Characterization of *Glossina pallidipes* midgut proteins as vaccine candidate(s) for Human African Trypanosomosis

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DECLARATION

I, Johnson Kang'ethe Kinyua, hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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ABSTRACT

There are currently no vaccines available against trypanosomosis and vector management as an option has problems with sustainability. Anti-vector and transmission blocking vaccine development is thus a novel strategy against vector borne diseases. Vaccines that target blood-feeding disease vectors, such as tsetse flies, mosquitoes and ticks, have the potential to protect against the many diseases caused by vector-borne pathogens. Vaccines developed against midgut proteins have been shown to reduce the fecundity of flies feeding on immunized animals. Midgut proteins isolated from the midgut of *Glossina pallidipes* were used to immunize rabbits and their efficacy as vaccine candidate(s) against the tsetse fly, and their potential to block transmission of *Trypanosoma brucei rhodesiense* assessed.

Two fractions, detergent (DET) and aqueous (AQ) fractions were separated using a non-ionic detergent (Triton X-114) and a series of bioassay experiments carried out using sera obtained from rabbits immunized with either of the two fractions. A group of flies was fed on *T.b. rhodesiense* innoculated antisera and the trypanosome establishment within the midgut studied. Antibody production was studied using immunodiffusion, immunoelectrophoresis and Western blot. Immunoaffinity chromatography and gel electrophoresis (1-D and 2-DE) were used to isolate and characterize the antigenic proteins from the crude midgut fractions. The longevity (survival), larviposition, mass of pupae and ability to transmit trypanosomes were assessed on flies fed on the antisera derived from rabbits immunized with either of the two fractions. A cDNA expression

library constructed from midgut proteins was immunoscreened and plasmids purified from the positive colonies. The plasmid DNA sequences were analyzed and the functional roles of the native antigens inferred from genomic and protein databases.

The results of the bioassay experiments indicated a significant reduction in the number of pupae for the flies fed on anti-AQ. The mortality rates of tsetse flies fed on sera from rabbits immunized with DET and AQ was 100% and 63% respectively as compared to 33% mortality for the controls in a period of 60 days. Assessment of fecundity indicated reduction of larviposition by about 30% for the flies fed on anti-DET and anti-AQ. Significant differences in abortions and pupal weights were also observed. The antigens were also shown to have a potential to interfere with the normal trypanosome establishment within the tsetse fly midgut. SDS-PAGE of immunoaffinity purified glycoproteins revealed glycoprotein antigens of M_r 28,200, 14,100 and 11,500 for the DET fraction while a glycoprotein antigens of M_r 85,000, 63000 and 12,500 was revealed for the AQ fraction. The cDNA sequence analysis revealed potential transmission blocking vaccine candidates against *Glossina pallidipes* which were inferred to include a serpin serine protease inhibitor (Ks1-M13), an infestin homolog reported as a novel thrombin inhibitor present in the midgut of the blood-sucking hemipteran *Triatoma infestans,* a proteolytic lectin (Ks2-M13) implicated in transformation of bloodstream form trypanosomes to procyclic forms, a protein (Ks4-M13) thought to be involved in embryonic development/oogenesis and in regulation of epidermal growth factor receptor signaling pathway and an exocyst complex

component Sec10 family that contains the Sec10 component of the eukaryotic golgi exocyst complex, which specifically affects the synthesis and delivery of secretory and basolateral plasma membrane proteins, an aspartate-Glutamate racemase (Ks7-M13) important in the protection of the peptidoglycan layer of cell walls from cellular proteases, a sulphotransferase (Ks8-M13) family involved in the sulphation of a diverse range of compounds including biogenic amines, steroids, hormones, bile acids and a variety of xenobiotics including drugs and carcinogens and a protein with ribonuclease activity. These proteins may be involved in pathways critical to the biology of the tsetse fly and the results suggest their potential for use in development of anti-tsetse and transmission blocking vaccines. This study proposes the use of these molecules in vaccine formulations to test their optimal efficacy as a control measure against African trypanosomosis, a neglected disease of the poor. The vaccine would hence be referred to as "TsetseGARD".

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Lastly, my profound gratitude goes to my wife Jane. Your constant inspiration pushed me through.

DEDICATION

To my wife Jane M. Kang'ethe

To my sons Morris, George, Charles and David

To my parents Naomi Njeri and Harrison Kinyua

You have ALL made a difference in my life.

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ABBREVIATIONS

AAT	African Animal Trypanosomosis
ANOVA	Analysis of variance
APS	Ammonium persulphate
AQ	Aqueous phase
ATP	Adenosine Triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
Con A	Concanavalin A
2-DE	2-dimensional gel electrophoresis
dCTP	Deoxycytidine Triphosphate
cDNA	Copy DNA
DALYs	Disability Adjusted Life Years
DEAE-cellulose	Diethylaminoethyl-cellulose
DET	Detergent phase
DFMO	Diflouromethylornithine
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediamine tetraacetic acid
ESTs	Expressed Sequence Tags
FAO	Food and Agricultural Organization

HAT	Human African Trypanosomiasis
HCl	Hydrochloric acid
IACUC	Institutional Animal Care and Use Committee
IgG	Immunoglobulin G
IEF	Isoelectric focussing
IPTG	Isopropylthiogalactoside
KCl	Potassium Chloride
KSPCA	Kenya Society for the prevention of Cruelty to Animals
LB	Luria-Bertani
mRNA	Messenger RNA
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
NaN_3	Sodium azide
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl flouride

Poly (A)+	Poly-Adenylate
RNA	Ribonucleic acid
SAS	Saturated ammonium sulphate
SDS	Sodium dodecyl sulphate
SM	Storage Medium
SRA	Serum resistance associated gene
TBS	Tris Buffered Saline
TCA	Trichloroacetic acid
TE	Tris-EDTA
Tris	Tris- (hydroxyl methyl-aminomethane)
TX-114	Triton X 114
WHO	World Health Organization

UNIT ABBREVIATIONS

Bp	Base pair
Cfu	Colony forming units
M	Molar
mg	Milligram
ml	Milliliter
min	Minutes
mM	Millimolar
mmol	Millimoles
nm	Nanomoles
μCi	Microcurie
μg	Micrograms
μl	Microliter
μM	Micromolar
g	Gravitation constant
g	grams
pH	-Log hydrogen ion concentration
pI	Isoelectric point
Rev/min	Revolution per minute
Sec	Seconds
U	Unit
v/v	Volume by volume
λ	Lambda

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

1. **Kinyua, J.K, Nguu, E.K., Mulaa, F. and Ndung'u, J.M (2005).** Immunization of rabbits with *Glossina pallidipes* tsetse fly midgut proteins: Effects on the fly and trypanosome transmission. *Vaccine* **23**; 3824-28

2. **J.K.Kinyua, F. Mulaa, E.K. Nguu and J.M. Ndung'u (2006)** Characterization of protective antigens from the midgut of *Glossina pallidipes. In press*

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1. Characterization of *Glossina pallidipes* midgut molecule(s) as vaccine candidate(s) against African trypanosomiasis. Abstract in the 3rd and 4th workshop of East Africa Network of trypanosomiasis

2. Immunization of rabbits with *Glossina pallidipes* midgut proteins: Effects on the fly and trypanosome transmission: Oral presentation in the 28th ISCTRC meeting in Addis Ababa between 26th and 30th September 2005

3. Exploitation of tsetse fly midgut proteins for Trypanosomosis control. American Society for Tropical Medicine and Hygiene (ASTMH) conference held in USA, Washington DC, 11th-15th December 2005.

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CHAPTER ONE

BACKGROUND INFORMATION

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Both male and female tsetse flies (Diptera:Glossinidae), are the cyclical vectors of the trypanosomes which cause African sleeping sickness in humans (HAT) and nagana in animals (AAT). It is conservatively estimated by the World Health Organization (WHO) that there could be 300,000-500,000 cases annually of HAT with 60 million people (WHO, 2000) at risk in 36 countries covering ~40% of Africa (11M km^2) (SWG, 2001). After devastating epidemic in the early 20^{th} century when a million people died of HAT, the disease almost disappeared in Africa by the 1960's. But we are now in the midst of another epidemic with increasing number of new infections and mortality (55,000 deaths in 1993; 66,000 in 1999) and a disease burden of 2.05M DALY (disability adjusted life years) (Barret, 1999). The breakdown of surveillance, allied to displacement of populations by war and natural disaster, are contributory factors to this new epidemic. Given that HAT affects hard-to-reach rural populations, and these war torn areas lack active surveillance, the disease prevalence numbers are undoubtedly a gross underestimation. The considered view is that the situation may worsen (Barret, 1999). In addition to public health impact of HAT, it has been estimated that AAT limits the availability of meat and milk products of large regions of Africa. It also excludes effective cattle rearing from ten million square kilometers of Africa (Steelman, 1976) with wide implications for land use; i.e.

constraints on mixed agriculture and lack of animal labor for ploughing (Jordan, 1986).

Control of the disease is mainly based on vector control and chemotherapy, which depends on a few drugs that were developed many years ago. The development of more efficacious drugs has not been vigorously pursued as it is evidently not profitable for the pharmaceutical industry (McNeil, 2000). Some of the widely used drugs such as melarsoprol, suramin and pentamidine are associated with adverse reactions and drug resistance (Anene, 2001). The only new drug, which has been introduced in the recent past, is eflornithine® (DL-α-difluoromethylornithine-DFMO), is unfortunately is not effective against *T. b. rhodesiense* infections, due to an innate resistance. Treatment using any of the drugs is costly, and requires long durations of hospitalization. According to Jordan (1986), overall prospects for the use of trypanocidal drugs are not bright because of increasing drug resistance. Moreover, some of the tsetse fly control strategies conducted in the past, such as bush clearing (tsetse habitat destruction) or the elimination of wild animals (trypanosome reservoir hosts), have been discarded for ecological and environmental reasons. Limitations are also imposed on the indiscriminate use of insecticides. According to Holmes (1997), vaccines, drugs, vector control, and healthcare measures must all be considered as elements of a multi-prolonged attack aimed at substantially reducing the toll of trypanosomosis.

Many attempts have been made to develop a vaccine against trypanosomosis. This has however, yielded very little success due to the parasite's ability to vary its surface glycoprotein coat thereby overwhelming the immune system. In view of such shortcomings, efforts seeking new control strategies against African trypanosomosis remain imperative.

Potential novel approaches at controlling African trypanosomosis include the development of vaccines which would target the tsetse fly vectors or other vaccines that would block transmission of parasite in the tsetse fly. Some protection of laboratory animals against *G. morsitans* has been reported when the flies are repeatedly fed on the animals (Brown and Cipriano, 1985; Matha *et al.*, 1986). This was reflected by an apparent increase in mortality of the flies over time, and the induction of relatively high titers of circulating antibodies, which cross-reacted with salivary gland homogenates. The same approach has proved tractable in control of the one host tick, *Boophilus microplus* (de la Fuente *et al.*, 2000 and de Vos *et al.*, 2000). The present study was therefore designed at developing a vaccine which, when administered to a host would either kill tsetse flies or block their ability to transmit trypanosomes.

1.2 LITERATURE REVIEW

1.2.1 Economic Importance of African trypanosomosis

Sleeping sickness and African animal trypanosomosis are transmitted through the bite of the tsetse fly (*Glossina spp.*), and renders approximately 10

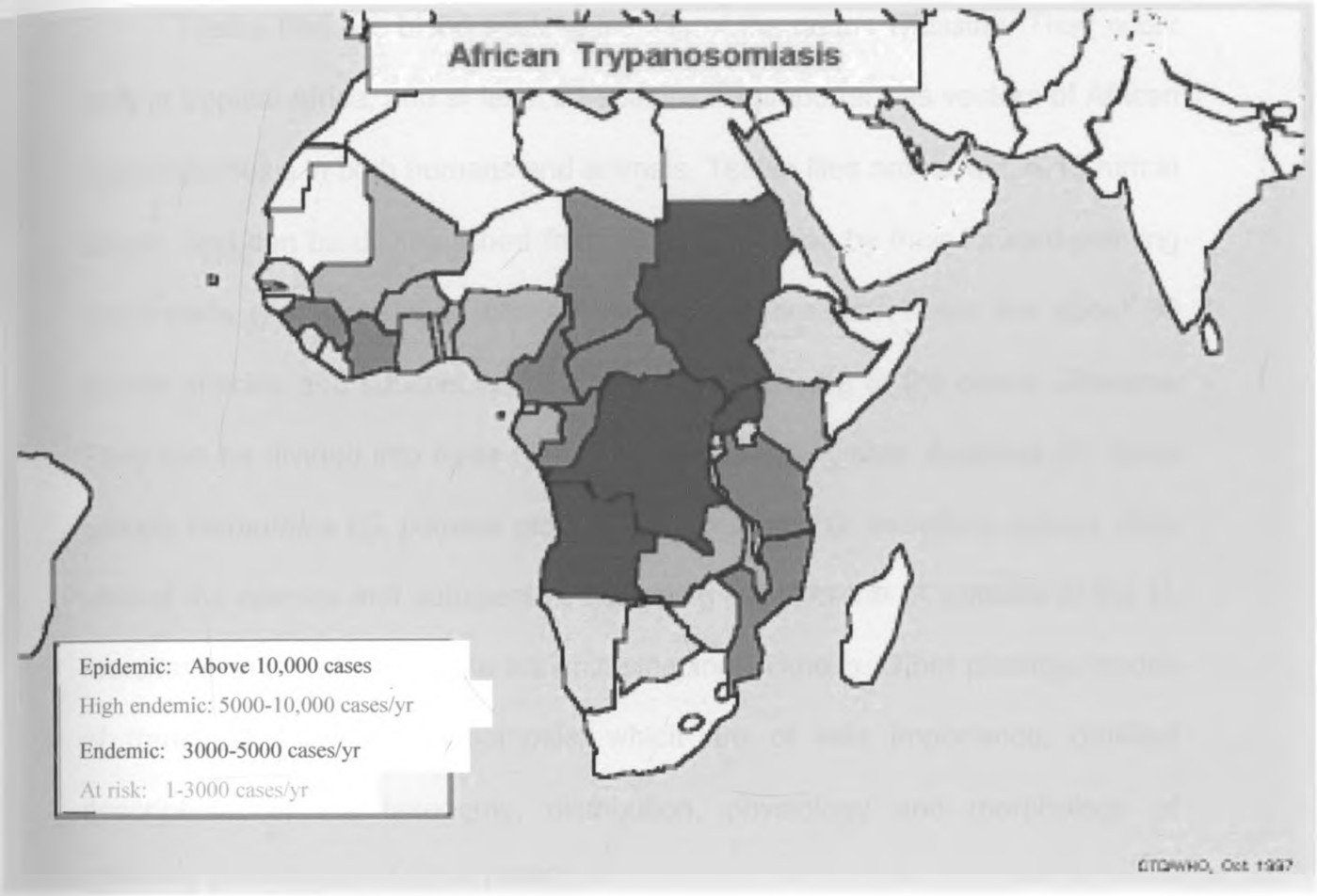
million km^2 of the African continent unsuitable for agriculture and human habitation if intervention measures are not undertaken (Molyneux and Ashford, 1983) (map 1). Tsetse control is of considerable priority in Africa due to the losses in human life and livestock. In eastern Africa, sleeping sickness has been confined to specific areas (known as foci), and many epidemics have ravaged these foci during recorded history (1900 onwards). The first reported major epidemic in East Africa was the Great Epidemic of 1900, which devastated the Busoga focus in Uganda and Kenya (Christy, 1903) where half a million people were estimated to have been infected during the epidemic (Hide *et al.*, 1996). *According to the World Health Organization (WHO), more than sixty million people, mainly living in rural areas of sub-Saharan Africa, are at risk of human African trypanosomosis (HAT). Some 45,000 new cases are reported each year, but this figure does not reflect the real epidemiological situation because surveillance is poor (covering only 5 to 7% of the people at risk) and the diagnostic tests that are widely used have low sensitivity. For an active surveillance program to be effective, it would have to cover 70% of the people at risk, which would cost some US$35 million per year, in addition to the annual US$38.5 million needed for drugs to treat 300,000 cases (FAO, 2000).*

The programme on African animal trypanosomiasis (PAAT) estimate that AAT causes approximately 3 million cattle deaths per year and farmers are required to administer approximately 35 million doses of costly trypanocidal drugs many of which fail because of resistance in parasites developed for these

chemicals (Geerts *et al.*, 2001). Economic losses in cattle production are estimated at US$ 1-1.2 billion annually and total agricultural losses are estimated at US$ 4.75 billion per year (FAO, www.fao.org/ag/againfor/programmes/en/paat/home.html).

