31kDa	240	AAK97208.1; *Culex quinquefasciatus*	502	34.0
TSGF-2	506	AAK97208.1; *Culex quinquefasciatus*	502	39.0
31kDa	240	ABI20162.1; *Phlebotomus duboscqi*	516	32.0
TSGF-2	506	ABI20162.1; *Phlebotomus duboscqi*	516	36.0
31kDa	240	AAF78901.1; *Lutzomyia longipalpis*	508	29.0
TSGF-2	506	AAF78901.1; *Lutzomyia longipalpis*	508	36.0
31kDa	240	EAA04809.4; *Anopheles gambiae*	511	30.0
TSGF-2	506	EAA04809.4; *Anopheles gambiae*	511	36.0

Any two or more proteins with a percentage similarity of 30% and above are significantly

similar to produce similar antigenic reactions. As seen from the table, the percentage

similarity of the selected proteins ranged between 29% and 39%.

CHAPTER FIVE

DISCUSSION

Tsetse salivary gland growth factor-2 and tsetse antigen-5 form part of the protein constituents of tsetse saliva and are therefore injected into the mammalian host during tsetse feeding. Both proteins have been shown to promote trypanosome establishment in the vertebrate host by modulating the host's immune response in a manner that favors the survival of the parasites (Van den Abbeele *et al*., 2010). This in turn implies a close correlation between trypanosome infection status of the host and its immune response in relation to these proteins besides other antigenic components of the trypanosomes. Therefore, by measuring the host's immune response against these proteins, one could expect to determine tsetse exposure in cattle and possibly their trypanosome infection status, if the proteins play a role in establishment of trypanosomes in the vertebrate host. However, this study demonstrated that both rTSGF-2 and rTag-5 fragments of TSGF-2 and Tag-5 respectively are not specific markers of exposure to tsetse bites in cattle and therefore they cannot be used to distinguish trypanosome infected from non infected cattle.

Following transformation of the competent cells (BL 21 DE3) with rTSGF-2 and rTag-5 recombinant pET 28a expression vector, a large number of colonies for the two proteins (on separate plates) were obtained. All the 8 colonies selected (4 for rTSGF-2 and 4 for rTag-5) expressed their respective proteins showing high transformation efficiency. This was probably because transformation was done using pure circular recombinant plasmids

unlike cases where ligation reactions are used with high chances of having ligation of non recombinant plasmids (re-circularization).

Recombinant tsetse salivary gland growth factor- 2 was successfully expressed in BL21 DE$_3$ *E. coli* strain. The rTSGF-2 protein was expressed in sufficient amounts and of the expected molecular size as previously observed by Li and Aksoy (2000), who cloned and expressed the same 810 base pairs gene fragment falling between 375 to 1094bp of the of the TSGF-2 cDNA.

Tsetse antigen-5 was expressed in very low amounts in the different *E. coli* bacterial expression hosts used at varying temperatures including BL21 DE3 and Rosetta *E. coli* strains. However, BL21 DE3 yielded better results as detected by anti- histidine antibodies, with an expression product of about 15kDa, a result similar to what was observed by Li *et al*., (2001). The low expression levels of rTag-5 could have been due to use of sub-optimal expression hosts and expression temperatures, despite the fact that different expression hosts, culture volumes (up to 1 liter) and expression temperatures were tried. Rapid degradation of the expressed products which may occur concurrently as expression is taking place, during or after lysate preparation could also be another reason explaining the very low expression levels observed with rTag-5, despite the addition of protease inhibitors during lysate preparation.

The rTSGF-2 was successfully purified using Ni-NTA agarose column and this was attributed to the high expression levels of this 6X His tagged protein. However, rTag-5 protein purification was not attempted due to the very low expression levels, despite the measures undertaken to optimize the expression and proper handling of the harvested

43

cells and samples thereafter. Such measures included working at lower temperatures of 4^0C and use of protease inhibitor cocktail (Sigma® P8849) during the lysate preparation, which were always done immediately after expression to minimize enzymatic degradation. These were in addition to the expression optimization steps undertaken as pointed out earlier in this section.

The Western blots of unpurified and purified recombinant rTSGF-2 and un purified rTag-5 were probed with pooled plasma from cattle exposed to tsetse flies from trypanosome endemic area of Uganda. Some of these animals were trypanosome positive while others negative. Plasma of animals from non tsetse endemic areas including calves that had not been exposed to tsetse bites was also tested. Negative control serum used was obtained from Sigma. Both rTSGF-2 and rTag-5 were recognized by plasma from all the groups of animals except the commercially obtained serum. This means that there were antibodies in the non exposed animals that could recognize the two proteins, suggesting possible exposure to bites of other arthropod vectors expressing antigenically similar proteins to the ones of interest in this study. This is confirmed by the ELISA results, which are also in agreement with this finding. Signals from Western blots were stronger with rTSGF-2 as compared to rTag-5 and this was attributed to the expression levels of rTSGF-2 which were high compared to that of rTag-5 which was low. Despite the very low expression levels of rTag-5, the plasma could recognize it meaning it is also immunogenic just like rTSGF-2. Non specific binding to *E. coli* proteins was observed with the non purified samples (the whole cell *E. coli* cell lysate, containing the proteins of interest) and this was probably because, field serum samples were used, where source animals could have been exposed to *E. coli* such that antibodies against certain *E. coli* proteins were also

contained in the plasma samples together with those against TSGF-2 and Tag-5. For reactive animals, there was no obvious difference in signal intensity between plasma from the different groups of animals, including cattle (and calves) from non tsetse endemic areas which had themselves probably never been exposed to tsetse bites and this suggested a possibility of maternal transfer of the reactive antibodies and/or actual exposure to other arthropod bites e.g. mosquitoes, which produce similar proteins.

The ELISA tests were carried out using rTSGF-2 antigen in an attempt to distinguish between tsetse exposed from non exposed cattle and trypanosome infected from non infected cattle. The results indicated no significant difference in OD values between tsetse exposed and non exposed cattle; and also no difference between trypanosome infected and non infected cattle (P 0.05 in both cases). This was probably because the different groups of cattle had been exposed to bites of other blood feeding arthropods like ticks, mosquitoes and sand flies which express antigenically similar proteins to rTSGF-2, leading to production of antibodies against such proteins which could cross-react with the protein under study. Somda (2010) found similar results when he demonstrated high anti saliva response in animals highly exposed to tsetse bites. From his study however, he also observed that cattle from a tsetse free area had high anti tsetse saliva responses. On further investigation he observed cross reactivity with the saliva of the horse fly. In the present study, BLAST searches were performed against TSGF-2 (Gene Bank accession number: AAD52851.1) and they revealed presence of significantly similar proteins to rTSGF-2 in other arthropod vectors including ticks under genus *Ixodes*, *Culex*, *Aedes* and *anopheles* mosquitoes, as well as *Lutzomyia* and *Phlebotomus* sand flies (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignments were carried out using the

ClustalW2 alignment tool to establish the extent of similarity between rTSGF-2 fragment and some of these proteins. The following proteins as referred to by their Gene Bank accession numbers were found to be similar as follows; 34.0 % sequence similarity to AAK97208.1 from *Culex quinquefasciatus*, 32% similarity to ABI20162.1 from *Phlebotomus duboscqi*, 29% to AAF78901.1 from *Lutzomyia longipalpis and* 30% similar to EAA04809.4 from *Anopheles gambiae* (see Table 1; Appendices II, III, IV,V and VI) among others. The percentage similarity of the above proteins fall at or above the protein similarity cut off of 30% (Rost, 1999; Yang and Honig, 2000). The significantly similar proteins to rTSGF-2 are basically adenosine deaminases with growth factor motifs, a group of proteins secreted by a wider range of organisms including non biting insects, molluscs and some vertebrates. This shows likelihood that this group of proteins is secreted by many other arthropods feeding on cattle. Tsetse salivary gland growth factor-2 falls in this category which partly explains the reactivity with plasma from non tsetse endemic areas.

The results from this study also deviates from those of a previous study by Somda (2010), who showed a strong positive association between the anti-saliva response and the risk of being infected by trypanosomes. Here, rTSGF-2 ELISA tests could not distinguish between trypanosome positive and negative cattle probably due to similar reasons as above, in addition to the fact that the Somda study was non specific to the different salivary constituents as it involved general anti-saliva response, not response to individual salivary proteins. Thus the difference observed in that study could have been a product of components other than TSGF-2 examined in this study. Identification and further exploration of other candidate proteins in saliva may give clues to components

that would be of diagnostic value. Noteworthy is that this study considered only a fragment of either of the two proteins, with rTSGF-2 being only 240 amino acids (gene region between 375-1094 base pairs of the TSGF-2 cDNA) as compared to the 506 amino acids of TSGF-2 (Li and Aksoy, 2000). This indicates a possibility that other regions of the TSGF-2 gene may produce specific and therefore more useful results in relation to establishment of tsetse exposure and trypanosome infection status in cattle.

Despite the fact that rTag-5 ELISA tests were not performed due to purification limitations, similar results to those of rTSGF-2 are likely to be observed for similar reasons as Tag-5 family of proteins is expressed by a wider range of arthropod vectors including sand flies, *Culicoides*, *Stomoxysis calcitrans* (Ameri *et al*., 2008) and tabanids (Xu *et al*., 2009), as compared to TSGF-2. But whether specific response would vary between trypanosome positive and negative cattle is best determined by carrying out the ELISA.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

- The rTSGF-2 was expressed and purified in sufficient quantities in this study while rTag-5 was only expressed in very low quantities.

- Both rTSGF-2 and rTag-5 were recognized by plasma from both tsetse exposed and non exposed cattle but not by commercially obtained negative control serum.

- The rTSGF-2 can neither be used to specifically distinguish tsetse exposed from non exposed cattle nor trypanosome infected from non infected cattle. Therefore it is not a reliable marker of exclusive exposure to tsetse bites in cattle. The usefulness of rTag-5 in this respect remains to be determined.

6.2 RECOMMENDATIONS

- The expression of rTag-5 should be optimized with subsequent purification and ELISA tests should be performed.

- Polyclonal antibodies against rTSGF-2 and rTag-5 should be produced in laboratory animals and assayed for specificity.

- Similar proteins secreted by other vectors such as some ticks, *Phlebotomus* sand flies, *Culicodes, Stomoxysis*, *Culex* mosquitoes and tabanids should be evaluated for recognition by antibodies produced against rTSGF-2 and rTag-5.

- Future studies should consider use of antigenic salivary proteins expressed from gene regions unique to *Glossina*.

REFERENCES

Abbeele JVD**, Caljon G, Ridder KD, Baetselier PD, Coosemans M, (2010).** *Trypanosoma brucei* modifies the tsetse salivary composition, altering the fly feeding behavior that favors parasite transmission. *PLoS Pathogens*, 6(6):e1000926.

Abbeele JVD, Claes Y, Bockstaele DV, Le RD, Coosemans M, (1999). *Trypanosoma brucei* spp. development in the tsetse fly: Characterization of the post-mesocyclic stages in the foregut and proboscis. *Parasitology*, 118: 469–478.

Adeniji KO, (1993). Ruminant livestock control and distribution in Africa. World *Review of Animal Production*, 28(2): 25-32.

Alves-Silva J, Ribeiro JM, Van Den Abbeele J, Attardo G, Hao Z, Haines LR, Soares MB, Berriman M, Aksoy S, Lehane MJ, (2010). An insight into the sialome of *Glossina morsitans morsitans*. *BMC Genomics*, 30;11:213.

Ameri M, Wang X, Wilkerson MJ, Kanost MR, Broce AB, (2008). An immunoglobulin binding protein (antigen 5) of the stable fly (Diptera: Muscidae) salivary gland stimulates bovine immune responses. *Journal of Medical Entomology*, 45(1):94–101.

Anderson JM, Oliveira F, Kamhawi S, Mans BJ, Reynoso D, Seitz AE, Lawyer P, Garfield M, Pham M, Valenzuela JG, (2006). Comparative salivary gland transcriptomics of sandfly vectors of visceral leishmaniasis. *BMC Genomics*, 7:52.

Arca B, Lombardo F, Francischetti IM, Pham VM, Mestres-Simon M, Andersen JF, Ribeiro JM, (2007). An insight into the sialome of the adult female mosquito *Aedes albopictus*. *Insect Biochemistry. and Molecular Biology*, 37(2):107–127.