In addition to their importance in public health, African trypanosomes cause nagana, a wasting and fatal disease in livestock. *Trypanosoma vivax* and *T. congolense* are regarded as major pathogens of cattle and other ruminants, while *T. simiae* causes high mortality in domestic pigs. Nagana has restricted agricultural development and has a profound effect on the economy of tropical Africa. Approximately 70% of the humid and sub-humid zones of sub-Saharan Africa are devoid of cattle and only through continued chemotherapy and tsetse control programs, cattle can graze on fringe of the tsetse habitat. Shortages of the financial and human resources necessary to implement or sustain a comprehensive control program characterize all endemic countries.

Map 1. Areas in Africa affected by Human African Trypanosomosis



Courtesy: WHO, 1997

1.2.2. The tsetse flies

Tsetse flies are blood sucking insects of the genus *Glossina*. They occur only in tropical Africa, and at least 21 species are important as vectors of African trypanosomosis in both humans and animals. Tsetse flies are robust, 6-15 mm in length, and can be distinguished from other biting flies by their forward-pointing mouthparts (proboscis) and characteristic wing venation. There are about 30 known species and subspecies of tsetse flies belonging to the genus *Glossina*. They can be divided into three distinct groups or subgenera: *Austenia* (*G. fusca* group), *Nemorhina* (*G. palpalis* group) and *Morsitans* (*G. morsitans* group). Only nine of the species and subspecies, belonging to either the *G. palpalis* or the *G. morsitans* group, are known to transmit sleeping sickness. Other possible modes of transmission of trypanosomosis, which are of less importance, detailed descriptions of the taxonomy, distribution, physiology and morphology of *Glossina* are given by Jordan (1986).

The female tsetse fly does not lay eggs but produces larvae, one at a time. The larva develops in the uterus over a period of 10 days and when fully grown is then deposited on moist soil or sand in shaded places, usually under bushes, fallen logs, large stones or buttress roots. It immediately buries itself and turns into a pupa. The fly emerges 22-60 days later, depending on the temperature. Females mate only once in their lifetime and, with optimum availability of food and breeding habitats can produce a larva every 10 days until it dies in 8-13 weeks.

All tsetse flies, both males and females, feed on blood, although there is species variation in their preferences for the source of blood. Most tsetse flies feed preferentially on animals, and only accidentally on humans or in the absence of their preferred hosts. While searching for food, tsetse flies are attracted by large moving objects (Green, 1988), by carbon dioxide, urine and certain colors while at close range.

1.2.3. Processing of bloodmeal by the tsetse fly

The metabolism of the bloodmeal in tsetse is reviewed by Bursell *et al.* (1974).

(i) The role of saliva:

Tsetse flies discharge saliva while probing the host for a bloodmeal. Tsetse saliva, like that of many other blood sucking insects, contains a powerful anticoagulant enzyme. The anticoagulant function of tsetse saliva was first suggested by Stuhlmann (1907), later demonstrated by Yorke and Macfie (1924) and identified as antithrobin (Lester and Lloyd, 1928). Vector saliva plays an important role in arthropod disease transmission by creating a suitable environment for the disease agent in both the host and the vector (Titus and Ribeiro, 1990). Saliva has vasodilatory and anticoagulant properties that make it easier for the fly to find and obtain its bloodmeal. Two platelet aggregation inhibitors have been identified from the saliva of *G. morsitans* (Mant and Parker, 1981). Saliva may also have immunosuppressive, anti-inflammatory properties that would prevent an adverse reaction in the host. The quantity of saliva

secreted by tsetse appears to increase as they become hungrier, but there is no evidence that infected tsetse salivates more copiously than non-infected ones.

(ii) The role of the midgut:

A number of studies have been carried out to investigate the enzymes involved in digestion of the bloodmeal. Stimulated by the feeding process, trypsin begins to be secreted from the anterior and posterior portions of the midgut (Akov, 1972). Laboratory experiments showed that trypsin secretion could be stimulated by feeding flies on goats and on various blood components except for washed bovine erythrocytes (Gooding, 1974). The quantity of secretion was correlated with amounts of protein and carboxypeptidase B in the posterior midgut (Gooding, 1977a), which led Gooding to conclude that the amount of protein, rather than the size of the meal ingested, determined the level of trypsin in the posterior midgut. Other proteolytic enzymes (carboxypeptidases, a chymotrypsin-like enzyme and aminopeptidases) have been isolated from *G. morsitans* and sub-species of *G. palpalis* (Cheeseman and Gooding, 1985).

Female tsetse flies ingest larger bloodmeals and have higher levels of digestive proteases to deal with it than males (Gooding, 1977a). Protein in the tsetse bloodmeal stimulates enzyme activity in the midgut. Specifically, trypsin inhibitors found in the bloodmeal serum (Gooding, 1977a) are believed to inhibit production of trypsin and carboxypeptidase B, in the fly midgut. Digestion of blood proteins appears to take place only in the posterior section of the midgut,

not the anterior, and involves the six enzymes identified by Gooding (1977a, b), which convert proteins to peptides and free amino acids.

Studies on bloodmeal digestion in tsetse, and subsequent metabolism of digestive products, have been carried out to determine the optimum frequency of feeding and the theoretical daily capacity for flight, and to link these physiological capabilities to tsetse behavior.

Female tsetse flies have a significant capacity to store nutrients destined for larval development. A bloodmeal taken in early pregnancy provides these nutrients for the developing larva, which is particularly important during the late stage of pregnancy when the larva is developing rapidly. During the first half of the 9-day reproductive cycle, lipids – mainly in the form of trigiycerides resulting from digestion of a bloodmeal – are synthesized and stored as triacylglycerol in the female's fat –body (Pimley, 1985). Normal growth of the larva is a function of optimum feeding throughout the pregnancy cycle (Moloo, 1976a, b).

1.2.4 The trypanosomes

1.2.4.1 Important species of trypanosomes

Trypanosomosis is caused by parasitic protozoa of the genus *Trypanosoma*. There are many species of *Trypanosoma,* all members of the section Salivaria in the family *Trypanosomastidae*, Order: *Kinetoplastidae*. There are four subgenera namely, *Duttonella, Nannomonas, Trypanozoon* and *Pycnomonas. Trypanosoma* are of the class kinetoplastida. a monophyletic group

of unicellular parasitic protozoa. The name is derived from the Greek *trypaô* (boring) and *soma* (body) because of their corkscrew-like motion. The two sub-species, *T. brucei rhodesiense* and *T. b. gambiense,* which cause human sleeping sickness, are in the subgenus *Trypanozoon.* The two species cause acute and chronic diseases respectively. The third subspecies, *T. b. brucei,* is only infective to animals and not humans, in which it causes a chronic disease referred to as nagana.

Trypanosomes have one nucleus, a single flagellum and a kinetoplast near the basal body of the flagellum. In a blood sample, the parasite species is identified by microscopic examination of the position of these organelles, the size and shape of the body and their movement between blood cells. Biochemical and molecular approaches are also used in the identification of trypanosomes. *Trypanosoma b. rhodesiense* is identified by its resistance to lysis by human serum. A more accurate Polymerase Chain Reaction (PCR) method, based on a serum resistance associated (SRA) gene, has been developed (Gibson, 2002).

The developmental cycle of trypanosomes in the vector ranges from 12 to 23 days depending on the species and temperature. They undergo cyclical development following the ingestion of an infected mammalian blood meal, culminating in to infective metacyclic trypanosomes in the salivary gland. Mechanical transmission of trypanosomes by other biting flies notably the tabanidae or tsetse is rare but could also occur.

1.2.4.2 Classification

Many attempts to rationalize the classification of trypanosomes have been made since the appreciation of their medical and veterinary importance. Perhaps the earliest development in this, were the independent suggestions by Duke and Roubaud (1913), that the classification of trypanosomes should be based on the mode of their development in the tsetse fly (Hoare, 1970b). This position was adapted by Bruce in 1915, although he proceeded to include *T. brucei, T. evansi* and *T. equiperdum* in the same group, having observed their affinity (Hoare, 1972).

Wenyon (1926) suggested two major groupings based on whether the trypanosomes completed their development in the anterior station or the posterior station of the insect vector. Until then, confusion had been caused by the common practice of assigning a new species each time a trypanosome was isolated from a new host. This broad division was adapted by Hoare (1970b) when he divided them into *Salivaria* and *Stercoraria* respectively. Thus, *Salivarian* trypanosomes complete their development in the salivary medium and transmission is innoculative during feeding. On the other hand, *Stercorarian* trypanosomes complete their development in the faecal medium of the posterior station and transmission is by contamination.

Three subgenera of the genus are included within the *Stercorarian* section: *Herpetosoma* (Hoare, 1972); *Megatrypanum* (Hoare, 1964) and *Schizotrypanum* (Hoare, 1972). Typically, these parasites are non pathogenic

with the exception of *T. (S.) cruzi* which causes Chagas' disease. *Salivarian* parasites are subdivided into subgenera: *Duttonella* (Chalmers, 1918), *Nannomonas* (Hoare, 1964), *Trypanozoon* (Hoare, 1972) and *Pycnomonas* (Hoare, 1964). These include species of immense medical and veterinary importance.

Trypanosoma vivax (subgenus *Duttonella*) is widely distributed in Africa and is also found in Central and South America, and the West Indies (Hoare, 1970b). Bruce *et al.* (1915) were the first to demonstrate that *T. vivax* was transmitted by tsetse flies. All tsetse fly species that have been investigated are capable of transmitting *T. vivax* (Buxton, 1955). Additionally, *T. vivax* is efficiently transmitted mechanically by biting flies (Hoare, 1970b).

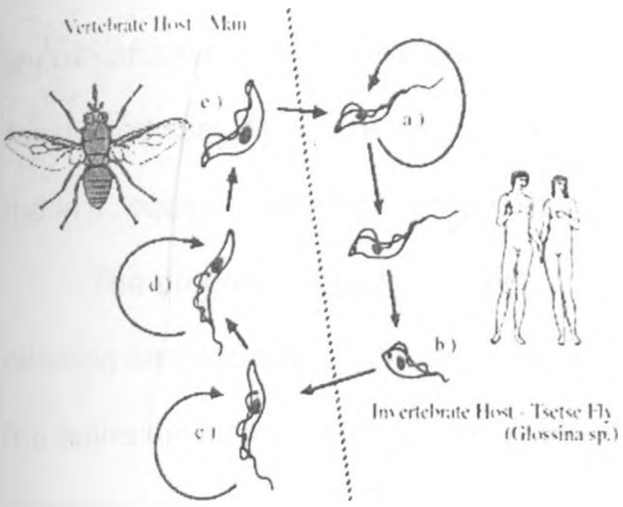
Within subgenus *Nannomonas*, are the most important pathogenic species affecting domestic animals in Africa (Hoare, 1970b). For a long time, two species namely; *Trypanosoma godfreyi* and *Trypanosoma congolense* were recognized within this subgenus. *Trypanosoma godfreyi* is pathogen of suidae, previously described (McNamara *et al.*, 1994). *Trypanosoma congolense* infects a variety of domestic animals, including bovines, equines, sheep, goats, camels, pigs and dogs (Hoare, 1970b). *Trypanosoma simiae* is a parasite of *suidae* and is distinguished from *T. congolense* in causing an acute and lethal disease in domestic pigs. However, non-lethal strains of *T. simiae* in pigs have been described (Mackenzie and Boyt, 1969), while *T. congolense* is sometimes asymptomatic (Killick-Kendrick and Godfrey, 1963).

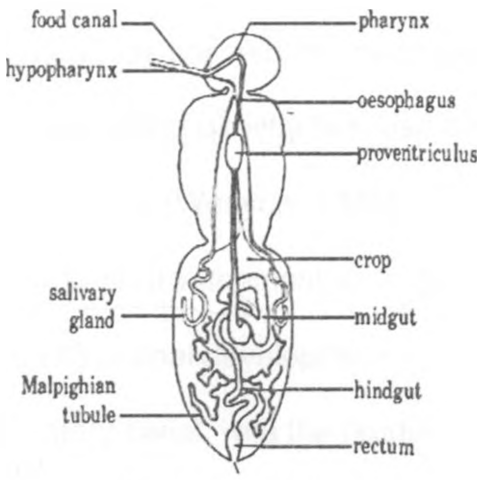
The subgenus *Trypanozoon* is composed of species which are morphologically indistinguishable and differ only in behavioral features, and in the disease they cause (Hoare, 1972). Three of the subspecies, *T.b. gambiense, T.b. rhodesiense* and *T.b. brucei* are restricted to Africa where they are transmitted cyclically by tsetse flies. *T.b. gambiense* and *T.b. rhodesiense* cause sleeping sickness in man, while *T.b. brucei* infects livestock and is not infective to humans. Two other members are transmitted non-cyclically, *T. evansi* mechanically by biting flies such as tabanids, and *T. equiperdum* directly between equines during coitus (Hoare, 1972).

1.2.4.3 The life cycle

Trypanosomes are typically digenetic protozoan parasites with life cycles alternating between a vertebrate host, where they exist in blood or tissues, and diverse haematophagous invertebrates. These hosts, mainly insects, act as intermediate hosts and vectors, transmitting the parasites to new vertebrate hosts. Tsetse flies are the only cyclical vectors of trypanosomes. Bloodstream forms are taken up during feeding by the intermediate hosts where they undergo development.

Fig. 1. The life cycle of trypanosomes





Courtesy: Serap Aksoy

a) Trypomastigote forms inoculated into vertebrate host in Tsetse fly blood meal. Here the extra cellular parasites undergo asexual reproduction by binary fission in the bloodstream, and, later in infection, lymph and spinal fluid.

b) Trypomastigote forms transform to a form capable of infecting the Tsetse fly, the so-called "Stumpy" form.

c) When taken up by the Tsetse fly in its blood meal into the fly's midgut, the parasites mitochondrion becomes active, and further cycles of binary fission take place.

d) The parasites migrate to the fly's proventriculus and salivary glands, where they transform to the epimastigote forms and further cycles of binary fission take place.

e) In the salivary glands, trypomastigote forms appear, capable of infecting the vertebrate host in the fly's blood meal ("Metacyclic" Trypomastigotes)

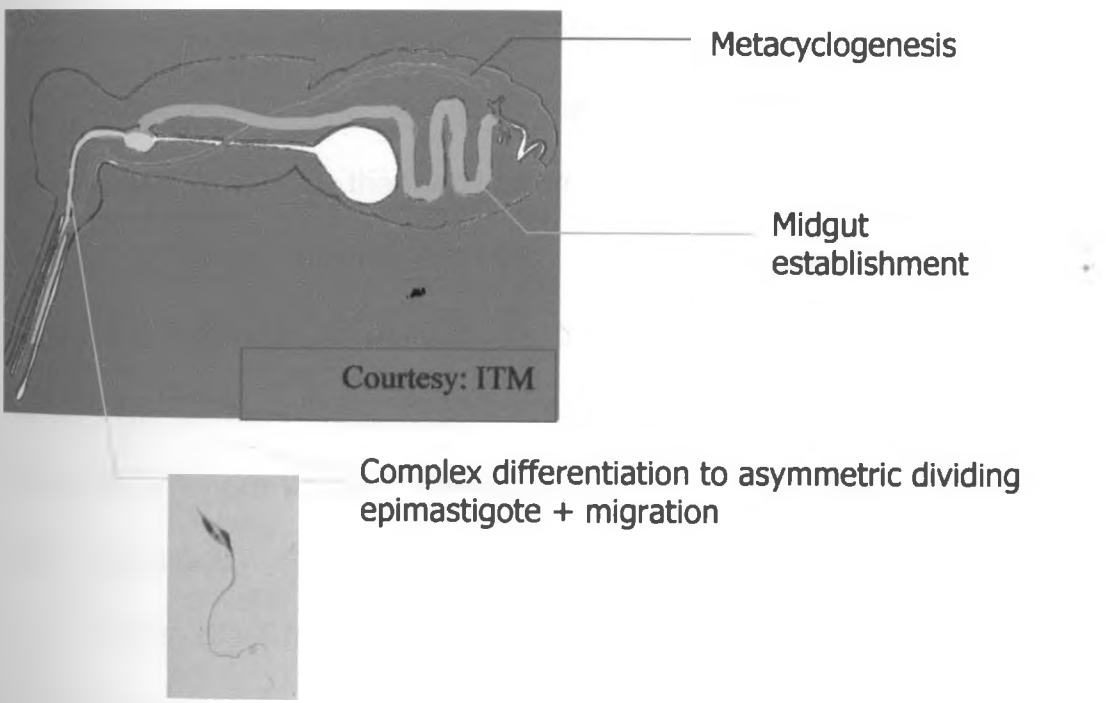
Transmission to the new host is effected either during feeding or in a contaminative manner via feacal material, depending on the site in the insect where infective forms develop. This process is by-passed in mechanically transmitted species, where trypanosomes are transmitted directly between hosts, and in *T. equiperdum* which is transmitted during coitus (Wenyon, 1926).

The progress of trypanosomes during cyclical development in tsetse flies following an infected feed is determined by the site of final development in the fly. The entire development is confined to the alimentary canal, with the production of infective forms (metacyclic trypanosomes) occurring in the hindgut, as in *Stercoraria*. Tsetse-transmitted Salivarian trypanosomes migrate to the proboscis or salivary glands where metacyclic trypanosomes are produced. *Trypanosoma rangeli* has a dual cycle in triatomine bugs maturing both in the hindgut and the salivary glands (Hoare, 1972).

Some species of trypanosomes, notably *T. evansi* and *T. vivax,* can be transmitted mechanically. Transmission like this is dependent on trypanosomes remaining transiently viable in the mouth parts of biting flies, and is facilitated by interrupted feeding (Foil *et al.*, 1987). The vampire bat (*Desmodus rotundus*) is also a mechanical vector for equine and bovine trypanosomosis in South and Central Africa, where a large proportion of large carnivores such as lions and hyenas were found infected despite not being a frequent source of bloodmeals for tsetse fly (Molyneux and Ashford, 1983).

Trypanosomes undergo morphological and physiological changes during the transition between vertebrate and invertebrate hosts. In blood and tissue, trypanosomes depend entirely on glycolysis for energy metabolism (Opperdoes, 1985). On entering the tsetse fly, they are exposed to an environment with little glucose and oxygen; in addition to glycolysis, they then assume a more economical way of generating energy, switching on the Krebs cycle and electron transport chain for the full utilization of pyruvate (Vickerman, 1970). In this insect stage, the mitochondrion is fully developed, with distinctly developed convolutions (cristae) as observed by electron microscopy.

Fig 2. The various stages of trypanosome development in the tsetse fly



1.2.4.4 *Peculiar features of trypanosomes*

The complex developmental cycle of trypanosomes is characterized by activation and repression of metabolic pathways, and is correlated with changes in organelle and surface membrane morphology (Vickerman, 1985). The understanding of these mechanisms is of potential value in the management of the diseases that trypanosomes cause, and in rational drug development.

An important feature in the success of the trypanosome parasite is its ability to change its surface antigens, the phenomenon of antigenic variation. Chronic infections are characterized by fluctuations in parasitaemia, with each peak representing the expression of a new variable antigen type (VAT). Trypanosomes use this mechanism of antigenic variation to survive against the host immune response (Vickerman, 1978).

Members of the family Trypanosomastidae have some biochemical peculiarities, which set them apart from other eukaryotes. Trypanosomastids are unique in having kinetoplast DNA, complex mitochondrial DNA molecules catenated into a single network. As described, in digenetic trypanosomes, the complexity of the mitochondrion varies depending on whether the parasite is in the vector (where it is fully developed) or the vertebrate host, where it may be fully regressed.

Kinetoplastidae have membrane-bound organelles known as glycosomes in which are located a number of glycolytic enzymes involved in the metabolism of glucose and glycerol, and of which there are about 200-300 in each cell

(Opperdoes, 1983). A number of glycolytic enzymes in the glycosome are organized into a large multienzyme complex and may form a rational target for drug design (Opperdoes, 1983).

All trypanosomes that are pathogenic to man lack the enzyme catalase, which catalyses the breakdown of hydrogen peroxide (Meshnick *et al.*, 1977). This renders them highly vulnerable to compounds that stimulate the production of hydrogen peroxide. Virtually all mammalian cells have glutathione, a tripeptide which they use to remove hydrogen peroxide and other by-products of aerobic metabolism. The absence of catalase makes trypanosomes completely reliant on their glutathione equivalent (a unique compound known as trypanothione) for hydrogen peroxide detoxification. Trypanothione is maintained in reduced state by the enzyme trypanothione reductase. Fairlamb *et al.* (1985) identified an unusual co-factor that is an absolute requirement for trypanothione reductase activity. This was identified as the polyamine spermidine, which was found covalently linked to trypanothione. This opened up new avenues for drug design. Difluoromethyl ornithine (DFMO), the only drug developed for sleeping sickness in 40 years (TDR news, 1992), acts by inhibiting ornithine decarboxylase, a key enzyme in polyamine synthesis.

1.2.4.5 Non-tsetse transmitted trypanosomes

The distribution of Salivarian trypanosomes has principally been determined by the distribution of tsetse fly vectors, which are confined to Africa south of the Sahara desert. However, two subgenera, *Duttonella* and *Trypanozoon*, have members that are transmitted non-cyclically. These include *T. evansi* and *T. equiperdum* (Trypanozoon), and *T. vivax* (*Duttonella*). *Trypanosoma vivax* and *T. evansi* are also transmitted by other biting flies and have spread beyond the confines of the tsetse belt (Hoare, 1972), while *T. equiperdum* is transmitted between equines during coitus. Like all *T. evansi* stocks studied so far, *T. vivax* stocks from South America are not transmissible cyclically by tsetse (Dirie *et al.*, 1993).

Trypanosoma evansi is probably a derivative of *T. brucei*, the result of survival outside the tsetse fly belt in Africa from where it spread to the Middle East, Asia and South America (Hoare, 1972). This species has remained relatively stable, forming a largely homogeneous group by isoenzyme analysis (Gibson *et al.*, 1983) and on the basis of kinetoplast DNA (Masiga and Gibson, 1990; Ou *et al.*, 1991; Lun *et al.*, 1992). *Trypanosoma evansi* is primarily a parasite of equines and camels but it is an important pathogen of other domestic animals including cattle in South and Central America and buffaloes in Asia. *T. evansi* is transmitted mechanically by haematophagous insects, particularly Tabanids (*Tabanus ssp.*) and stable flies (*Stomoxys ssp.*) (Foil, 1989).

Trypanosoma equiperdum is a tissue parasite and bloodstream forms are rarely seen. It is typically monomorphic, widely distributed outside Africa, and has been reported in Asia, Europe, North and South America, and North and Southern Africa (Hoare, 1972). Hoare (1972) considered that *T. equiperdum* could have arisen from an equine strain of *T. evansi*, which adapted itself to tissue parasitism. Lun *et al.* (1992) propose that either *T. evansi* arose via *T. equiperdum*, losing first minicircle diversity and then maxicircles as it adapted to life outside the tsetse belt, or that the two trypanosome species represent divergent lines from a single ancestral strain of *T. brucei*.

Trypanosoma vivax is capable of transmission cyclically by tsetse, and mechanically by biting flies (Hoare, 1970b). The extent of mechanical transmission of *T. vivax* in Africa is unknown, but it is distributed widely in cattle in South America where transmission is non-cyclical (Gardiner, 1989). It is thought to have been introduced to South America with cattle shipments from West Africa almost two centuries ago (Hoare, 1972; Gardiner, 1989). Indeed, comparative studies of isoenzymes profiles (Murray, 1982; Dirie *et al.*, 1993) and DNA hybridization (Dickin and Gibson, 1989) show similarities between *T. vivax* parasites from South America and West Africa. A distinct group of *T. vivax* isolates from East Africa has been described on the basis of pathogenicity (Wellde *et al.*, 1983), and biochemical characteristics (Murray, 1982; Gardiner, 1989). Dependence on non-cyclical transmission outside the tsetse belt could have resulted in the loss of tsetse transmissibility.

1.2.5 Clinical manifestation of Human African trypanosomosis

The initial clinical sign in humans is a rubber tender swelling (chancre) at the site of the bite followed within weeks, by an intermittent fever that is usually serious in *T. b. rhodesiense* patients, and a lymphadenophathy, that is characteristic in *T. b. gambiense* cases. Other symptoms include general malaise such as headache, pain in joints, weight loss, and itching; oedema of the face; anaemia particularly with *T. b. rhodesiense*; advanced conditions are characterized by epileptic attacks, coma and damage to the heart (X-rays show hypertrophy of the heart muscle, particularly with *T. b. rhodesiense*). *Trypanosoma cruzi* causes Chagas' disease, which is clinically manifested in adults, but most likely arises from childhood infection. It is this chronic disease, which is characterized by neurological disorders (including dementia), megacolon, megaoesophagus, and damage to the heart muscle. Acute forms of the disease are rare but present as severe aneamia, muscle pain and neurological disorders. The chronic disease is associated with damage to nerves, causing cessation of gut muscle contractions, irregular heart beats and destruction of nervous system motor centers. *T. cruzi* is capable of crossing the placenta and so chronically infected mothers can infect their babies, who may succumb to a very acute form of the disease.

1.2.6 Trypanosomosis management strategies

Several approaches have been used in the control of African trypanosomosis, ranging from those aimed at the disease-causing organism to those targeting the vector. Each of the available methods has its' own strengths and limitations. Some of the interventions used in the past, such as bush clearing (tsetse habitat destruction) or elimination of wild animals (trypanosome reservoir), have been discarded for ecological reasons. Indiscriminate use of insecticides through aerial or ground spraying has impacted negatively on the environment. At present the following less controversial interventions are available or being pursued:

1.2.6.1 Chemotherapeutic control of Human African Trypanosomosis

Only a few drugs are effective against sleeping sickness, and little progress has been made in developing new ones in the last 5 decades (Croft, *et al.*, 1997). Pentamidine and suramin, which were developed before the 1920s, remain the drugs of choice for treatment of early-stage *T. b.gambiense* and *T. b. rhodesience* disease respectively, while the arsenic-based melarsoprol, developed in 1949, remains the primary drug for treatment of late-stage sleeping sickness (Table 1). DL-α-difluoromethylornithine (DFMO), an inhibitor of ornithine metabolism in trypanosomes, has been used in the treatment of late-stage disease. The use, effectiveness and limitations of these drugs are given below:

1. Pentamidine

Pentamidine, an aromatic diamidine that is soluble in water, has been used since 1940. Two forms of the drug have been used: Pentamidine isethionate (relative molecular mass (M_r) =593) and the closely related pentamidine methane sulfonate with a relative molecular mass M_r = 533. The production of the latter salt has been discontinued. Pentamidine is a reversible inhibitor of trypanosomal s-adenosyl-l-methionine decarboxylase, thereby reducing the synthesis of polyamines (Bitonti *et al.*, 1986a). Other possible modes of action reviewed elsewhere (Sands *et al.*, 1985), are kinetoplast fragmentation or inhibition of glycolysis, or of DNA, RNA, protein and phospholipid synthesis, or of amino acid transport of cation efflux. Trypanosomes contain a pentamidine transport system, which raises the intracellular concentration of the drug to many times the plasma concentration, thus increasing its selectivity (Damper and Patton, 1976). Pentamidine acts relatively slowly as trypanosomes can be detected in the blood and lymph nodes for up to 48 h after the first injection (Van Hoof *et al.*, 1944).

Despite having been discovered more than half a century ago, the price of pentamidine increased considerably in the last few years (from US $ 1 to around US $ 30 per 300-mg vial) as the AIDS epidemic created a new and lucrative market for the treatment and prophylaxis. Fortunately, trypanosomosis patients have been excluded from this law of supply and demand, as the manufacturer agreed to provide pentamidine isethionate free of charge for trypanosomosis

patients, through the World Health Organization from 2001 and 2005. The status henceforth is unclear.

2. *Suramin*

Suramin has a molecular weight (M_r) of 1429, and is soluble in water. Suramin is a sulfated naphthylamine, and has been used since the early 1920s for the treatment of early –stage *T. b. rhodesiense* HAT.

Its mode of action is poorly understood, as it inhibits numerous enzymes, including L-α –glycerophosphate Oxidase (Gutteridge, 1985), RNA polymerase (Hawking, 1978) and many others that probably have no relation to its trypanocidal effect: hyaluronidase, urease, hexokinase, fumerase and trypsin. It is relatively a slow trypanocidal drug with trypanosomes disappearing from blood and lymph nodes 12-36 h after the first injection (Hawking, 1978).

Table 1: An overview of trypanocidal drugs used in treatment of sleeping sickness. The table indicates the type of drug and the species of trypanosome affected.

Drug	Species	Indicator	Year of 1st use	Comments
Pentamidine Isethionate	*T.b. gambiense*	Stage 1	1940	
Suramin sodium salt	*T.b rhodesiense*	Stage 1	Early 1920s	
Melarsoprol (Mel B)	*T.b. gambiense* *T. b. rhodesiense*	Stage 2	1949	Increased treatment failure (Resistant strain?)
Eflornithine	*T.b gambiense*	Stage 2	1981	Difficult use.
Nifurtimox	*T.b. gambiense* *T.b.rhodesiense?*	Stage 2	1977	Not registered for HAT; Compassionate use only Case series only, toxicity and action on *T.b. rhodesiense* is poorly documented

Suramin, because of its poor CNS penetration, is not used for late-stage trypanosomosis, although it clears the hemolymphatic system of trypanosomes (with a temporary improvement of symptoms). The drug is used for that purpose

in patients with CSF anomalies, in the hope that this pre-treatment will reduce the toxicity of melarsoprol, especially the Jarish-Herxheimer reactions (Buyst, 1975).

Suramin is produced by Bayer and sold as 1 g vials under the trade name Bayer205®. It costs approximately US $ 7 per 1-g vial but it has also been part of the free donations to WHO.

3. Melarsoprol

Melarsoprol, also known as Mel B or Arsobal, was synthesized half a century ago (Friedhem, 1951), through the addition of BAL (British Anti – Lewisite, dimercaprol), a heavy metal chelator, to the arsenic of melarsen oxide. It has a molecular weight of 398, and is dissolved in propylene glycol. The cellular target for melarsoprol has been shown to be trypanothione; its irreversible binding to trypanothione results in a compound called Mel T (Fairlamb *et al.*, 1989).

Trypanothione synthesis is also reduced by ornithine decarboxylase inhibitors, so that theoretically eflornithine and melarsoprol could have synergistic effects. Melarsoprol is also a potent inhibitor of pyruvate kinase, a key glycolytic enzyme (Flynn and Bowman, 1969).

Melarsoprol remains the most active trypanocidal drug available. Despite the poor CSF penetration, trypanosomes in the CSF become slower moving and fewer in number only 5-6 h after the first injection, and disappear from the blood

stream and the lymph nodes even more rapidly, within 30-120 min (Whittle and Pope, 1972).

Drug induced encephalopathy is the most important complication of melarsoprol treatment. Two types of encephalopathy, reactive and haemorrhagic have been described by pathologists and clinicians (Adams *et al.*, 1986). Polyneuropathy is the other common (10 % patients) and potentially severe adverse effects of melarsoprol (Gherardi *et al.*, 1990).

Melarsoprol is sold under the trade name of Arsobal® by Rhone Poulenc Rhorer Doma in Paris. The current price is approximately FF25 per 5-ml ampoule. Thus, a course of nine injections of Melarsoprol for an adult will cost approximately US $ 45. The drug has been provided for free courtesy of the WHO.

4. Eflornithine

Ornithine decarboxylase is the enzyme that catalyzes the conversion of Ornithine to Putrescine. The reaction is the first and the rate–limiting step in the synthesis of the polyamines spermidine and spermine (Bacchi *et al.*, 1980). Polyamines are essential for the growth and multiplication of all eukaryotic cells; they affect nucleic acid synthesis and contribute to the regulation of protein synthesis (Pegg and McCann, 1982). Eflornithine (ά- difluoromethyl ornithine), also known as DFMO' is a selective and irreversible inhibitor of Ornithine decarboxylase, which thus decreases putrescine and spermidine concentrations.

It is a 'suicide inhibitor', being a substrate of its target enzyme (Mc Cann *et al.*, 1986). In animal models, its curative effect against trypanosomes can be reversed by the administration of Putrescine, spermidine and spermine (Nathan *et al.*, 1981).

Eflornithine has been the only newly registered molecule for the treatment of HAT over the last 50 years. It has been used successfully in the treatment of 2nd stage *T.b. gambiense* patients since the 1980s, but seldom for *T.b. rhodesiense* HAT, which is more resistant to this drug than *T.b. gambiense* HAT.

Eflornithine causes significant toxicity, and in reports by Pepin *et al.* (1987) five out of 26 patients (19%) died after treatment with the drug. However, in retrospect, this was related probably more to the desperate condition of the patients than the inherent toxicity of the drug. In a larger trial, only four of 207 (2%) patients died during or shortly after treatment; three being children less than two years of age and a woman who was also infected with HIV/AIDS whose level of consciousness deteriorated during treatment (Milord *et al.*, 1992). The most frequent adverse effect associated with eflornithine is bone marrow depression.