Balaan N, Waithaka HK, Njogu AR, Goldman R, (1995). Intracellular antigens (microtubules- associated proteins copurified with glycosomal enzymes)- Possible vaccines against trypanosomiasis. *Journal of Infectious Diseases*, 172:845-850.

Barral A, Honda E, Caldas A, Costa J, Vinhas V, Rowton ED, Valenzuela JG, Charlab R, Barral-Netto M, Ribeiro JM, (2000). Human immune response to sand fly salivary gland antigens: a useful epidemiological marker*? Amerian journal of Tropical Medicine and Hygiene,* 62: 740–745.

Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ, and Krishna S, (2003). The trypanosomiases. *Lancet,* 362:1469– 1480.

Biebinger S, Rettenmaier S, Flaspohler J, Hartmann C, Peña-Diaz J, Elizabeth L, Wirtz, Hotz HR, Barry1 JD, Clayton C, (1996). The PARP promoter of *Trypanosoma brucei* is developmentally regulated in a chromosomal context. *Nucleic Acids Research,* 24: 1202–1211.

Bossche VD, Akoda K, Kubi C, Marcotty T, (2006a). The transmissibility of *Trypanosoma congolense* seem to be associated with its level of resistance to isometamedium chloride. *Veterinary Parasitology*, 123: 365-367.

Brightwell R, Dransfield RD, and Kyoku C, (1991). Development of low cost tsetse trap and odor baits for *Glossina pallidipes* and *G. longipennis* in Kenya. *Medical and Veterinary Entomology*. 5: 153-164.

Caljon G, Abbeele JVD, Sternberg JM, Coosemans M, Baetselier D P, Magez S, (2006). Tsetse fly saliva biases the immune response to Th2 and induces anti-vector antibodies that are a useful tool for exposure assessment. *International Journal of Parasitology*, 36: 1025–1035.

Caljon G, Broos K, Goeyse I D, Ridder K D, Sternberg JM, Coosemans M, Baetselier PD, Guisez Y, Abbeele JVD, (2009). Identification of a functional Antigen 5-related allergen in the saliva of a blood feeding insect, the tsetse fly. *Insect Biochemistry and Molecular Biology*, 39(5-6):332–341.

Calvo E, Dao A, Pham VM, Ribeiro JM, (2007). An insight into the sialome of *Anopheles funestus* reveals an emerging pattern in anopheline salivary protein families. *Insect Biochemistry and Molecular Biology*, 37(2):164–175.

Campbell CL, Vandyke KA, Letchworth GJ, Drolet BS, Hanekamp T, Wilson WC, (2005). Midgut and salivary gland transcriptomes of the arbovirus vector *Culicoides sonorensis* (Diptera: Ceratopogonidae). *Insect Molecular Biology*, 14(2):121–136.

Cappello M, Li S, Chen X, Li CB, Harrison L, Narashimhan S, Beard CB, Aksoy S, (1998). Tsetse thrombin inhibitor: bloodmeal-induced expression of an anticoagulant in salivary glands and gut tissue of *Glossina morsitans morsitans*. *Proceedings of the National Acadademy of Sciences of the U. S. A*, 24;95(24):14290-14295.

CDC, (2006). Parasites and Health; Laboratory identification of parasites of Heath concern. *www.dpd.cdc.gov/DPDX/HTML/Para_Heath.htm* 24th.OCT 2006.

Croft SL, Barret MP, and Urbina JA, (2005). Chemotherapy and trypanosomiases and leishmaniasis. *Trends Parasitology*, 21:508–512.

Enyaru JC, Matovu E, Nerima B, Akol M and Sebikali C, (2006). Detection of *T.b. rhodesiense* trypanosomes in humans and domestic animals in south east Uganda by amplification of serum resistance-associated gene. *Annals of the New York Academy of Sciences*, 1081:311

Fatic BM, (2011). Human Parasitology, 218, ISBN 8120342925

Feldmann U, Hendrichs J, (2001). Integrating the sterile insect technique as a key component of area wide tsetse and trypanosomiasis intervention. PAAT technical and scientific series 4 OA/WHO/IAEA/OA.

http://blast.ncbi.nlm.nih.gov/Blast.cgi *(Accessed on 24th May 2012 at 6:00 PM)*

ILCA (International Livestock Centre for Africa), (1979). Trypanotolerant Livestock in Westand Central Africa. ILCA monograph 2. ILCA Addis Ababa.

Kalu AU, (1995). Prevalence of trypanosomiasis among trypanotolerant cattle of Nigeria. *Preventive Veterinary Medicine*, 24(2): 97-103.

Kanost MR, (1999). Serine proteinase inhibitors in arthropod immunity. *Developmental and Comparative Immunology*, 23(4-5):291-301.

Kanost MR, Jiang H, Yu XQ, (2004). Innate immune responses of a lepidopteran insect, Manduca sexta. *Immunological Reviews*, 198:97-105.

Kennedy PGE, (2006). Diagnostic and neuropathogenesis issues in human African trypanosomiasis. *International Journal for Parasitology.*36 (5):505–512.

Kettle DS, (1995). Medical and Veterinary Entomology, 2nd Edition. CAB International publishers. ISBN 0 85198 3 Hbk pg 225-243.

Kovar L, Kopecky J, and Rihova B, (2001). Salivary gland extract from *Ixodes ricinus* tick polarizes the cytokine profile toward Th2 and suppresses proliferation of T lymphocytes in human PBMC culture. *Journal of Parasitology*, 87:1342-1348.

Kuzoe F, Schofield C, (2005). Strategic Review of Traps and Targets for Tsetse and African Trypanosomiasis Control. Geneva: World Health Organization. TDR/IDE/TRY/05.1.

Kuzoe FA, (1993). Current situation of African trypanosomiasis. *Acta Tropica.* 10: 153-162.

Lane RS, Moss RB, Hsu YP, Wei T, Mesirow ML, Kuo MM, (1999). Anti-arthropod saliva antibodies among residents of a community at high risk for Lyme disease in California. *American Journal of Tropical Medicine and Hygiene*, 61: 850–859.

Leak SGA, Peregrine S, Mulatu W, Rawlands GJ, and D'ieteren GDM, (1996). Use of insecticide impregnated targets for the control of tsetse flies (*Glossina spp*.) and trypanosomiasis occurring in cattle in an area of south west Ethopia with a high prevalence of drug resistant trypanosomes. *Tropical Hygiene and International Health*. 1. 599-609.

Li S, Aksoy S, (2000). A family of genes with growth factor and adenosine deaminase similarity are preferentially expressed in the salivary glands of *Glossina m. morsitans*. *Gene*, 252:83-93.

Li S, Kwon J, Aksoy S, (2001). Characterization of genes expressed in the salivary glands of the tsetse fly, *Glossina morsitans morsitans*. *Insect Molecular Biology*, 10(1):69–76.

Lubega GW, Byarugaba, DK, Prichard RK, (2002). Immunization with a tubulin rich preparation from *Trypanosoma brucei* confers broad protection against African trypanosomosis. *Experimental parasitology*, 102:9-22.

Matovu E, Mhairi LS, Geiser F, Reto B, Mäser P, Wallace LJM, Burchmore RJ, Enyaru JCK, Barrett MP, Kaminsky R, Seebeck T, Koning HP, (2003). Mechanisms of Arsenical and Diamidine Uptake and Resistance in *Trypanosoma brucei. Eukaryotic Cell*, 1535-9778.

Milne TJ, Abbenante G, Tyndall JD, Halliday J, Lewis RJ, **(2003).** Isolation and characterization of a cone snail protease with homology to CRISP proteins of the pathogenesis-related protein superfamily. *Journal of Biolological Chemistry*, 15;278 (33):31105-31110

Murray PK, Murray M, Wallace M, Morrison WI, McIntyre WIM, (1979). 15th Meeting of ISCTRC, Banjul, Gambia1977, OAU/STRC. 116: 470.

Nascimento RJ, Santana JM, Lozzi SP, Araujo CN, Teixeira AR, (2001). Human IgG1 and IgG4: the main antibodies against *Triatoma infestans* (Hemiptera: Reduviidae)

salivary gland proteins. *American Journal of Tropical Medicine and Hygiene*, 65: 219–226.

Nobile M, Noceti F, Prestipino G, Possani LD, (1996). Helothermine, a lizard venom toxin, inhibits calcium current in cerebellar granules. *Experimental Brain Research*, 10(1):15–20.

Nolan DP, Jackson DG, Windle HJ, Pays A, Geuskens M, Michel A, Voorheis HP, and Pays E, (1997). Characterisation a novel stage specific invariant surface protein in *Trypanosoma brucei* containing an internal, serine rich, repeatitive motif. *Journal of Biological Chemistry*, 272: 29212-29221.

Ocaido M, Otim CP, Okuna NM, Erume J, Ssekitto C, Wafula RZO, Kakaire D, Walubengo J, and Monrad J, (2005). Socio-economic and livestock disease survey of agro-pastoral communities in Serere County, Soroti District, Uganda. *Livestock Research for Rural Develop.* 17 (8).

Palosuo K, Brummer-Korvenkontio H, Mikkola J, Sahi T, Reunala T, (1997). Seasonal increase in human IgE and IgG4 anti-saliva antibodies to *Aedes* mosquito bites. *Internatioal Archives of Allergy and Immunology*, 114: 367–372.

Pays E, (1997). The trypanosome surface. Preceedings of the third franciqui colloquium 26: 27 May 1997, Brussels. deBoek Universite.

Poinsignon A, Cornelie S, Remoue F, Grebaut P, Courtin D, Garcia A, Simondon F, (2007). Human/vector relationships during human African trypanosomiasis: initial screening of immunogenic salivary proteins of *Glossina* species. *American Journal of Tropical Medicine and Hygiene*, 76.

Poinsignon A, Remoue F, Rossignol M, Cornelie S, Courtin D, Grébaut P, Garcia A, and Francois Simondon, (2008). Human IgG Antibody Response to *Glossina* Saliva: An Epidemiologic Marker of Exposure to *Glossina* Bites. *American Journal of Tropical Medicine and Hygiene*, 78: 5 750-753.

Rasooly R, and Balaan NB, (2004). Trypanosome microtubule associated protein p15 as a vaccine for the prevention of African sleeping sickness. *Vaccine.* 22:1007-1015.

Remoue F, Alix E, Cornelie S, Sokhna C, Cisse B, Doucoure S, Mouchet F, Boulanger D, Simondon F, (2007). IgE and IgG4 antibody responses to Aedes saliva in African children. *Acta Tropica*, 104: 108–115.

Remoue F, Cisse B, Ba F, Sokhna C, Herve J-P, Boulanger D, Simondon F, (2006). Evaluation of the antibody response to Anopheles salivary antigens as a potential marker of risk of malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 100: 363–370.

Ribeiro JM, (1995) Blood-feeding arthropods: live syringes or invertebrate pharmacologists? *Infectious Agents and Disease*, *4: 143–152.*

Ribeiro JMC, Francischetti IMB, (2003). Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annual Review of Entomology*, 48:73–88.

Ribeiro JMj, Evans PM, MacSwain JL, Sauer J, (1992). *Amblyomma americanum*: characterization of salivary prostaglandins E2 and F2 alpha by RP-HPLC/bioassay and gas chromatography-mass spectrometry. *Experimental Parasitology*, 74(1):112-116.

Rogers DJ, Williams BG, (1993). Monitoring trypanosomiasis in space and time. *Parasitology* 106 (Suppl): S77–S92.

Rost B, (1999). Twilight zone of protein sequence alignments. *Protein Engineering*. 12(2):85-94.

Sambrook J, Fritsch EF, Maniatis T, (1989). Molecular cloning, A laboratory manual, 2nd edition. Protocol II; 1.82. Cold Spring Harbor Laboratory Press.

Service MW, (2006). The encyclopedia of arthropod transmited infections of man and domestic animals. *Animal Trypanosomiasis*, 33-46.

Simo G, Herder S, Cuny G, Hoheisel J, **(2010).** Identification of subspecies specific genes differentially expressed in procyclic forms of *Trypanosoma brucei* subspecies. *Infection, Genetics and Evolution*, 10(2):229-237.

Somda MB, (2010). Proceedings of the American Society of Tropical Medicine and Hygiene, www.astmh.org, Abstract no. 283.