Eflornithine is sold by Marion–Merrel-Dow under the trade name Ornidyl R. A two week course of intravenous eflornithine costs approximately US$150. Currently the drug has been donated to World Health Organization.

5. Nifurtimox

Nifurtimox is a 5- nitrofuran that has been used since mid 1970s in the treatment of American trypanosomosis (Chagas disease) (Brenner, 1979). It inhibits trypanothione reductase, resulting in the production of superoxide and peroxide, trypanothione being the major polyamine–containing analog of glutathione in trypanosomes, which acts as a cellular protectant against free radicals (Fairlamb, 1990a). The drug is normally produced by Bayer, and sold under the trade name Lampit® or Bayer 2502®.

Nifurtimox is not registered for use in HAT. It could represent an effective therapeutic alternative, at least for *T.b. gambiense*. However, its efficacy and safety needs to be evaluated in controlled, randomized clinical trials. Nifurtimox is very difficult to obtain outside countries endemic for Chagas disease. However, 200,000 tablets have been made and donated by Bayer to WHO and DNDi for clinical trials.

6. Diminazene

Diminazene aceturate is an aromatic diamidine, soluble in water, with a molecular weight of 587. Its mode of action is at least partially through irreversible inhibition of trypanosomal s-adenosyl-L-methionine decarboxylase, an enzyme involved in the synthesis of polyamines (Bitonti *et al.*, 1986a). It also selectively blocks kinetoplast DNA synthesis (Newton and Le Page, 1967) and is said to kill trypanosomes more slowly than suramin (De Read *et al.*, 1966).

Diminazene is produced by Hoechst and sold under the trade name Berenil®, with "restricted veterinary usage" clearly written on the package. A 1.05g Sachet costs approximately US $ 1.00, so that a course of three injections (7 mg/Kg /injection) would cost only US $ 1.50.

1.2.6.2 The future: New drugs. Therapeutic Perspective; Research & Development Priorities

The alarming small number of effective, affordable and well tolerated drugs for HAT calls for more research and development into new protocols (particularly combination treatments), together with mechanisms that will facilitate the clinical development of promising compounds.

(i) Combination treatments

Combination treatments involving existing drugs are thought to delay the occurrence of resistance. Several combinations of drugs have been shown to have synergistic activity against *T. b. brucei* in murine models. If synergistic, they might also allow a reduction in the number of dosages required and hence of the toxicity of the drugs. However, only a few combinations have been used in humans, and the knowledge of efficacy and safety of these protocols is too limited to recommend their systematic use.

(ii) Drugs under development

Several drugs hold the potential to become effective treatment for sleeping sickness. DB289, a diamidine derivative, is orally administered and has been shown to be active against African trypanosomes *in vitro* as well as in animal models in the first- stage HAT. Clinical trials for this drug are under way, and additional derivatives of the same class of compounds are under investigations for activity in late- stage HAT (C. Burri, pers. comm.). Even if all clinical trials are successful, DB289 will not reach the market for at least five more years.

Megazol is a nitro-imidazole synthesized in 1968 but discarded due to its mutagenicity (Ames positive). This compound, which partially crosses the blood – brain barrier in primates, has been re- investigated successfully in animal models in the past. However, recent studies have shown the drug to be genotoxic and its further development shelved.

No other substance is anywhere near pre- clinical or clinical development. Therefore, classes of compounds to treat HAT particularly the second-stage of the disease are in great need. The current Research and Development agenda includes, according to priority (i) Testing combinations of current trypanocidal drugs *in vitro* for drug synergism and in phase II and III clinical trials.

(ii) Pursuing the clinical development of the oral DB 289

(iii) Pursuing the development of DB compounds,

Partners from the public sector (WHO, Governments, Academia) and from the private sector (Industry, NGO) are already engaged in joint initiatives to maintain

the production of the current drugs and to develop new therapeutic protocols. In the long-term, the only way to ensure the ongoing ability to treat HAT patients is by stimulating ambitious research programs for new compounds.

1.2.6.3 Vector Control

The most widely used methods of tsetse control involve either trapping or killing the flies using insecticides. Despite the proven success of targets and traps, the sustainability of these approaches is questionable, generally because of the difficulties associated with their deployment and maintenance in inaccessible areas. More recently, pour-ons and dipping technologies have been used to control tsetse flies. Specially developed formulations of synthetic pyrethroids, suitable for direct application to cattle have been used for their ability to control tsetse flies. Cooper's Spot On, Ciba Geigy's Ectopor, and Bayer's Bayticol are examples of these pour-on formulations, and some success has been achieved through this approach (Baylis and Stevenson, 1998).

(i) Insecticidal Spraying

Jordan (1986) stated in the preface of his book that it is only removal of the vector that a disease-free environment can be created. With the absence of a vaccine for trypanosomosis and with increasing drug resistance to available trypanocidal drugs, control of the tsetse fly remains the most theoretically desirable means of controlling the disease. Despite the known limitations, the

use of trypanocidal drugs, generally administered by the livestock owners themselves, is the major method by which animal trypanosomosis is controlled today in most African countries. It has frequently been stated that we now have the technical means of either controlling or eradicating tsetse flies. The major difficulty lies in successfully implementing control in a sustainable way. Ikede (1986) therefore suggested that emphasis should be on the application of existing knowledge to the problems of livestock production rather than research. Based on the population dynamics of tsetse, the most successful attempts at controlling tsetse flies are likely to be at the extreme limits for survival of the fly (Rogers, 1979).

Until the mid -1970s the insecticides (Table 2) used were almost exclusively organochlorine compounds, including DDT and dieldrin. Most organophosphorous compounds and carbamates are more toxic than organochloride compounds or endosulfan. In the 1970s environmental considerations led to a search for alternative insecticides, especially synthetic pyrethroids, to which tsetse flies are highly susceptible.

Table 2: The various insecticides used for the control of tsetse flies (*Glossina spp.*)

Organochlorines	Pyrethroids	Avermectins
DDT	Natural pyrethrum	Ivermectin
Dieldrin	Permethrin	
BHC (Gammexane)	Deltamethrin	
Propoxur (a methylcarbamate)	Alphamethrin	
Dimethyl phthalate (indalone)	Flumethrin	
Diethyl toluamide (DEE repellent)	Lamda-cyhalothrin	
Endosulfan	Cyfluthrin – 'SOLFAC/Baofly'	
Ethylhexane-diol (Formula 22)	Alpha-cypermethrin (Cypermethrin) • 'Flectron' eartags • 'ECTOPOR'	

Susceptibility to insecticides by tsetse can vary from one species to another, and between the different stages (age, sex, physiological state) of a

species. Teneral male and female flies and older, fed males are generally similar in susceptibility to organochlorine insecticides, but fed pregnant females are much less susceptible by a factor of four to nine (Irving, 1968). Toxicity of insecticides can also be temperature dependent. Endosulfan has a positive temperature coefficient of toxicity whilst that of deltamethrin is negative (Smith *et al.*, 1994). Deltamethrin is more toxic than endosulfan at any temperature, and can be up to 300 times more toxic at 10 °C but the degree of greater toxicity varies widely with temperature. DDT, dieldrin and BHC are potent and persistent lipophilic insecticides, which can penetrate the cuticles of insects. Pyrethroids have low mammalian toxicity, whereas carbamates and organophosphates (developed after organochloride use was restricted) are highly toxic to mammals and other vertebrates.

Pyrethroids exhibit lethal and "knock-down" effects. In insects, pyrethrins combine with lipids in the nerve cell membranes and disrupt cationic conductance. This leads to excitation of the nervous system, which induces knock-down and then paralysis (Laveissière *et al.*, 1984b). Deltamethrin kills insects after a brief contact (<5 milliseconds) and it is environmentally safe since it is rapidly degraded in soil. Experiments have shown deltamethrin with longer knock-down effect than some other pyrethroids, permethrin, bioresmethrin and tetramethrin, and the duration of knock-down longer in male *G.m.morsitans* than in females (Quinlan and Gatehouse, 1981). These experiments show that

sublethal doses of insecticides can reduce lipid accumulation from a bloodmeal, which could render flies more susceptible to death from starvation.

(ii) Traps and targets

Tsetse flies are *k*-strategists, having a low rate of reproduction: they produce a single egg at a time and one egg every 9-10 days. The eggs and larva produced are retained in the female uterus until just before the 3rd-stage larva is ready to pupate, and this high level of parental 'care' ensures a high probability of each individual surviving. This differs from most insects, which are *r*-strategists, producing large numbers of eggs each having a low chance of survival to adulthood. Because of the stability of tsetse populations and low reproductive rate, little sustained mortality pressure (additional to natural mortality) needs to be exerted on a population to bring about extinction (Weidhaas and Haile, 1978). From estimates of the degree of trapping necessary to control a variety of insects, Weidhaas and Haile (1978) concluded that tsetse would require the lowest daily trapping rates, because of their low reproductive capacity and long-lived adults.

Most development of this method of tsetse control has concentrated on improved and cheaper designs of the target and odour attractants in order to attract as many tsetse as possible and to increase the number of tsetse actually landing on a target. More efficient odour attractants would allow fewer targets to be deployed per km^2 and would reduce costs. At present attractants are most

effective for *morsitans* group tsetse whilst further improvements are needed for attractants of *palpalis* group flies. Almost no work has been done with attractants for fusca group tsetse. Because of the differences in the habits and habitats of the three groups or subgenera of tsetse, the control using impregnated targets and traps is only successful against species of the *palpalis* group (Laveissière *et al*, 1981) and the *morsitans* group (Willemse, 1991). The control of *fusca* group tsetse, which are less attracted by synthetic phenols or other attractants, is more difficult. They generally occur in areas without cattle and are therefore economically less important for Africa as a whole, although locally they can be important vectors of trypanosomosis to cattle.

(iii) Application of insecticides to livestock

The principle is simply that tsetse coming to feed on cattle or other treated domestic livestock will be killed by picking up a lethal deposit of insecticide on the ventral tarsal spines on pre-tarsi whilst feeding. Alternatively, they will be repelled by the insecticide and will therefore not attempt to feed. Whilst a repellent effect may protect treated livestock, control of the tsetse population depends upon: i) a relatively large proportion of feeds being taken from domestic rather than wild animals; ii) a sufficient proportion of the livestock population being treated; and iii) a sufficiently low level of reinvasion. The treated livestock will then be equivalent to moving insecticide-impregnated targets, complete with built-in odour attractants. As cattle tend to aggregate in herds, a higher number of

treated livestock may be required than the number of cloth targets that would be required in an area. None of these parameters has yet been adequately defined and there are, therefore, no clear guidelines to determine how many cattle need to be treated in a given area. There are rough estimates of the number of the cattle requiring treatment in relation to the total number of potential hosts in an area, and of the theoretical time that would be taken for the tsetse population to be eradicated. In practice, control rather than eradication is likely in most situations, due to problems with reinvasion from untreated areas.

(iv) Non-insecticidal methods of tsetse control

For environmental reasons there is greater interest at present in searching for methods of tsetse control that either use lower quantities of insecticide or are based on techniques which do not require them at all. Whilst some techniques such as the sterile male release method have been used successfully in some programmes, others are still very much in the research stage, and may prove to pose insurmountable problems.

Biological control of tsetse, as for other insects, would be an attractive option as an alternative to the use of insecticides. Successful biological control of a number of insect pests has been achieved; for example the cottony-cushion scale (*Icerya purbasi*) pest of citrus fruits was controlled by a coccinellid beetle predator, *Rodolia cardinalis* (Van den Bosch and Messenger, 1973). Approaches that can be classified as biological control include genetic control, the use of

natural enemies (parasites, predators, bacteria and fungi), bush clearing and elimination of wild hosts. Sustained attempts to control tsetse have only been made using the latter two methods, which are now out of favor for environmental reasons.

(a) Bush clearing

Theoretically, habitat alteration as a means of tsetse control could be more sustainable than other methods, particularly if the altered habitat is maintained in a state unsuitable for tsetse by appropriate land use such as cultivation. The disappearance of *G. m. submorsitans* from much of northern Nigeria, where it appears unable to exist above certain densities of human population indicates that it is particularly sensitive to habitat changes (Jordan, 1986). *Palpalis* group of tsetse, on the other hand, seems able to coexist with high human populations and to inhabit small areas of woodland and gallery forest. Bush clearing has been widely employed in the past, and more recently, it has been a component of tsetse control schemes based on other techniques in order to make an unsuitable tsetse habitat so that reinvasion will be prevented.

Despite the apparent technical success of some trials with habitat modification, Lloyd *et al.* (1933) concluded that the method was unsuitable as a control measure due to the expense and speed of reinvasion. Arguments against bush clearing are that it can encourage soil erosion, decrease soil fertility and adversely affect water supplies.

(b) Elimination of wild animal hosts

In the 19th century it is likely that in Zimbabwe and South Africa, hunting resulted in an unintended contraction of tsetse distribution before the dramatic events of the rinderpest pandemic (Phelps and Lovemore, 1994). Wild animal elimination was introduced thereafter as a deliberate measure to control tsetse in Zimbabwe from 1919. Initially attempts were made to eliminate all potential hosts in an area. However this strategy was refined after tsetse bloodmeal analysis techniques were used to determine the predominant host species. Instead of elimination of all large wild animals in an area, there was more selective hunting of only those animals constituting a significant part of the tsetse diet.

Despite its theoretical feasibility, elimination of wild animals in areas where cattle are kept is unlikely to be useful. In Zimbabwe, cattle and donkeys were highly attractive to *G.m.centralis*, thus, this species could not be eradicated by the use of selective wild animal elimination alone in areas where those hosts were common (Robertson, 1983). Furthemore, it is unlikely that there will be a large population of wild animals in areas where domestic livestock are kept, and those that are present are likely to be hidden and difficult to eliminate. Removal of wild animals in these areas might increase the probability of domestic livestock being fed upon. In such a situation, it would be necessary to remove all domestic livestock before attempting to control the tsetse population through elimination of wild hosts.

(c) Biological control-natural enemies

Parasites and predators of tsetse flies were long ago observed in the field, leading to various experiments to investigate their usefulness for control purposes. Nolan (1977) tabulated pathogens of tsetse other than arthropods with references and abstracts.

(i) Hymenopteran and dipteran parasites

Two species of a parasitic wasp of the genus *Nesolynix* formerly classified as Syntomosphyrum; Boucek (1976), *Nesolynix glossinae* and *N. albiclavus*, have been recorded as parasites of tsetse puparia. The former has an east-west distribution across Africa from Senegal to the Indian Ocean, whilst the later has a north-south distribution along the eastern side of the continent. Both species occur in Kenya, Tanzania and Malawi. *Nesolynix glossinae* was first found by Fiske in Uganda in 1913 parasitizing puparia of *G.f. fuscipes*. The second species (named by Kerrick, 1961) in puparia of *G.m.morsitans* was found in Malawi (Waterston, 1916). In western Africa, *N.glossinae* was first reported by Nash (1947).

ii) Parasitic mites

Ectoparasitic mites of the family Erythraeoidea (probably *Leptus* species) have been reported on *G.fuscipes* in Kenya (Krampitz and Persons, 1967). There is no indication that mite infestations are harmful to tsetse or that they have a potential as control agents.

iii) Helminth parasites

Nematode parasites of tsetse from the family Mermithidae have been recorded since the beginning of the century. The first detailed description was from *Mermis spp.* parasitizing *G.p.gambiensis* in Liberia (Foster, 1963a) but the first record was from a wild *G. f. fuscipes* from Entebbe Uganda, in 1910. Subsequently, nematodes were found parasitizing *G.m.centralis* in the Democratic Republic of Congo (Rodhain *et al.*, 1913), Tanzania (Thomson, 1947) and Zambia (Lloyd, 1913).

The likely method of infestation is thought to be 2nd-stage larvae, which migrate to the surface of the soil and climb up grass and vegetation in wet weather. They then penetrate the body of the host through the inter-segmental integument, using the buccal stylet and a chitin solvent (Christie, 1936).

iv) Fungi

The effects of entomopathogenic fungi in *G. m. morsitans* have been studied in Kenya by Kaaya (1989b). The fungi *Beauveria bassiana*, *Matarhizium anisopliae*, *Paecilomyces fumosoroseus* and *P. farinosus* were all pathogenic, particularly *B. bassiana* and *M. anisopliae*, which killed up to 100% of infected adult flies. Male tsetse flies were more susceptible to infection than females and puparia did not appear to become infected. Among the problems of using fungi for tsetse control are those of a suitable control formulation and method of

dispersal (Kaaya, 1989b). The fungi *Absidia repens* and *Penicillium lilacinum* have been isolated from puparia of *G. f. congolensis* in the Central African Republic and proved pathogenic in experiments. They may therefore contribute to natural mortality, though it appears that entry points such as wounds are necessary for the puparia to become infected (Vey, 1971).