Stark KR, James AA, (1998). Isolation and characterization of the gene encoding a novel factor Xa-directed anticoagulant from the yellow fever mosquito, *Aedes aegypti*. *Journal of Biological Chemistry*, 14;273(33):20802-20809.

Uilenberg G, (1998). A field guide for the diagnosis, treatment and prevention of animal African trypanosomiasis. *Food and Agriculture Organisation of the United Nations*

Vale GA, Hall DR, and Gough AJE, (1998a). The olifactory response of tsetse flies, *Glossina spp*. (Diptera glossinidae), to phenols and urine in the field. *Bulletin of Entomological Research*, 78: 293-300.

Vickerman K, Preston TM, (1976). In: ''Biology of the kinetoplastida, *(ed.W.C.A. Lumsden and D.A. Evans). New York academic press. 1:35*.

Wallengren J, Håkanson R(1992). Effects of capsaicin, bradykinin and prostaglandin E2 in the human skin. *The British Journal of Dermatology*, 126(2):111-117.

Woo PTK, (1969). The hematocrit centrifugation technique for detection of trypanosomes in blood. *Can Jurnal of Zoology*; 47: 921-923.

www.fao.org/docrep/006/X0413E/X0413E05.htm: Accessed on 2nd August 2012.

www.ncbi.nlm.nih.gov/pubmed/10195279: Accessed on 3rd August 2012.

Xu X, Yang H, Ma D, Wu J, Wang Y, Song Y, Wang X, Lu Y, Yang J, Lai R, (2008). Toward an understanding of the molecular mechanism for successful blood feeding by coupling proteomics analysis with pharmacological testing of horsefly salivary glands. *Molecular and Cell Proteomics*, 7(3):582–590.

Yamazaki Y, Hyodo F, Morita T, (2003). Wide distribution of cysteine-rich secretory proteins in snake venoms: isolation and cloning of novel snake venom cysteine-rich secretory proteins. *Archives of biochemistry and biophysics*. 2003; 412(1):133–141.

Yamazaki Y, Morita T, (2004). Structure and function of snake venom cysteine-rich secretory proteins. *Toxicon.* 44(3):227-231.

Yang AS, Honig B, (2000). An integrated approach to the analysis and modeling of protein sequences and structures. II. On the relationship between sequence and structural similarity for proteins that are not obviously related in sequence. *Journal of Molecular Biology*. 18;301(3):679-89.

Zebra EN, (1988). Insecticidal activity of pyrethroids on insects of medical importance. *Parasitology*, *Today* 4: 53-57.

APPENDICES

Appendix I: Cattle tsetse exposure status as determined by rTSGF-2 ELISA test.

Non tsetse exposed Adults	Tsetse exposure Status	Non tsetse exposed calves	Tsetse exposure status	Tsetse exposed tryp –ve cattle	Tryp. Infection status	Tsetse exposed tryp +ve cattle	Tryp. Infection Stataus
0.425	+	0.397	+	0.4155	+	0.363	+
0.357	+	0.4555	+	0.399	+	0.8715	+
0.3545	+	0.3625	+	0.392	+	0.3745	+
0.515	+	0.3875	+	0.9391	+	0.3815	+
0.455	+	0.399	+	0.391	+	0.3615	+
0.3865	+	0.895	+	0.3965	+	0.3895	+
0.3815	+	0.315	+	0.3715	+	0.378	+
0.3995	+			0.3785	+	0.388	+
0.4615	+			0.513	+	0.3885	+
0.395	+			0.378	+	0.3995	+
0.564	+			0.3705	+	0.3805	+
0.965	+			0.458	+	0.421	+
0.399	+			0.875	+	0.3835	+
0.381	+			0.3725	+	0.3585	+
0.375	+			0.461	+	0.997	+
0.366	+			0.381	+	0.363	+
0.4545	+			0.3605	+	0.4685	+
				0.378	+	0.4835	+
				0.363	+	0.3845	+
				0.413	+	0.3545	+
				0.396	+	0.418	+
				0.537	+	0.376	+
				0.49	+	0.3745	+
				0.376	+	0.375	+
				0.383	+	0.389	+
				0.375	+	0.365	+
				0.394	+	0.3685	+
				0.381	+	0.399	+