Mass-rearing of tsetse for release after contamination with *B. bassiana* has been proposed, and termed the 'lethal insect technique' (LIT) (Mahamat *et al.*, 1997). Such tsetse would probably die shortly after release but would spread the contaminating fungus.

v) *Bacteria*

The bacterium *Serratia marcescens* applied to rabbits' ears killed tsetse that fed on them and has been isolated from wild tsetse (Onoviran *et al.*, 1985) in which it invades the hoemocoel. The bacteria multiply in the infected hosts blood and body cavity and produce a general septicaemia that kills within a few days. However, it is an unlikely candidate for biological control, as it may cause mastitis in cows and pneumonia and septicaemia in humans.

(vi) Sterile Insect Technique

An approach that is of an area-wide in nature is the sterile insect technique (SIT) which has been successfully applied in the control of several important pests including the screwworm fly in the USA and the Mediterranean fruit fly. SIT is a genetic population suppression approach and involves

sustained, systematic releases of irradiated sterile male insects among the wild population. The insects to be released are mass-reared in large-scale insectaries. Males are sterilized by irradiation and then taken to the selected areas and released by air. Releasing of sterile males in high numbers over a period of 3-4 generations, after having reduced population density by other techniques (trapping, insecticide spraying, etc), can lead to eradication of the target population (Vreysen *et al.*, 2000). The point is reached when there are so few fertile insects remaining that fertile mating does not occur and the population is eliminated. For maximum efficacy, the sterile males released must exceed the number of fertile wild male flies by a considerable margin. Commonly the sterile males are given an initial bloodmeal before release so as to reduce the risk of them subsequently transmitting trypanosomes. While the low reproductive rate of tsetse makes this highly desirable approach, it has been criticized due to the relatively large upfront costs that would be associated with the implementation. However, the recent successful eradication of *G. austeni* from the island of Zanzibar by SIT has demonstrated the feasibility and applicability of this technology in integrated tsetse control programs (Vreysen *et al.*, 2000).

Successful colonization of important tsetse species is a prerequisite for an SIT program and was initially difficult partly due to low reproductive rate of the tsetse fly. As a female tsetse fly stores sperm in its spermathecae in sufficient quantity to last for the whole of its reproductive life, females inseminated by a sterile male will produce no offspring. Consequently the population will eventually

die out provided it is not sustained by immigration from neighboring or contiguous areas. Therefore this method is only suitable for geographically isolated areas.

(vii) Control of tsetse flies by genetic manipulation

Much effort has gone into development of DNA transformation systems for the medically and agriculturally important vectors. This process of genetic transformation in many insects relies on the microinjections of transposable elements present on circular DNA molecules (plasmids) into eggs. The transposable elements insert themselves randomly into insect DNA resulting in germ line transformation whereby the transgene is passed on to every individual cell of the transgenic insect. Marker genes carried by the transposable element help identify transgenic individuals. It has been possible to introduce foreign genes into medically and agriculturally important insect vectors including the major human malaria vectors, *Anopheles* mosquitoes (Pereira *et al.*, 2002). By using tissue specific expression systems, anti-pathogen gene products can then be expressed where they can affect parasite viability, i.e. gut, salivary glands or in haemolymph. Recently, several immune effector molecules have been successfully expressed in transgenic mosquitoes and their adverse impact on parasite transmission has been demonstrated (Ito *et al.*, 2002). It is thought that these genetically engineered refractory insects can be driven into natural populations to replace their susceptible counterparts and, hence, reduce disease transmission. The principles of this approach are being widely debated among

scientists at large, and to evaluate the efficacy and feasibility of this strategy, various international committees have been established (Aultman *et al.*, 2000).

While genetic transformation systems are now available for various vector insects, the viviparous nature of the reproductive biology of tsetse has hampered the application of such germ-line transformation technology. However, in addition to transmitting trypanosomes, tsetse flies have established symbiotic relationships with maternally transmitted bacteria as described below, which live naturally in close proximity to trypanosomes. It has been possible to exploit these organisms to express gene products that can affect trypanosomes in the midgut. The following are examples of genetic manipulation;

(a) The tsetse symbionts

Similar to other insects with a single diet throughout their entire developmental cycle (such as blood, plant sap or wood), tsetse flies rely on microbial symbionts for additional nutrients that are not found in their restricted diet and which they are unable to synthesize. Micro-organisms with different ultrastructural characteristics have been reported from various tissues of tsetse (Shaw and Moloo, 1991; Wigglesworth, 1929). These organisms have been of interest since they intimately interact with the host physiology, for example, their elimination renders flies sterile and furthermore, they have been implicated in vector competence of tsetse. Based on the 16S rRNA sequence characterization, the symbionts of tsetse represent three distinct microorganisms (Aksoy, 2000).

Two of these organisms are members of the enterobacteraceae: the obligate mutualistic primary (P)-symbiont genus *Wigglesworthia* (Aksoy, 1995b) and the falcultative secondary (S)-symbiont genus *Sodalis* (Dale and Welburn, 2001). The third symbiont present in some tsetse species is related to *Wolbachia pipientis* (Cheng *et al.*, 2000). In some of the older literature, the term Rickettsia-like organisms (RLO) was used to refer to both *Sodalis* and the *Wolbachia*-related symbionts.

(b) Exploiting the commensal symbiont Sodalis for foreign gene expression

Given their close association with their host's biology and their localization in tissues, such as the gut, the beneficial microbes can be used for drug and vaccine delivery (Hooper and Gordon, 2001), or for expression of foreign genes, designed to block the development of other pathogens (Aksoy *et al.*, 2001). Since transgenic approaches involving egg manipulation are difficult due to viviparous reproductive biology of tsetse, transgenic symbionts provide an alternative method for examining gene functions *in vivo*. Since the symbionts live in close proximity to the developing trypanosomes in the midgut, anti-pathogenic products expressed and secreted from these cells could adversely affect parasite transmission.

The availability of an *in vitro* culture system for *Sodalis* has allowed for the development of a genetic transformation system to introduce and express foreign gene products in these cells (Beard *et al.*, 1993). The broad host range replicon *oriV* derived from a *Pseudomonas aeruginosa* plasmid was used to construct a

shuttle vector in the *E. coli* cloning vector pBR325 and was then introduced into *Sodalis* where it was found to replicate extra chromosomally. The transformed cells were selected by virtue of resistance acquired to multiple antibiotics encoded by this plasmid. The *in vitro* manipulated recombinant *Sodalis* has been found to be successfully acquired by the intrauterine progeny by microinjection into the female parent hemolymph. The recombinant symbionts have been shown to be passed on to the F1 as well as their offspring where they successfully express the marker gene product, green fluorescent protein (GFP) (Cheng and Aksoy, 1999). Through such an approach, the insect cells are not transformed as in germ-line transformation, but instead foreign genes are expressed in the symbiotic bacteria (somatic transformation). The relative ease of genetic transformation and gene expression in bacteria makes this a desirable system for transgenic approaches.

(c) Effector genes that can confer refractoriness traits

No matter which transformation system is chosen for foreign gene expression, the discovery of gene products that can have an adverse effect on pathogen development is a crucial step in this technology. The identification of monoclonal antibodies (mABs) with parasite-transmission-blocking characteristics and their expression as single-chain antibody gene fragments in symbionts provides a vast array of potential antipathogenic products. Towards this end, transmission blocking antibodies targeting the major surface protein procyclin of the insect-stage procyclic trypanosomes have already been reported

(Nantulya and Moloo, 1988). Recently it has been possible to express and secrete a single-chain antibody gene product in the transformed symbionts of reduviid bugs *in vivo* (Durvasula *et al.*, 1999). Hence, expression of single chain antibody gene fragments in *Sodalis* specific for these epitopes is now desirable. With transmission blocking mABs, it is possible to have a multitude of potential antiparasitic targets, which can be expressed simultaneously in the symbionts to prevent the development of resistance against any one individual target and also to enhance efficacy.

(d) Implication of transgenesis for existing control strategies

Among the techniques used for reducing the tsetse populations are odor-baited traps and targets, pour-ons, ultra-low volume aerial spraying, and the SIT which can each be used, singly or in combination, to maintain cleared barriers between treated and untreated areas (Serap *et al.*, 2006). Transgenics have the potential to improve two aspects of the current SIT technology and enhance its efficacy for future programs.

The first is development of vector reflactory strains. Since the large numbers of male flies released can potentially contribute to a temporary increase in disease transmission, the incorporation of reflactory traits into SIT release strains will greatly enhance the efficacy of this approach, especially in sleeping sickness endemic foci. During the current field SIT programs, male tsetse flies are fed on a bloodmeal containing a trypanocide before release. The second is the use of *Wolbachia* mediated cytoplasmic incompatibility (CI) as a method of

One such novel approach is by use of anti-tsetse and/or transmission blocking vaccines. Brown and Cipriano (1985) showed that repeated feeding of *G. morsitans* on laboratory animals was accompanied by increased mortality of the flies, and the production of relatively high titres of circulating antibodies, which cross-reacted with salivary gland homogenates. Similar results were obtained when *G. palpalis* were fed on rabbits (Matha *et al.*, 1986).

Nevertheless, the field of vaccine development has been faced with major problems and challenges associated with development of population immunity through the use of vaccines. These challenges include among others:

i) Lack of vaccines against certain priority infectious diseases

ii) Poor efficacy of currently available vaccines

iii) Poor thermostability of available vaccines.

Despite this, the most effective and sustainable way of controlling trypanosomosis would be a safe and cost effective vaccine (Newman and Powell, 1995). Therefore the search for a sustainable anti-trypanosome vaccine has continued and several attempts have been reported.

(i) Antigenic variation in the mammal

Trypanosome infection results in a humoral immune response followed by a rapid fall in parasite numbers. Survival of the parasite is ensured by antigenic variation, the continuous switching of the variant surface glycoprotein (VSG), the only antigen accessible to antibodies on the organism's surface. This is organized as the surface coat, containing more than $>10^7$ VSG molecules. Only

one VSG is expressed by each trypanosome at a time, except during switching, and in a few cases in *in vitro* culture when double expressers have been detected. Double expressers are selected against by the host, perhaps because mixed coats are structurally abnormal.

It is unlikely that the VSG has any specific biochemical role, as reflected in the diversity in its variable domain. Its importance resides in its dense packing on the cell surface (to block access of antibodies to other invariant surface molecules), also, its effect on charge (thus thwarting non-specific immune mechanisms) and perhaps also in its immunogenicity (helping maintenance of parasiteamia at sublethal levels).

A small proportion of trypanosomes, which have switched to new variable antigenic types (VAT), continue multiplying until they in turn, invoke an immune response. It is generally thought that, in a clonal infection, several hundred different VATs can arise. Despite this large number, there is a degree of order in their expression, probably resulting mainly from an order of gene switching, and it is believed that, for optimum parasite transmission, this is in delicate balance with the kinetics of the immune response.

(ii) Antigen expression by metacyclic trypanosomes

Expression of the VSG gene ceases in the tsetse fly, and is assumed only at the infective metacyclic stage of development. In many respects, the patterns of expression in the metacyclic population are different from those in the blood-stream. This metacyclic population uses only a small and specific subset of the

VSG repertoire, known as the Metacyclic- Variant Antigen Type (M-VAT) repertoire, which consists of 12 VATs in *T. congolense* and no more than 27 in *T.b. rhodesiense*.

Remarkably, the M-VAT repertoire is highly predictable in content, regardless of the VAT(s) expressed by the trypanosomes originally ingested by the fly. Such predictability does not easily fit with the general concept that VSG expression is essentially a system of great diversification. The M-VSG repertoire does change slowly, however, probably ruling out its exploitation for vaccine development.

(iii) The tsetse-trypanosome interface

It is evident that certain factors influence trypanosome growth, and perhaps also development, in the tsetse fly, in spite of the presence of a growth factor (GF) receptor on the procyclic form. Uptake of blood by the fly stimulates the appearance of two lectins, which bind and are detrimental to trypanosomes (Welburn and Maudlin, 1989). This may be a primitive form of defense against infection, which is circumvented for trypanosomes by a complex relationship between the fly and the endosymbiont rickettsiae-like organisms that reside in the fly. *In vivo* investigation of the lectins is difficult, but indications are that, late in the infection, the lectins may actually be required for the development of the trypanosomes to the epimastigote and metacyclic stages. It is now possible to purify the lectins, and *in vitro* experiments will help to clarify their role and reveal whether novel growth factor receptors on the trypanosome should be sought.

Molecules found on the surface of the procyclic trypanosome could be exploited for immunological control of parasite transmission (from fly to mammal). A series of monoclonal antibodies directed against a complex antigen on the surface of procyclic *T. congolense* have strong agglutinating activity against procyclic cells, and when incorporated into routine fly blood meals, one antibody markedly inhibited trypanosome transmission.

Similar results have been obtained with an antibody against an uncharacterized antigen of *T. brucei* (Nantulya & Moloo, 1988). The complex antigen of *T. congolense* shows a gel separation pattern and strong immunogenicity similar to those of procyclin, a surface protein of *T. brucei* (Richardson *et al.*, 1988). The function of this protein and its exploitation for control are areas worth further investigation.

(iv) The challenge of the VSG: variable surface glycoprotein

VSG proteins cannot be used as vaccine targets due to the high degree of their variability. The host clears all of the parasites bearing the first particular VSG but does not generate a response to the few that have switched. At the genomic level VSG switching occurs through two mechanisms. VSG expression sites are located at the telomeric ends of chromosomes. Individual genes can be copied and then displace the currently expressed copy of the VSG, or alternatively the active telomeric site can be silenced, and another activated. Only one expression site, and thus only one VSG is expressed on a trypanosome at a time.

On the parasite surface, the VSGs are physically switched both spontaneously and by immune pressure. The switch is mediated by phospholipase C, which rapidly cleaves the glycophosphoinositol (GPI) anchor that binds the VSG to the parasite and allows another VSG to take the former's place. The rapid switching of VSGs and the large number of different VSGs, postulated to be over a 1000 unique types, plus variants that result from recombination of genes, allow the trypanosome to constantly evade the immune response and hence the host defense mechanisms. The antigenic switching found in African trypanosomosis thus makes vaccine development a major challenge, with limited options.

(v) Flagellar pocket antigens

The flagellar pocket of the trypanosome is an area where receptors are used for specific host macromolecule uptake. It is conjectured that these antigens are highly conserved amongst flagellated protozoa. As such a vaccine against the flagellar pocket proteins of *T. brucei* might also confer protection against other trypanosome species. Most studies have used a murine model that is highly affected by disease-based immunosupression. However, in one study a flagellar pocket antigen from *T. b. rhodesiense,* with bovine serum albumin as the carrier and alum as the adjuvant, was used to inoculate 90 cattle in Kenya (Radwanska *et al.*, 2000). The rate of infection was reduced from 13% to 0.9%. This experiment is of particular note because it was carried out in an environment of natural exposure with a naturally infectable species of tsetse.

One specific example of a flagellar pocket protein is trypanosomal transferrin-binding protein (TFBP), whose gene is present in multiple versions, and to which antibodies are produced in chronic infection. Borst *et al.* (1987) proposes that those antibodies hinder the multiplication of trypanosomes, and proposes that a vaccine stimulating antibody production against the repertoire of TFBPs might hinder trypanosome proliferation. He also reports that several other proteins are present in the flagellar pocket, and could also serve as targets for vaccine development.

(vi) Congopain

Cysteine proteases of microorganisms can degrade host proteins such as immunoglobulins and complement factors. They can also modulate cytokine activities, and are suspected to interfere with antigen presentation and processing. Some cattle species in Africa are more resistant to infection than others. Studies have shown that a *T. congolense* cysteine protease (congopain) could be playing a role in the different levels of tolerance. The more resistant cattle generate a stronger IgG response to congopain than the less resistant ones. Attempts are being made to use congopain antigens in a vaccine which would generate antibodies capable of neutralizing the enzyme activity (Foy *et al.* (2002).

(vii) Intracellular Antigens

Members of the Trypanosomatidae family have subpellicular microtubules cross-linked to each other and to the plasma membrane by unique trypanosomal

microtubule-associated proteins (MAPs). The trypanosomal MAP (p52) has been used in an antigen preparation with the enzymes aldolase and GAPDH (which the protein co-purifies with) as a vaccine in mice and rats. When animals were immunized (Lubega *et al.*, 2002) three times over a period of three weeks with p52 isolated from *T. b. brucei*, 100% protection was achieved. Serum from immunized animals also cross-reacted with *T. b. rhodesiense* and *T. b. evansi*, which gives hope for cross protection, at least among trypanosome species, and possibly for the whole family.

1.2.6.5 Development of anti-vector and transmission blocking vaccines

Controlling blood-sucking arthropods by immunization of hosts with antigens of arthropod tissue ("concealed" antigens) is gaining increasing attention (Willadsen *et al.*, 1995; Kinyua, *et al.*, 2002, 2005). The use of immunogens directed against internal antigens is appealing, since they are probably not introduced into the host during feeding and would avoid potential cutaneous hypersensitivity reactions in the host tissue (Wikel, 1988). The most promising approaches to vaccine induction of anti-arthropod immunity may be digestive tract derived antigens because of their accessibility to antibodies in the bloodmeal (Wikel, 1988).

Immunological control of ectoparasites has been demonstrated via the successful TickGARD vaccine, which is based on Bm86 antigen (Willadsen *et al.*, 1995). This vaccine protects cattle by reducing *B. microplus* survival, fecundity

and subsequent egg viability. It is not certain that application of this vaccine protects herds from the incidence of anaplasma or babesiasis. Nevertheless controlling tick infestation still protects cattle from hide damage, weight loss, and irritation, and so the vaccine has commercial benefit.

Efforts are also underway for the development of anti-mosquito vaccines. A midgut protein, AgMucl, an abundant, highly glycosylated protein on the luminal midgut epithelium isolated from *Anopheles gambiae* has been used to vaccinate mice. A significantly higher mortality from mosquitoes fed on immune mice as compared with control was observed (Lal *et al.*, 2001). Moreover, since *Plasmodium* ookinetes bind to luminal midgut epithelium carbohydrate residues in order to penetrate the midgut (Zieler *et al.*, 1999), AgMucl has been suggested as a potential target antigen for transmission blocking vaccine (Lal *et al.*, 2001).

The lynchpin for the future in anti-vector immunization is the identification of molecular targets within the vector tissues. In 1939, William Trager first demonstrated anti-vector immunity when he immunized guinea pigs and rabbits with tick extracts and induced a lethal immune response against ticks, blood feeding on the same immune animals (Trager, 1939). The few current identified anti-vector antigens provide a hint at the potential diversity of anti-vector molecular targets. Immune attack on the peritrophic matrix proteins (peritrophins 44 and 95) of the sheep blowfly, *Lucilia cuprina*, is thought to block digestion and absorption of the bloodmeal, and consequently starve the larvae (Wijffels *et al.*, 1999). Bm91, from the tick *Boophilus microplus*, is an angiotensin-converting

enzyme (ACE) homologue (Jarmey *et al.*, 1995). Mammalian ACE is involved in regulating vascular concentrations of angiostensin, but other ACE homologues have been found in *Drosophila melanogaster*, *Anopheles stephensi* and other insects where the enzyme seems to be involved in reproduction, and specifically with male fertility (Isaac *et al.*, 1999). Bm 86 is another anti-vector antigen that was discovered in *B. microplus*, but has no known homologue. It contains several epidermal growth factor-like domains, and seems to be critical to the normal physiology of tick midgut cells, which are possibly involved in endocytosis (Willadsen *et al.*, 1993). Antigens of the midgut are the most often used as the targets of anti-vector vaccine strategies, probably because midgut antigens on the luminal epithelial surface are in direct contact with the ingested bloodmeal. However, such an approach may be limiting when one takes into account studies describing antibodies that cross into the vector haemocoel (Aligham *et al.*, 1992). One recent report on the advantage of this phenomenon demonstrated that monoclonal antibodies against *Anopheles gambiae* salivary glands, when ingested via bloodmeal, are capable of blocking *Plasmodium sp.* sporozoite invasion of the salivary glands (Brennan *et al.*, 2000). If many organs and tissues are susceptible to immune attack, the possibility of identifying numerous anti-vector antigen targets arises. For example blood feeders can ingest greater than five times their body weight in blood and must rapidly diurese and process the bloodmeal for survival and reproductive success (Romoser, 1996). Bloodmeal diuresis is the work of malphigian tubules, which may have antibody-targetable

molecular transporters and/or channels such as the aquaporins (Echevarria *et al.*, 2001). Likewise, vector neuro-endocrine pathways that control critical processes such as molting (which are often the target of current chemical control strategies) may prove to be effective anti-vector immune targets (Elvin and Kemp, 1994). Ultimately the discovery of anti-vector immunological targets will further research into basic vector and insect physiology.

Control of any vector-borne disease with an anti-vector vaccine is dependent on specifics of each pathogen lifecycle, and the biology of the vector. These specifics must be considered for each disease system. Although diseases with zoonotic reservoirs may be theoretically difficult to control using these strategies, when administered only to human hosts, they may be more easily controlled if it is also possible to immunize the zoonotic reservoir hosts. Vaccines that target a vector's reproductive fitness are likely to be most effective when applied towards vectors that are not very fecund, such as the viviparous tsetse fly. Finally, it may be possible to control some vectors by attacking important symbionts within the vectors, either by immune-targeting critical antigens of the micro-organism or vector antigens that the microorganism relies upon to survive (Beard *et al.*, 2001). Triatomid bugs and tsetse flies which transmit chagas and African trypanosomosis respectively, each harbor important symbionts in their guts.

1.2.6.5.1 Tsetse genome studies: contribution to vaccine development

Current genome sequencing efforts will greatly aid the search for critical antigen homologues among insects. At the recent past, a total of 21,427 Expressed Sequence Tags (ESTs) were produced from the midgut of adult *Glossina morsitans morsitans* and grouped into 8,876 clusters or singletons potentially representing unique genes (Aksoy *et al.*, 2006). Putative functions were ascribed to 4,035 of these by homology (Sanger *Glossina morsitans* GeneDB database). Identification of putative immunity genes and their preliminary characterization provides a resource for the experimental dissection of tsetse-trypanosome interactions. The work on *Glossina* genome is spearheaded by the International *Glossina* Genomics Initiative (IGGI), (Aksoy *et al.*, 2005) which commenced in 2004. Its aim is to generate a full genome sequence for the genus *Glossina*. The availability of genomics information can lead to the development of new control strategies aimed directly at the fly or at its parasite transmission ability (vector competence). The data from ongoing studies indicate that knowledge on tsetse-trypanosome interactions during establishment of infections in the fly is central for the development of such strategies (Levashina *et al.*, 1999).

Obviously, the potential benefit of anti-vector and anti-ectoparasite immunity to both disease control and commercial industry is great. The fact that advancement in this field has been slow despite the potential benefits, suggests that the complexity of this science is great as well. Current understanding of

molecular immunology within both the host and the vector, and the application of rapid and interactive molecular screening techniques, should help us sort out these complexities more quickly.

New tools as in application of molecular biological techniques in insect science have increased over the past decade. Tools have emerged that would allow scientists to rapidly discover anti-vector targets. Genome analysis and database searches should enable quick identification of current anti-vector antigen homologues among many insects. Along the same lines, analyses of critical biochemical pathways in other species may lead to the discovery of homologous pathways in insects and to their potential as targets of immunological methods.

1.2.6.5.2 Mode of action for the anti-vector immune factors

Numerous facts and hypothesis have been articulated as to the mode of action of anti-vector immune factors. It has been shown that, spectrums of immune factors are ingested with the vector's bloodmeal that could eventually lead to the death or physiologic impairment of the insect. Antibodies, lectins, complement, and immune effector cells (cytotoxic T-lymphocytes, NK cells and macrophages) are some of the factors that may act separately or synergistically to impair the insect. Antibodies have been the most consistently examined immune effectors in vectors. Many studies have shown that antibodies directed against midgut antigens can reduce vector survival, fecundity, or infection from

parasites and viruses (Willadsen and Billingsley, 1996). Moreover, antibody fragments can also readily cross into the haemocoel and therefore have access to many other vector tissue antigens. Antibodies by themselves can bind to their targets and may sterically hinder enzyme activity or protein ion-channels, or simply block pores as is thought to be the case with antibodies against the peritrophins of *L. cuprina* (Wijffels *et al.*, 1999). In conjunction with complement, antibodies can also direct membrane-attack complexes to the surfaces of cells or they can direct T-cell activity towards the cell surfaces to which they are binding (Roitt *et al.*, 1996). Plant lectins have long been known to inhibit insect digestion and reduce insect survival, probably by the same mechanism as antibodies (Peumans and Van Damme, 1995).

Complement and cellular immune effectors have not been thoroughly studied for their effects against the vector, although both are known to be enzymatically active at least during the first few hours following bloodmeal ingestion (Grotendorst and Carter, 1987). Complement in conjunction with antibodies, mannose-binding protein, or by itself, can form membrane-attack complexes on the cellular surface to open pores in cell membranes (Roitt *et al.*, 1996). Cellular immune effectors can release many cytotoxic agents such as perforins, granzymes, and reactive oxygen intermediates (Moretta, 1997), all of which may damage the vector. However, very little published experimental evidence exists as to whether cellular immune factors can contribute to vector damage. The addition of granulocytes to Bm 86 serum did not increase tick

damage or mortality. It has been shown that leukocytes can opsonize malaria gametocytes for up to several hours post-ingestion in the mosquito midgut (Lensen *et al.*, 1997).

Protein immunization often stimulate a type II immune response that is dominated by antibody production and type II cytokines that primarily stimulate B-cell proliferation. However, cellular immunity is variable but often weak (Gurunathan *et al.*, 2000). However, the immune response from protein vaccination is dependent on antigen dose, the number of injections, and recognition of certain immunostimulatory epitopes within the heterogenous antigen mixture. Some animals may respond to a series of vaccinations with type 1 immune response and type II cytokines that stimulate T-cell proliferation.

1.3 Rationale of the study

Antibodies, complement and other cellular factors ingested in the tsetse bloodmeal could be directed at molecular targets in the tsetse gut, in an attempt to disrupt the biological activities of the vector (Willadsen and Billingsley, 1996). The host's ability to produce antibodies against the antigens of ticks and other ectoparasites was recently exploited in development of a vaccine against *B. microplus* ticks. The vaccine, which is based on a midgut membrane glycoprotein (Bm86), was made in Australia under the name TickGARD^{TM} (Willadsen *et al.*, 1995). The glycoprotein (Bm86) binds to the gut digest cells and inhibits endocytosis. The vaccines, which work through antibodies, complement and

other effector mechanisms (Garcia-Garcia *et al.*, 1998), have also been tested in Brazil (Hungerford *et al.*, 1995), Argentina, Columbia, Cuba (under the name GAVAC™ Plus) and Mexico (de la Fuente, 1995). Similarly, the death rates of mosquitoes fed on rabbits vaccinated with midgut antigens from *Anopheles stephensi* were higher than those of mosquitoes fed on unvaccinated rabbits (Alger and Cabrera, 1972). It was then suggested that the antibodies induced by vaccination either mechanically damaged the gut of the vector, inhibited enzymes, or interfered with bacterial flora. Thoracic muscles of stable flies were damaged, and tsetse flies (*G. m. morsitans*) fed on the rabbits died; some survivors exhibited other effects of vaccination, including paralysis of legs, interference in deposition of endocuticle and reduced post-emergence growth (Schlein and Lewis, 1976). A recent study by Kinyua *et al.* (2002) has shown that *Amblyomma variegatum* ticks fed on rabbits immunized with integral and peripheral midgut proteins show reduced engorgement and egg batch weights. The eggs laid by these ticks also show reduced hatchability, further reflecting the effects of vaccination.

Development of vaccines that utilize 'novel' or 'concealed' immunogens has been an effective strategy for induction of anti-tick immunity (Rinding *et al.*, 1994). Tsetse molecules linked to receptor-mediated endocytosis of digested bloodmeal components might be good targets for an anti-tsetse vaccine. In the present study, *G. pallidipes* midgut derived immunogens were investigated in an attempt to identify vaccine candidate(s) molecules against tsetse flies. *Glossina*

pallidipes is by far the most important tsetse species in Africa, infesting most of the areas where the disease occurs, and is important in the transmission of both animal and human trypanosomosis. Furthermore effective antigens obtained from *G. pallidipes* could also be effective against other *Glossina species.*

The process of maturation of *T. congolense* in tsetse is initiated by lectins secreted in the fly midgut (Welburn and Maudlin, 1989). When lectin activity in the midgut was inhibited, the trypanosomes remained as procyclic forms, indicating that midgut lectins secreted by tsetse flies were responsible for triggering maturation of procyclic *T. congolense* and *T. b. brucei.* In related studies, it has been demonstrated that antibodies block oocytes from invading midgut cells in mosquito (Lal *et al.*, 1994). The parasites bind to specific receptors within the midgut. In tsetse flies antibodies bound to such receptors could potentially reduce or inhibit transmission and establishment of trypanosomes, providing a unique opportunity to develop a vaccine that would prevent transmission of trypanosomes by tsetse flies.

Tsetse flies' midgut proteins were evaluated in this study for their efficacy as transmission blocking vaccine candidates of *T. b. rhodesiense,* the causative organism of acute human African trypanosomiasis. The investigations of anti-tsetse and transmission blocking vaccines could eventually lead to the development of vaccine(s) against the parasite and the vector, a strategy that was given high priority by the WHO/TDR Scientific Working Group on African Trypanosomiasis (WHO, 2001).

1.4 Aims and objectives:

(i) General objective

The general objective of the study is to isolate, identify and characterize *G. pallidipes* midgut protein(s) used to immunize rabbits against *G. pallidipes* and determine their potential as transmission blocking and ati-tsetse fly vaccine candidate(s) against *T. b. rhodesiense* infection

(ii) Specific objectives:

1) Isolation and fractionation of midgut protein(s) and immunization of rabbits;
2) Assessment of fecundity, survival and mortality rates of tsetse flies fed on immunized rabbits;
3) Assessment of transmission blocking efficacy of the midgut proteins against *T. b. rhodesiense;*
4) Purification and characterization of protective proteins(s);
5) Expression and identification of recombinant antigen(s) from gut cDNA library.

CHAPTER TWO

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 Reagents and Chemicals

The reagents and Chemicals used for this study were of analytical grade. They were obtained from Sigma, UK; Qiagen, Germany; BD Biosciences Clontech USA; Eppendorf, Germany; Amersham, England; Serva, Germany; BDH, England; Pharmacia, Uppsala, Sweden; Boehringer-Mannheim GmbH Biochemica, Germany; BioRad, Richmond, USA; Pierce, Rockford, USA.

2.2 Tsetse flies, Rabbits and Trypanosomes

A pathogen-free strain of *Glossina pallidipes* maintained at the Trypanosomiasis Research Center (TRC), the formerly Kenya Trypanosomiasis Research Institute (KETRI) insectary was used. The colony had been established using pupae obtained from the IAEA, Seibersdorf, Vienna, Austria. The experimental flies were maintained at 25 ±1°C and 80±5 % relative humidity and fed *in vitro* on rabbit serum.

Three months old New Zealand White rabbits weighing approximately 2 kg were obtained from the TRC Small Animal Unit. They were treated with broad spectrum antibiotics and coccidiostats, and maintained on commercial pellets and water *ad libitum*. The animals were housed in individual cages and managed in accordance with guidelines set by the Kenya Veterinary Board and the TRC's Institutional Animal Care and Use Committee (IACUC). Fifteen Swiss white mice

and a clone of *T. b. rhodesiense* (KETRI 3741) isolated from Busoga, Uganda in 1972 were used for infectivity studies.

2.3 Fractionation of midgut proteins

The proteins used to immunize rabbits were isolated from partially fed adult flies according to a method by Bordier, 1981. The flies were starved for 2 days, dissected and their midguts removed and put in liquid nitrogen. The midguts were solubilized as follows: Ten grams (10g) of midguts were washed four times by centrifugation (1000 x *g*, for 30mins, at 4°C) with phosphate buffered saline (PBSE) (0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.0) containing 1mM EDTA and 1mM phenylmethyl sulphonyl fluoride (PMSF) as protease inhibitors. The pellets from the final washing were sonicated and homogenized (30sec, speed 5, 30sec. pauses) in a polytron tissue homogenizer (Kinematic GmbH, Switzerland) and centrifuged (40,000 x *g* for 2 hrs at 4°C). The pellet obtained was re-suspended in PBS containing 1% pre-condensed Triton X-114 and allowed to stand on ice for 1h, then mildly sonicated before centrifugation (40,000 x *g*, 2hrs, 4°C). For the separation of proteins in the supernatant, a cushion of 6% (w/v) sucrose in 0.1M phosphate buffered saline (PBSE) pH 7.2, containing 0.06% TX-114, was placed at the bottom of a 1.5 ml conical eppendorf centrifuge tube. The clear sample was overlaid on this sucrose cushion, and the tube incubated for 3min at 30°C. The tube was centrifuged for 3min at 3000 x *g* at room temperature in an eppendorf centrifuge. After centrifugation the detergent (DET) phase appeared as an oily

droplet at the bottom of the tube. The upper aqueous (AQ) phase was removed from the tube and 0.5% fresh TX-114 added. After dissolution of surfactant at 0°C the mixture was overlaid on the sucrose cushion used previously, incubated for 3 min at 30°C for condensation and centrifuged on the previous detergent phase. At the end of separation, the AQ phase was rinsed with 2% TX-114 in a separate tube without the sucrose cushion. The DET phase of this last condensation was discarded.