Appendix II: ClustalW2 Sequence alignment results of the of 31kDa TSGF-2 fragment (rTSGF-2) with TSGF-2 and AAK97208.1

```
31kDa        ------------------------------------------------------------
TSGF-2       MLGIKYIIILGLFGAITSAVDQEYSDSRNKLIKKERRHSLGGKLELTEKEKKVNRIFMRH 60
AAK97208.1   --MWKKVVIFLLIWDSAYSAKLISRLDRESILDAEKKHRTGGNAYLTEREAQANDIVTRL 58


31kDa        -----------------------------------------------------GSVSPEWII 9
TSGF-2       KHNELSVAFNDSSQNAPAMHFFKAKHIIENSMVFKFLKKMPKGGALHLHALGSVSPEWII 120
AAK97208.1   RSKILLEGIANSTGFAPAMHFFQAKPLIESSPIFRMLKAMPKGSVLHLHNTAAVSSKWVI 118
                                                          .:**.:*:*

31kDa        KNITYLPGLKKCINRANAPVLTFRPNAEPHMCKFDYTDVNDERKSEN--AEEYDKKLAAA 67
TSGF-2       KNITYLPGLKKCINRANAPVLTFRPNAEPHMCKFDYTDVNDERKSEN--AEEYDKKLAAA 178
AAK97208.1   KNLTYRSEAKLCEVNG-TVFFTVR---QSKFCDSEPQKSITKLRAQNASAEAFDLWLESF 174
             **:**  .   *  *   .. : .:*.*    :.::*. :   .   : :::*  ** :*   * :

31kDa        FNLFTPTPEKDYPTINTVWGRFVQMFFGLWDALHYLPAAKAFYWRLLEELYEDNVMYAEL 127
TSGF-2       FNLFTPTPEKDYPTINTVWGRFVQMFFGLWDALHYLPAAKAFYWRLLEELYEDNVMYAEL 238
AAK97208.1   INLKLRDPELMHTDVNTVWNDFQQMFDASKDLLMYKPFFEDYHRQMLREFYDDNVQYIEL 234
             :**    **  :. :****. * *** .  * * * *  : :: ::*.*:*:*** * **

31kDa        RVELFRLYTENGRLDREQSILQLW-EVTEQFKKEHPDFLGVRLIYTVLRGSKPGELQEYY 186
TSGF-2       RVELFRLYTENGRLDREQSILQLW-EVTEQFKKEHPDFLGVRLIYTVLRGSKPGELQEYY 297
AAK97208.1   RASLSKVYDANGKDYNEFEIVKIISDIVDSFKKDHPDFFGVKIIYAKHRSIDNETVESFL 294
             *..* ::*  **:   .*  .*::: ::.:.***:****:**::**:    *.  .    ::.:

31kDa        SRLKNYTTRQPDKLVGFDMVGPEDSDLRLLSFADNLIELSDKTKFFFHAGETNS------ 240
TSGF-2       SRLKNYTTRQPDKLVGFDMVGPEDSDLRLLSFADNLIELSDKTKFFFHAGETNSYG-GTD 356
AAK97208.1   EKFIALNQEFPDLVVGFDLVGQEDINNPLILFTDQLCKFEKTAPYFFHAGETNGYGSEAD 354
             .::    . . ** :****:** ** :   *: *:*:* ::...: :********.


31kDa        ------------------------------------------------------------
TSGF-2       LNLVDAILLNTTRIGLGYALPKHPVLMKIVKAREIPVEVCPISNQVLHLVNDLRNHPGAI 416
AAK97208.1   LNLVDAVLLNSRRIGHGYSLYKHPVLWKMVKQKGIALEICPLSNQVLRLVTDLRNHPAVF 414


31kDa        ------------------------------------------------------------
TSGF-2       LLANNIPMVISNDAPAFWGVQGLSYDYYYTIMSLASNKAGLSTLKQLVFNSIKYSALPEE 476
AAK97208.1   YVSESVPIVIAPDDPGFWDSAAVGFDYYYALMSLAPHSAGIGFLKQIVWDSVKYSTLTEP 474


31kDa        ------------------------------
TSGF-2       EKKTAEKNLEKQWNQFIDNILKDTEFNISK 506
AAK97208.1   ERTQYAELLQPKWEAFLDFIIASKVLNY-- 502
```

Key: 31kDa: rTSGF-2 amino acid sequence, TSGF-2: TSGF-2 full length amino acid sequence, AAK97208.1: Protein from *Culex quinquefasciatus*.   "*" shows areas of amino acid homology.

Appendix III: ClustalW2 Sequence alignment results of the of 31kDa TSGF-2 fragment

(rTSGF-2) with TSGF-2 and ABI20162.1

```
31kDa        ------------------------------------------------------------
TSGF-2       ---------MLGIKYIIILGLFGAITSAVDQEYSDSRNKLIKKERRHSLGGKLELTEKEK 51
ABI20162.1   MFPRLIVWLLAASAVHAVLDISNIKPKRDYENFLQKYAEYADDEVDRSVGSDITLSLKEK 60


31kDa        ------------------------------------------------------------
TSGF-2       KVNRIFMRHKHNELSVAFNDSSQNAPAMHFFKAKHIIENSMVFKFLKKMPKGGALHLHAL 111
ABI20162.1   FVNQYLMDLKTEELKAGLKNPSQFIPSNHFFSVLDRINSSEIFKIIRRMPKGAILHAHDT 120


31kDa        GSVSPEWIIKNITYLPGLKKCIN-RANAPVLTFRPNAEPHMCKFDYTDVNDERKSENAEE 59
TSGF-2       GSVSPEWIIKNITYLPGLKKCIN-RANAPVLTFRPNAEPHMCKFDYTDVNDERKSENAEE 170
ABI20162.1   ALCSTDYVVS-ITYRDHLWQCADPKTGALQFRFSKESPKNTDTCQWTPVSEERKNQGEEQ 179
             .  *.::::. ***    * :* : ::.*  : *  ::  :   . ::* *.:***.:. *:


31kDa        YDKKLAAAFNLFTPTPEKDYPTINTVWGRFVQMFFGLWDALHYLPAAKAFYWRLLEELYE 119
TSGF-2       YDKKLAAAFNLFTPTPEKDYPTINTVWGRFVQMFFGLWDALHYLPAAKAFYWRLLEELYE 230
ABI20162.1   YNSKLRSQLSLYNTDPINRSRDVDSIWNDFMGLFGVNFGLLTYAPVWKDYYKQFLKEMME 239
             *:.** : :.*:.. * :     ::::*. *: :*    :. * * *. * :* ::*:*: *


31kDa        DNVMYAELRVELFRLYTENGRLDRE-QSILQLWEVTEQFKKEHPDFLGVRLIYTVLRGSK 178
TSGF-2       DNVMYAELRVELFRLYTENGRLDRE-QSILQLWEVTEQFKKEHPDFLGVRLIYTVLRGSK 289
ABI20162.1   DGVQYLELRGTLPPLYDLDGKIYNEEQVVEIYYNVTEEFKKENSTFIGAKFIYAPVRFVN 299
             *.* * ***  *  ** :*:: .* * :   ::***:****:. *:*.::**: :*  :


31kDa        PGELQEYYSRLKNYTTRQPDKLVGFDMVGPEDSDLRLLSFADNLIELSDKTKFFFHAGET 238
TSGF-2       PGELQEYYSRLKNYTTRQPDKLVGFDMVGPEDSDLRLLSFADNLIELSDKTKFFFHAGET 349
ABI20162.1   ATGIKTLTTTVKQLHERFPDFLAGFDLVGQEDKGGPLIGFSRELLELPESINFFFHSGET 359
             .  ::   : :*:   * ** *.***:** **..  *:.*: :*:**.:. :****:***


31kDa        NS----------------------------------------------------------- 240
TSGF-2       NSYGGTDLNLVDAILLNTTRIGLGYALPKHPVLMKIVKAREIPVEVCPISNQVLHLVNDL 409
ABI20162.1   NWNGMTDDNLIAAVTLGTKRIGHGYALFKHPRVLKQVKKDKIAIEVCPISNQVLRLVADM 419
             *


31kDa        ------------------------------------------------------------
TSGF-2       RNHPGAILLANN-IPMVISNDAPAFWGVQGLSYDYYYTIMSLASNKAGLSTLKQLVFNSI 468
ABI20162.1   RNHPGSILLANKKYPMVISSDDPSFWEATPLSHDFYMAFMGLASYHQDLRMLKQLAINSL 479


31kDa        -------------------------------------
TSGF-2       KYSALPEEEKKTAEKNLEKQWNQFIDNILKDTEFNISK 506
ABI20162.1   EYSSMTLEEKTNAMKLWEAEWEKFIKELETEVFSLLE- 516
```