2.4 Gel filtration chromatography of the DET phase

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the detergent isolated midgut proteins (DET fraction) gave a very dark background. To improve on resolution, the amount of the free detergent in the DET phase was reduced by gel filtration on Sephadex G-15 (Pharmacia, Upsalla, Sweden). The gel was swollen in excess buffer (10mM PBSE, pH 7.2, 0.02% NaN_3) and then degassed. The column was packed with the gel and then equilibrated with the buffer. The sample was then applied on the bed surface in a volume of about 5% of the gel volume. The first eluted sample material (about 30ml) was concentrated (200µg/ml) in polyethylene glycol 8000, (PEG), (Sigma, UK) before dialysis (12 kDs cut off) against 10mM PBSE, pH 7.2 at 4°C overnight. The dialyzed material was analyzed by SDS-PAGE (Laemmli, 1970) as described below.

2.5 Sample analysis by SDS-PAGE and 2-Dimensional gel electrophoresis (2-DE)

SDS-PAGE and 2-DE were done to determine the protein composition of the midgut fractions. SDS-PAGE was performed according to a modified method of Laemmli (1970). The gels consisted of 3% stacking gel and 5-20% gradient separation acrylamide gel. Samples were dissolved in sample buffer (124mM Tris-HCl buffer, pH 6.8, containing 0.4% SDS, 1.25% glycerol, 10% dithiothreitol (DTT) and 0.1% bromophenol blue). Samples were then heated for ten minutes at 56 °C before loading and run at constant voltage of 200 V for 3.5hrs using Tris-glycine pH 8.3 with 45.5 mM Tris and 411 mM glycine as the running buffer. Broad range molecular weight markers (Sigma, UK) were run along with samples for estimation of molecular weights. Protein bands were stained using silver nitrate Modified from Morrissey, 1981 (Appendix 1).

Two-dimensional gel electrophoresis was carried out according to O'Farrel *et al.* (1977). Protein samples (DET and AQ-affinity purified proteins) were separated (separately) by isoelectric focusing (IEF) using Sigma wide-range (pH range 3.5-10.0) ampholytes.

The process involved two steps:

(i) Prefocussing: carried out at 15 min at 200 V, 30 min at 300 V and 30 min at 400 V. In this step the pharmalyte carrier ampholytes were focused to generate a stable linear gradient (3.5-10.0).

(ii) Sample application and running: sample application was carried out and pre-focusing done for 2 hr at 400 V after which a continued focusing was done at 800 V overnight.

The first dimension rod gel was loaded and run through an SDS-PAGE (5-20%) (Laemmli, 1970). Individual proteins were resolved as discrete spots, which were visualized after silver staining modified from Morrissey, 1981 (Appendix 1).

2.6 Isolation of glycoproteins from the DET and AQ fractions through Con A- Sepharose affinity chromatography

The glycoprotein composition of the protein fractions was determined by lectin affinity chromatography, performed using Con A sepharose (Pharmacia) column (1.0 cm, i.d, x 10cm) to ascertain glycosylation of the isolated fractions. The column was equilibrated with Con A-buffer (0.01M Tris-HCl, pH 7.5 containing 1mM $CaCl_2$) and elution carried out, first using Con A-buffer then 200mM α-methyl-D-mannopyranoside in con A buffer. The column was run at 4 °C at a flow rate of 0.14ml/min. The eluted proteins were concentrated (200µg/ml) by dialysis in polyethylene glycol 8000, (PEG), and dialyzed against 10mM PBSE, pH 7.2 at 4°C overnight. The dialyzed proteins were analyzed using SDS-PAGE.

2.7 Protein assay

Protein estimation of the midgut protein fractions was done using the Bicinchoninic acid (BCA) protein assay method (Pierce, Co., Rockford, IL, USA) according to the manufacturer's protocol. Bovine serum albumin (BSA) (Sigma, UK) was used as the protein standard. Absorbance was measured at 562 nm using a model DU-50 Beckman spectrophotometer (Beckman, Palo Alto, CA, USA).

2.8. Immunization of rabbits with *G. pallidipes* midgut proteins

Two groups of six rabbits each were immunized with DET and AQ protein samples. A third group (control) was immunized with adjuvant only. Before immunization, 30ml of blood was bled from the rabbits' marginal ear vein and pre-immune sera prepared. The experimental rabbits received 1.5 mg (0.75mg/ml) immunogen each with saponin as the adjuvant (Bonford, 1980). Three booster injections, each containing 750, 500 and 500 µg proteins (in 2ml PBS) without the adjuvant were administered to each rabbit at an interval of 10 days. The rabbits were immunized and boosted through intradermal and intramuscular routes. The rabbits were bled before each booster injection and serum collected by centrifugation (1000 x *g*, 15 min., 4°C).

2.9 Assessment of protective ability of immunization against the tsetse flies and trypanosome establishment

Bioassay experiments were done to assess the effects of immunization on mortality, infectivity and fecundity of tsetse flies.

2.9.1. Mortality

Three groups of 60 male tsetse flies each were fed *in vitro* for 60 days on serum obtained from DET and AQ immunized and control rabbits and the mortality rate for the flies determined by collecting dead flies daily during the experiment. The number of dead flies for the treatments was assessed against the the dead in the control experiment. A graph of mortality against time was done for DET, AQ and control.

2.9.2. Infectivity

The anti-DET, anti-AQ and control sera were inoculated with a clone of *T.b. rhodesiense* (KETRI 3741). Three groups (DET, AQ and Control) of 60 male flies each were fed initially with infective serum and subsequently maintained on uninfective serum (*in vitro* feeding) for a period of 35 days. 5 flies from each group were dissected at 7 day intervals and the establishment of parasites in the midgut, proboscis and salivary glands assessed.

To assess the ability of the flies to transmit trypanosomes, fifteen infected flies whose infection was confirmed by probing them on warm glass slides were individually fed on a mouse (5 mice/group of DET, AQ and control respectively). Infection of mice fed upon by infected flies was determined through microscopy

and PCR (Gibson, 2002). This was intended to determine whether the trypanosomes found in the tsetse fly were infective to mice.

2.9.3. Fecundity

In order to determine whether feeding of flies on immunized rabbits had any adverse effect on their reproductive performance, 300 female flies, 100 flies per group were fed on immunized rabbits for a period of 60 days. Larviposition, pupal weights, abortion and percentage hatchability were assessed.

2.9.4. Data analysis

Chi square (X^2) tests (Snedector and Cochran, 1980) were carried out on data collected from assessed parameters (mortality, larviposition, pupal weights etc.). The Chi square was used to determine the significant dissimilarity matrices among the various parameters assessed. Squared Euclidean distance was used to compute distance matrices using SPSS.

2.10 Immunological characterization of the protective proteins (antigens)

2.10.1 Antibody detection by immunodiffusion

The procedure by Ouchterlony (1968) was used to determine the presence of specific antibodies against the proteins used for immunization. In a standard agar diffusion plate, small circular wells were placed circumferentially around a larger circular well. The antigen (15µg) was poured into a central well and the plate pre-incubated for 1-3 days, serial dilutions (10µg, 5µg, 2.5µg, 1.25µg and 0.625µg) of the antibody was put into the peripheral wells and the plates incubated for 24 h.

The precipitin bands at the point of equilibrium between the antigen and the antibody were visualized after staining with Coomassie Brilliant Blue.

2.10.2 *Antibody detection by immunoelectrophoresis*

Melted agar (3.5 ml, 2%) was poured onto the glass slides on a leveled surface. Two agar glass slides with two wells each were separately filled with the antigens (15µg) (DET and AQ).

The glass slides were placed in the electrophoresis tank and each end of the glass slide connected to the buffer chamber with filter wicks. The tank was closed and a current of about 8 mA applied per each glass slide. The electrophoresis was run for 1 hr. A trough was created in the agar between the two wells, filled with antiserum (30µg) and incubated overnight in a humid chamber. The slides were stained with coomassie blue.

2.10.3 *Isolation of immunoglobulin G (IgG) by ion exchange chromatography.*

The following procedure was done in order to separate the immunoglobulins from other contaminating proteins. Pooled sera from the experimental animals in each group were double diluted with distilled water and precipitated with equal volumes of saturated ammonium sulfate (SAS), pH 7.2. After centrifugation (1000 x *g*, 4°C, 10 min) the precipitate was dissolved in an equal volume of PBS, pH 7.2 and precipitated twice more with a saturated ammonium sulfate solution. The last precipitate was brought to the initial volume of serum with distilled water. The

immunoglobulin solution was centrifuged again (1000 x *g*, 4 ºC, 15 min) to remove any remaining precipitate and then loaded on to a DEAE-cellulose (4ml cellulose/ml of serum) column previously equilibrated in PBS buffer, pH 8.0. The column was washed with two column volumes of the buffer and the eluted IgG concentrated (200µg/ml) by ammonium sulphate fractionation as described above.

2.10.4. Western blot

A modified Western blot procedure of Towbin *et al.* (1979) was used to determine the antigenic composition of the DET and AQ protein fractions. Gut antigens of fed tsetse were resolved in 5-20% gradient SDS-PAGE. Using a Semi-dry blotter electrophoresis transfer unit (Sigma, UK), the separated proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Whatman 3 MM filter paper was used in the sandwich for the blotting process after thoroughly soaking them in Towbins transfer buffer (48mM Tris-HCl, pH 8.3, 29mM glycine, 20% methanol (v/v); 0.37% SDS (SDS is optional for transfer of denaturing gels). A constant current of 0.8 mA per cm^2 of nitrocellulose membrane was applied to the set up for 2 h at 27ºC. At the end of transfer the protein bands on the nitrocellulose membrane were revealed with Rouge ponceau stain (50% Rouge ponceau in 3% trichloroacetic acid), the positions of the protein standards marked with a pencil and then destained with PBS then 0.3% Tween 20 (PBS-T). The nitrocellulose sheet was incubated (in PBST) for 2.5 h at room

temperature or overnight at 4°C to block the remaining protein-binding capacity (Batteiger *et al.*, 1982).

The sheets were then incubated in whole rabbit antiserum, 0.5µg/ml in PBS-T for 4 h at room temperature with constant shaking, after which they were washed for 15 min, each in 4 consecutive baths of PBS-T. They were then incubated in secondary antibody (goat anti-rabbit antibody conjugated to horseradish peroxidase) (Bethyl Laboratories, USA) at 1:1000 dilution in PBS-T for 2 h. The antibodies were washed off as described above and the sheets rinsed briefly in PBS to remove Tween 20, followed by washing in 10mM Tris-HCl, pH 6.8 for 5 min. Staining was done by treating the sheets in developing solution (four parts 10mM Tris-HCl, pH 6.8, one part 0.3% 4-chloro-1-naphthol in ice cold methanol and 0.33µl/ml 30% hydrogen peroxide). The reaction was stopped by rinsing the strips with distilled water.

2.10.5. Purification of protective antigens by affinity Chromatography

Antigens from both the AQ and DET protein fractions were purified by affinity chromatography. The required amount of freeze-dried powder of CNBR-activated Sepharose 4B was swollen for 15 min in 1 mM HCl and washed on a sintered glass filter (porosity G3) with the same solution. A total of approximately 200ml of buffer per gram dry gel was added in several aliquots, the supernatant being sucked off between successive additions. 1 gram freeze dried powder gave a gel volume of 3.5 ml.

The purified IgGs were mixed with the coupling buffer [$NaHCO_3$ buffer (0.1 M, pH 8.3) containing NaCl (0.5 M)]. The antibody solution was mixed (about 5-10 mg protein per ml of gel) with the gel suspension overnight at 4ºC in an end-over-end mixer. The remaining active groups were blocked with a blocking agent (0.2 M glycine, pH 8.0) for 2 h at room temperature. The excess adsorbed proteins and the blocking buffer were washed with coupling buffer followed by acetate buffer (0.1 M, pH 4) containing NaCl (0.5 M) and by coupling buffer.

The protein-gel mixture was loaded into a column (1cm i.d x 10cm) and washed with several volumes of the coupling buffer. A sample (30mg) of DET and AQ fractions were loaded into separate columns. The unbound proteins were washed prior to elution with a lower concentration of elution buffer (10mM glycine-HCl, pH 2.5). The bound proteins were eluted with 0.1 M Glycine-HCl, pH 2.5 and detected at OD_{280}. The eluent was concentrated (200µg/ml) with polyethylene glycol (PEG, 8000) and dialyzed against 10mM PBSE, pH 7.2. The protein analysis was done by SDS-PAGE and 2-Dimentional gel electrophoresis as earlier described (Laemmli, 1970; O'Farrel *et al.*, 1977). The proteins were visualized by silver staining.

2.11 Identification of genes coding for protective antigens

2.11.1 Construction of *G. pallidipes* midgut cDNA library

The methods described below were used for the synthesis of a cDNA library of the tsetse midgut proteins. The expression library was subsequently screened with anti-DET and anti-AQ serum.

2.11.1.1 Total RNA extraction from the midgut tissues

The method described below was used to extract total RNA from the tsetse fly midgut for the preparation of cDNA library. A total RNA extraction kit (Qiagen, UK) was used according to the manufacturer's instructions. Tsetse midgut tissue sample (30 mg) was ground thoroughly with a mortar and pestle under liquid nitrogen and the tissue powder decanted into RNAse free liquid nitrogen-cooled, 2ml microcentrifuge tube. Liquid nitrogen was allowed to evaporate and the appropriate volume of buffer (RLT) added. The tissue was homogenized by passing lysate at least 5 times through a 20-gauge needle fitted to an RNAse-free syringe.

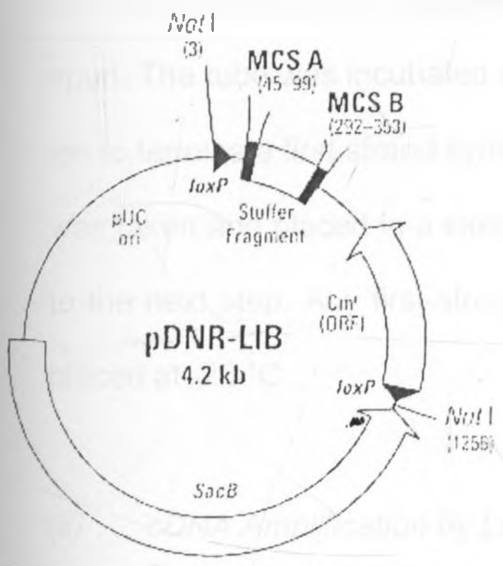
The tissue lysate was centrifuged for 3 minutes at maximum speed in a microcentrifuge. The supernatant was carefully transferred in to a new microcentrifuge tube and used in subsequent steps. One volume (600 µl) of 70% ethanol was added to the cleared lysate, and immediately mixed by pippeting. The sample (700 µl) was added into RNeasy mini column placed in a 2 ml collection tube. The tube was closed gently, and centrifugation done for 15 s at

8000 x *g*. The flow through was discarded. Buffer RW1 (700 µl) was added to the RNeasy column. The tube was closed gently and centrifuged for 15 s at 8000 x *g* to wash the column and the flow-through was discarded together with the collection tube. The RNeasy column was transferred into a new 2 ml collection tube and 500 µl RPE buffer added onto the RNeasy column. The tube was closed gently, and centrifuged for 15 s at 8,000 x *g* to wash the column and the flow through discarded. Another 500 µl RPE buffer was added to the RNeasy column. The tube was closed gently, and centrifuged for 2 min at 8,000 x *g* to dry the RNeasy silica-gel membrane. To elute, the RNeasy column was transferred to a new 1.5 ml collection tube and 30-50 µl RNase-free water pipetted directly on to the RNeasy silica-gel membrane. The tube was closed gently, and centrifuged for 1 min at 8,000 x *g* to elute. The eluate containing the total RNA was kept at -20°C until required.

2.11.1.2 *cDNA Synthesis by long distance (LD) PCR*

Creator™ Smart™ cDNA Construction kit (Clontech, BD Sciences) was used with the following procedures:

Fig. 3. pDNR-LIB Donor Vector (Clonetech, Canada): The recombinant vector with the cDNA inserts was used to transform the *E.coli* bacteria.