**Key: 31kDa: rTSGF-2 amino acid sequence, TSGF-2: TSGF-2 full length amino acid sequence, ABI20162.1;
Adenosine deaminase-like from *Phlebotomus duboscqi*. "*" shows areas of amino acid homology.**

Appendix IV: ClustalW2 Sequence alignment results of the of 31kDa TSGF-2 fragment (rTSGF-2) with TSGF-2 and EAA04809.4

```
31kDa       ------------------------------------------------------------
TSGF-2      -----MLGIKYIIILGLFGAITSAVDQEYSDSRNKLIKKERRHSLGGKLELTEKEKKVNR 55
EAA04809.4  MAFHLVVTILLLLGVTESHALTLSRTKTYDQLRAAIKESEAAYGLGGKVHLNPDERQADR 60


31kDa       ---------------------------------------------------------GSVS 4
TSGF-2      IFMRHKHNELSVAFNDSSQNAPAMHFFKAKHIIENSMVFKFLKKMPKGGALHLHALGSVS 115
EAA04809.4  ILRAMKKDELKVVDRARPVAG--MHFFDAKPFIENSTVFEALKLMPKGAILHLHNSAAVS 118
                                                                    .:**

31kDa       PEWIIKNITYLPGLKKCINRANAPVLTFRPNAEPHMCKFDYTDVNDERKSENAEEYDKKL 64
TSGF-2      PEWIIKNITYLPGLKKCINRANAPVLTFRPNAEPHMCKFDYTDVNDERKSENAEEYDKKL 175
EAA04809.4  SSWVIKNLTYRAEAKLCNVDDRYYFTVRPLNFCPENQTRPINQLRKERK-DDIDTFDIWL 177
            ..*:***:**   * *      .  . .    * *.  .    .::..*** :: : :* *

31kDa       AAAFNLFTPTPEKDYPTINTVWGRFVQMFFGLWDALHYLPAAKAFYWRLLEELYEDNVMY 124
TSGF-2      AAAFNLFTPTPEKDYPTINTVWGRFVQMFFGLWDALHYLPAAKAFYWRLLEELYEDNVMY 235
EAA04809.4  ESFISMKLKPTRIGYASTDELWLDFESCFDAMKGFLQYKPFFEAYHWQLLHEFYNDRVYY 237
             : :.:    ... .*.: : :* * . * .: . *:* *  :*::*:**.*:*:*.* *

31kDa       AELRVELFRLYTENGRLDRE-QSILQLWEVTEQFKKEHPDFLGVRLIYTVLRGSKPGELQ 183
TSGF-2      AELRVELFRLYTENGRLDRE-QSILQLWEVTEQFKKEHPDFLGVRLIYTVLRGSKPGELQ 294
EAA04809.4  IELRMSFAKVFDAAGREYGPPEVAYLLHRIVDDFRRQHPAFQGVKLILAKHRNMTDGELE 297
             ***:.: :::   **      :     * .:.::*:::** * **:** :  *. . ***:

31kDa       EYYSRLKNYTTRQPDKLVGFDMVGPEDSDLRLLSFADNLIELSDKT---KFFFHAGETNS 240
TSGF-2      EYYSRLKNYTTRQPDKLVGFDMVGPEDSDLRLLSFADNLIELSDKT---KFFFHAGETNS 351
EAA04809.4  KALQLYDSLSTTFPGFVVGFDLVGQEDINRSLKSFSSLLVQPPVSSGPPKYFFHAGEIAG 357
            :  .  .. :*  *. :****:** ** :  * **:. *::  . .:    *:****** .

31kDa       ------------------------------------------------------------
TSGF-2      YG-GTDLNLVDAILLNTTRIGLGYALPKHPVLMKIVKAREIPVEVCPISNQVLHLVNDLR 410
EAA04809.4  YFTEADENVIDAVLLDTKRIGHGYALMKHPILWHAVQHKQIAIEVCPISNQVLGLVRDLR 417


31kDa       ------------------------------------------------------------
TSGF-2      NHPGAILLANNIPMVISNDAPAFWGVQGLSYDYYYTIMSLASNKAGLSTLKQLVFNSIKY 470
EAA04809.4  NHPASFYVAQNIPIVITSDDPGFWDAVGVSFDYYYAFMAIAP-HSGLGFLKQLVWDSIRY 476


31kDa       -----------------------------------
TSGF-2      SALPEEEKKTAEKNLEKQWNQFIDNILKDTEFNISK 506
EAA04809.4  SSLSDGERQNITATMEKQWALFVRQVVSANSSATE- 511
```

Key: 31kDa: rTSGF-2 amino acid sequence, TSGF-2: TSGF-2 full length amino acid sequence, EAA04809.4: Protein from *Anopheles gambiae*. "*" shows areas of amino acid homology.

Appendix V: ClustalW2 Sequence alignment results of the of 31kDa TSGF-2 fragment (rTSGF-2) with TSGF-2 and AAF78901.1

```
31kDa        ------------------------------------------------------------
TSGF-2       --------MLGIKYIIILGLFGAITSAVDQEYSDSRNKLIKKERRHSLGGKLELTEKEKK 52
AAF78901.1   MFSQLVVWLLATSTVCLAWDNSWIMDMKYERYSQRRSYYLAEEEDRSVGSDIELTAKEQV 60


31kDa        -----------------------------------------------------------G 1
TSGF-2       VNRIFMRHKHNELSVAFNDSSQNAPAMHFFKAKHIIENSMVFKFLKKMPKGGALHLHALG 112
AAF78901.1   VNERLMELKMTELKNGLQDPAGFIPWNHIFDVLYRINSSELFHIIQKMPKGGILHAHDTA 120
                                                                        .

31kDa        SVSPEWIIKNITYLPGLKKCINRAN-APVLTFRPNAEPHMCKFDYTDVNDERKSENAEEY 60
TSGF-2       SVSPEWIIKNITYLPGLKKCINRAN-APVLTFRPNAEPHMCKFDYTDVNDERKSENAEEY 171
AAF78901.1   LCSTDYVIS-LTYEPNLWQCADPTTGAFQFLFSREAPTNTDTCTWTLVADERAKQGEENY 179
               *.:::*. :** *.* :* : :. * : * :* .: . :* * *** .:. *:*

31kDa        DKKLAAAFNLFTPTPEKDYPTINTVWGRFVQMFFGLWDALHYLPAAKAFYWRLLEELYED 120
TSGF-2       DKKLAAAFNLFTPTPEKDYPTINTVWGRFVQMFFGLWDALHYLPAAKAFYWRLLEELYED 231
AAF78901.1   NSALRSQLSMYNTNPIMHNRDVDSIWRQFMGIFGVNGGLLTYAPVWKAYYLQFLKEMFAD 239
             :. * : :.::...* . :::::* :*: :* . * * *. **:* ::*:*:: *

31kDa        NVMYAELRVELFRLYTENGRLDREQSILQL-WEVTEQFKKEHPDFLGVRLIYTVLRGSKP 179
TSGF-2       NVMYAELRVELFRLYTENGRLDREQSILQL-WEVTEQFKKEHPDFLGVRLIYTVLRGSKP 290
AAF78901.1   GVQYLELRTTLPPLYDLDGKTYNEVEIMQIYYDATKEFKKQNPTFIGAKIIYAPVRVVDD 299
             .* * ***. * ** :*: .* .*:*: ::.*::***::* *:*.::**: :* .

31kDa        GELQEYYSRLKNYTTRQPDKLVGFDMVGPEDSDLRLLSFADNLIELSDKTKFFFHAGETN 239
TSGF-2       GELQEYYSRLKNYTTRQPDKLVGFDMVGPEDSDLRLLSFADNLIELSDKTKFFFHAGETN 350
AAF78901.1   AGIPALMAKVRELHEKFPDFMAGFDLVGQEDKGRPLIAFSREILKLPNSIDFYFHAGETN 359
             . : ::::: : ** :.***:** **.. *::*: ::::*.:. .*:*******

31kDa        S---------------------------------------------------------- 240
TSGF-2       SYGGTDLNLVDAILLNTTRIGLGYALPKHPVLMKIVKAREIPVEVCPISNQVLHLVNDLR 410
AAF78901.1   WDGMTDDNLIDAVLLGTKRIGHGYAVLKHPRVLKEVKRNKIAIEVCPASNQVLRLVADYR 419


31kDa        ------------------------------------------------------------
TSGF-2       NHPGAILLAN-NIPMVISNDAPAFWGVQGLSYDYYYTIMSLASNKAGLSTLKQLVFNSIK 469
AAF78901.1   NHPGSVLLANKEYPVVISSDDPSFWEAKPLSHDFYMAFLGLASSRQDLRLLKQLAINSIK 479


31kDa        -----------------------------------
TSGF-2       YSALPEEEKKTAEKNLEKQWNQFIDNILKDTEFNISK 506
AAF78901.1   YSAMSPREKLQAMQMWEAEWKKFIDGFNA-------- 508
```

Key: 31kDa: rTSGF-2 amino acid sequence, TSGF-2: TSGF-2 full length amino acid sequence, ABI20162.1; Adenosine deaminase from *Lutzomyia longipalpis*. "*" shows areas of amino acid homology.

Appendix VI: Qiaexpressionist™ purification procedures

Protocol 1: Preparation of cleared *E.coli* lysates under denaturing conditions

- **Materials**

- Cell pellet

- Buffer B

- 2X SDS-PAGE sample buffer (0.09M Tris.Cl, pH 6.8, 2% SDS, 20% glycerol, 0.02% bromo-phenol blue and 0.1M DTT)

1. Thaw the cell pellet for 15 minutes on ice and resuspend the cells in lysis buffer B.

2. Stir cells for 15 minutes.

3. Centrifuge lysate at 10,000g for 20-30 minutes at 4°C to pellet the cellular debris and save supernatant.

4. Add 5µL of 2X SDS-PAGE sample buffer to 5 µL of supernatant and store at -20°C for SDS-PAGE analysis. Proceed to purification under denaturing conditions (Protocol 2).

Protocol 2: Purification of His-tagged proteins from *E.coli* under denaturing conditions

Materials

- Cleared lysate from 20-200ml culture

- Ni-NTA resin

- Buffer A-E

Procedure

1. Add 1ml of the 50% Ni-NTA slurry to 4ml lysate and mix gently by rocking.

2. Load lysate-resin mixture carefully into an empty column with the bottom cap still attached.

3. Remove the bottom cap and collect the flow through for SDS-PAGE analysis.

4. Wash twice with 4ml buffer C.

5. Elute the recombinant protein 4 times with 0.5ml buffer D followed by 4 times with 0.5ml buffer E. Collect fractions and analyse by SDS-PAGE.

Reagents for purification under denaturing conditions

Buffer B

- 10mM NaH_2PO_4 13.8g $NaH_2PO_4.H_20$

- 10mM Tris-Cl   1.2g Tris base

- 8M Urea   480.5g

- Adjust pH 8.0 using NaOH

Buffer C

- 100mM NaH_2PO_4 13.8g $NaH_2PO_4.H_2O$

- 10mM Tris-Cl   1.2g Tris base

- 8M Urea   480.5g

- Adjust pH 6.3 using HCL

Buffer E

- 100mM NaH_2PO_4 13.8g $NaH_2PO_4.H_2O$

- 10mM Tris-Cl   1.2g Tris base

- 8M Urea   480.5g

- Adjust pH 4.5 using HCl

Appendix VII: Sample loading buffer composition

0.5M Tris-HCl, pH 6.8, 10% (v/v) glycerol

10% (w/v) SDS

5% (v/v) 2-mercaptoethanol

1% (w/v) bromophenol blue

Appendix VIII: Transfer buffer composition

25mM Tris, 192mM glycine, 20% methanol pH 8.3).

Appendix VIII: pET28a Expression Vector map