MCS A

loxP

TTA TCA GTC GAC GGT ACC GGA CAT ATG CCC GGG AAAT TCG GCC ATT ACG GCC TGC AGG ATC C Stuffer fragment.

(i) *First-Strand cDNA Synthesis*

The following reagents were combined in a sterile 0.5-ml microcentrifuge tube; 1-3 µl RNA sample (0.025-0.5 µg poly A+ or 0.05-1.0µg total RNA) (For the control

reaction, 1 µl [1 µg] of the control RNA was used), 1µl (10µM) SMART IV Oligonucleotide, 1µl (10µM) CDS 111/3' PCR Primer.

Deionized H_20 was added to a total volume of 5 µl. The contents were mixed and the tube spun briefly in a microcentrifuge. The tube was incubated at 72°C for 2 min, cooled on ice for 2 min and spun briefly to collect the contents at the bottom. The following were added to each reaction tube; 2.0 µl 5X First-Strand Buffer (250mM Tris pH 8.3, 30mM $MgCl_2$, 375mM KCl), 1.0 µl DTT (20 mM), 1.0 µl dNTP Mix (10mM), 1.0 µl Powerscript Reverse Transcriptase (5U/µl) to a total volume of 10.0 µl. The contents of the tube were mixed gently by pipetting and briefly the tube was spun. The tube was incubated at 42 ˚C for 1 hr in an air incubator and then placed on ice to terminate first strand synthesis. A 2-µl aliquot from the first-strand synthesis was taken and placed in a clean, prechilled, 0.5 ml tube. The tube was placed on ice to the next step. Any first-strand reaction mixture that was not used right away was placed at -20 ˚C.

(ii) cDNA Amplification by LD PCR

The thermal cycler was preheated to 95ºC. The following components were combined in the reaction tube; 2 -µl First-Strand cDNA (**from Step 2.11.1.2**), 10 µl 10X Advantage 2 PCR Buffer, 2 -µl 50X dNTP mix, 2-µl 5'PCR Primer (10µM), 2 -µl CDS 111/3' PCR Primer (10µM), 2-µl 50X Advantage® 2 Polymerase Mix, 80-µl De-ionized H_2O to a total volume of 100 -µl. The contents were mixed gently by flicking

the tube. The tube was then centrifuged briefly to collect the contents at the bottom of the tube. The tube was capped and placed in a preheated (95˚C) thermal cycler. Thermal cycling was carried out using GeneAmp 2720 as follows; 1 cycle (95ºC, 20 sec) and 25 cycles (95ºC, 5 sec, 68ºC for 6 min). A 5-µl sample of the PCR product was analyzed alongside 0.1-µg of 1-kb DNA size markers, on a 1.1% agarose/EtBr gel.

(iii) Removal of protein contamination by proteinase K digestion

A 50 µl sample of amplified ds cDNA (2-3 µg) was pipetted in a sterile 0.5-ml tube and 2 µl proteinase K (20 µg/µl) added. The remaining ds cDNA was stored at -20 ˚C (up to three months). The contents were mixed and the tube briefly spun. The mixture was incubated at 45ºC for 20 min and the tube briefly spun. De-ionized H_2O (50 µl) was added to the tube and 100 µl of phenol:chloroform:isoamylalcohol (25:24:1) added and mixed by continuous gentle inversion for 1-2 min. The tube was centrifuged at 14,000 rpm for 5 min to separate the phases and the top (aqueous) layer was moved to a clean 0.5 µl tube. The interface and lower layers were discarded. 100 µl of chloroform:isoamylalcohol (24:1) was added to the aqueous layer and the contents mixed by continuous gentle inversion for 1-2 min. The tube was centrifuged at 14,000 rpm to separate the phases and the top (aqueous) layer was moved to a clean 0.5 ml tube. The interface and lower layers were discarded. Ten µl of 3 M Sodium Acetate, 1.3 µl of Glycogen (20 µg/µl) and 260 µl of room-temperature 95 % ethanol were added and immediately centrifuged at 14,000 rpm for

20 min at room temp. The supernatant was carefully removed with a pipette without disturbing the pellet. The pellet was washed with 100µl of 80% ethanol and air dried (10 min) to evaporate off residue ethanol. 79 µl of deionized H_2O was added to resuspend the pellet.

2.11.1.3 Restriction of cDNA by Sfi Digestion and size fractionation

In a fresh 0.5 ml tube, 79 µl cDNA, 10 µl 10X *Sfi* 1 buffer, 10 µl *Sfi* 1 Enzyme (20U/µl) and 1 µl 100X BSA were added, mixed well and incubated at 50°C for 2 hrs; two µl of 1% xylene cyanol dye was then added to the tube and mixed well. For the cDNA Size Fractionation of the restricted cDNA, sixteen 1.5 ml tubes were labeled and arranged in a rack in order. The CHROMA SPIN-400 Columns were prepared for drip procedure. The column was inverted several times to completely resuspend the gel matrix. The air bubbles were removed from the column. A 1000 µl pipetter was used to re-suspend the matrix gently to avoid generating air bubbles. The bottom cap was removed and the column let to drip naturally. The column was attached to a ring stand and the storage buffer was let to drain through the column by gravity flow until the surface of the gel beads was just exposed in the column matrix. The flow rate was approximately 1 drop/40-60 sec. The volume of 1 drop was approximately 40 µl. Column buffer (700 µl) was added carefully and gently (along the column inner wall) to the top of the column and allowed to drain out. A mixture of *Sfi* 1-digested cDNA (100 µl) and xylene cyanol dye were added to the top-center surface of the matrix and allowed to absorb fully into the surface of the matrix. With 100 µl of column

buffer, the tube containing the cDNA was washed and this material gently applied to the surface of the matrix. The buffer was allowed to drain out of the column until there was no liquid left above the resin.

The rack containing collection tubes was placed under the column, so that the first tube was directly under the column outlet. Column buffer (600µl) was added and immediately collection of single-drop fractions begun (approximately 35µl per tube) in tubes #1-16. Each tube was capped after collection of each fraction. The column was recapped after collection of fraction #16.

The profile of the fractions was checked by gel electrophoresis before proceeding with the experiment. On a 1.1% agarose/EtBr gel, 3µl of each fraction was electrophoresed (separately) in adjacent wells, alongside 0.1µg of a 1-kb DNA size marker. The gel was run at 150V for 10 min. The peak fractions were determined by visualizing the intensity of the bands under UV. The first four fractions containing cDNA were pooled into a clean 1.5-ml tube.

The following reagents were added to the tube with 4 pooled fractions containing the cDNA (140µl) and mixed gently by rocking the tube back and fourth; 1/10 vol. Sodium Acetate (3M; pH 4.8); 1.3µl Glycogen (20mg/ml) and 2.5 vol. 95% ethanol (-20°C). The tube was placed in liquid nitrogen overnight, and then centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was carefully removed with a pipette without disturbing the pellet. The tube was briefly centrifuged to bring all remaining liquid to the bottom. All the liquid was carefully

removed and the pellet allowed to air dry for about 10 min. The pellet was resuspended in 7µl of deionized H_2O and mixed gently.

The procedure proceeded to section 2.11.1.4, or the cDNA stored at -20°C until the ligation step.

2.11.1.4 Ligation of ds cDNA into a plasmid vector, pDNR-LIB (Fig. 3)

The table below shows the various constitutions done on cDNA ligation mixtures. Three 0.5-ml tubes were labeled and reagents added as follows:

Ligation using three different ratios of cDNA to vector			
Component	Ligation A (µl)	Ligation B (µl)	Ligation C (µl)
cDNA	0.5	1.0	1.5
pDNR-LIB (0.1 µg/µl)	1.0	1.0	1.0
10X Ligation Buffer	0.5	0.5	0.5
ATP (10 mM)	0.5	0.5	0.5
T4 DNA Ligase (400U/µl)	0.5	0.5	0.5
De-ionized H_2O	2.0	1.5	1.0
Total volume (µl)	5.0	5.0	5.0

The reagents were mixed gently to avoid producing air bubbles and the tubes were briefly spun to bring contents to the bottom of the tube. The unused cDNA was kept at 4 °C for later use.

The tubes were incubated at 16°C overnight. 95µl of sterile DEPC-treated H_2O and 1.5 µl of glycogen were added to each of the above mixtures and mixed well with a pipette tip. Ice-cold 95% ethanol (280µl) was added and mixed by gently rocking the tube back and fourth.

The tube was placed in liquid nitrogen for 1-2hr and then spun in a microcentrifuge at 15,000 rpm for 20 min at room temperature. The ethanol layer was carefully removed without disturbing the pellet, then air dried. Each pellet (A, B, and C) was then re-suspended in 5µl of sterile DEPC-treated H_2O.

2.11.1.5 Transformation of E.coli with recombinant Plasmids

LB broth (970µl) was added to 14-ml polypropylene tubes labeled A, B, and C and to positive and negative control tubes. Competent cells (2µl) were added to each ligation reaction tube (A, B, and C from above section) and to positive and negative controls and mixed thoroughly with a pipette tip. The cells were heat shocked by heating at 42 ˚C for 90 sec and cooled in ice.

The entire volume was transferred to the pre-labeled polypropylene tubes containing 970µl LB broth (prepared above) and incubated with shaking (225 rpm) for 1hr at 37°C. During the incubation, three 1.5-ml polypropylene tubes A, B, and C were labeled. Tubes for positive and negative controls were also labeled. The LB broth (50µl) was added to each of these tubes.

At the end of the 1hr incubation, 1µl of each transformation mixture was removed and added to the appropriate tube containing 50µl of LB broth and mixed

gently by swirling. The remaining transformation mixture was stored at 4°C. The aliquot (50-µl) was then spread separately on a prewarmed 90-mm LB agar plate containing 30µg/ml of chloramphenicol. The inoculum was allowed to soak into the plates for 10min, plates inverted and incubated at 37°C overnight. The plates were examined the following day.

The percentages of recombinant clones in each transformation were determined by analyzing the DNA from 15 independent clones in each transformation. The inserts were screened for by performing PCR directly on colonies (see section 2:11:2:2). The desired transformation mixtures were pooled to generate the original, unamplified cDNA library.

2.11.1.6 Determination of titer for the plasmid libraries

LB/Chloramphenicol (LB/Cm) plates were pre-warmed at 37°C (or 30°C) for 1-2hr. An aliquot of the library was thawed and placed on ice. One µl of the library was added to 1ml of LB broth in a 1.5-ml microcentrifuge tube and mixed gently by vortexing. This was Dilution A ($1:10^3$). One µl from Dilution A was added to 1ml of LB broth in a 1.5-ml microcentrifuge tube and mixed by gently vortexing. This was Dilution B ($1:10^6$). One µl was removed from Dilution A to 50µl of LB broth in a 1.5-ml microcentrifuge tube and mixed by gentle vortexing. The entire mixture was spread onto a prewarmed LB/Cm plate.

Fifty µl and 100-µl aliquots were removed from Dilution B and spread onto separate LB/Cm plates. The plates were left at room temperature for 15-20min to

allow the inoculum to soak into the agar. The plates were inverted and incubated at 37°C (or 30°C) overnight.

The number of colonies was counted to determine the titer (cfu/ml). The titer was calculated according to the formulas:

- Colony # Dilution A*10^3*10^3=cfu/ml
- (colony # Dilution B/plating volume)*10^3*10^3*10^3=cfu/ml

2.11.1.7 Amplification of plasmid Libraries

2.11.1.7.1 Determining the number of plates required for amplification

The following calculation was used to determine the number of plates to use.

(# of independent clones x 3)= # clones

# of clones to screen/colonies per plate = # of plates

- To determine how much of the library stock to spread on each plate.

  # clones/ library titer =µls of library to plate

- The agar plates were allowed to dry at room temperature for 2-3 days, or at 30˚C for 3 hr, prior to plating cells.

2.11.1.7.2 Library amplification protocol

The library was plated directly on selective medium (LB/Cm plates) at a high enough density so that the resulting colonies were nearly confluent (20,000-30,000 cfu per 150-mm plate). Enough cfu were plated to obtain at least 2-3X the number of independent clones in the library.

The plates were inverted and incubated at 37˚C for 18-20 hr. Five ml of LB + 25% glycerol were added to each plate and the colonies scraped into the liquid. All the re-suspended colonies were pooled in one flask, mixed thoroughly and 1-ml aliquots of the library culture stored in liquid nitrogen. For use within one week, aliquots were stored at 4˚C.

2.11.2 Polyclonal antibody screening of the cDNA library (E. coli colonies)

2.11.2.1 Immunoscreening

One µl of the library was diluted in 1 ml of LB media and mildly vortexed. One µl was then mixed with 50 µl of LB media and plated on pre-warmed LB/Cm agar plates. The plates were incubated for 4-5 hrs until the colonies were just visible (pinprick sized). Nitrocellulose membranes were moistened in distilled water and placed onto plates using forceps. The plates were incubated overnight at 37°C. The plates were removed and the top agar allowed to harden at 4°C for 10 min.

For orientation, marks were made by stabbing through the filter into the agar with an inked needle. The filters were removed carefully, incubated in denaturation buffer (0.5 M NaOH, 1.5 M NaCl, 10% SDS), neutralized in 0.5 M Tris-HCl, pH 6.8, 1.5 M NaCl and blocked in TBS-5% Blotto for 1 hr at room temperature on rocker or 4°C overnight. The plates were stored at 4°C until needed. The filters were transferred to an antibody probe. Four ml of anti-AQ and anti-DET diluted serum was used per 90 mm filter. The filters were incubated 2-3 hrs at room temperature or overnight at 4°C on rocker. The filters were then washed three times, 10 min each in TBS-Tween,

transferred to peroxidase conjugated second antibody and incubated 1-2 hrs at room temperature. The filters were washed three times, 10 minutes each in TBS-Tween and once in TBS only. Positive clones were visualized by incubating the filters with 3% 4-Chloro-1-naphthol (Sigma) in ice cold methanol and 0.33µl/ml 30% Hydrogen peroxide (H_2O_2). The reaction was stopped by rinsing the filters with distilled water

2.11.2.2 Molecular screening

Six isolated positive colonies were picked with sterile toothpicks and inoculated each into 50µl of TE buffer in separate 1.5-ml microcentrifuge tubes. The tubes were boiled for 5 min and a 25µl PCR reaction was set with 1µl boiled colony lysate, 2.5 µl 10X Advantage 2 PCR buffer, 0.5 µl dNTP Mix (10 mM each), 0.5 µl Sense primer (M13 sequence primer), 0.5 µl Anti-sense primer (M13 sequence primer), 19.5µl De-ionized water and 0.5µl 50X Advantage 2 Polymerase Mix to make a total volume of 25 µl. The DNA was amplified with 1 cycle (94°C, 30 sec); 25 cycles (94°C, 30 sec, 68°C, 2 min) and 1 cycle of 68°C, 5min.

2.11.2.3 Isolation of plasmid DNA

Isolation of the plasmid DNA was done using an eppendorf FastPlasmid™ mini kit according to the manufacturer's instructions as follows:

Fresh bacterial culture (1.5 ml) was pelleted at maximum speed (at least 12,000 x *g* or 13,000 rpm) for 1 minute in a 2 ml culture tube. The supernatant was decanted, taking care not to disturb the bacterial pellet. Ice cold (0-4° C) complete lysis solution (400 µl, with lysozyme/RNase mixture) was added and mixed thoroughly to the

bacterial pellet by constant vortexing at the highest setting for a full 30 seconds (the step is critical for obtaining optimum yield). The lysate was incubated at room temperature for 3 minutes and then decanted or pipetted to a spin column. The spin column was centrifuged for 30-60 seconds at 12000 x *g* speed. Diluted wash buffer (400 µl, with isopropanol) was added to the spin column and centrifuged for 30-60 seconds for 12000 x *g*. The spin column was removed from the centrifuge and the filtrate decanted from the spin column waste tube. The spin column was returned into the waste tube and centrifuged for 12000 x *g* for 1 minute to dry the column. The spin column was transferred into the collection tube and 50µl of elution buffer added to the center of the spin column. The column was centrifuged for 30-60 seconds, the column removed and discarded. The eluted DNA was immediately used or stored at -20° C for later use.

2.12. Sequencing of the Plasmid DNA

The isolated recombinant plasmids were sent to Macrogen (Seoul, Korea) where their inserts were sequenced and raw sequenced data returned for further analysis.

2.13. Database Search and Sequence Analysis

Sequence analysis against EST databases was performed using the BLAST search program in National Center of Biotechnology Information (NCBI, Bethesda, MD). The sequences were first processed by identifying any possible vector sequences by Vecscreen (NCBI) and removal done by Bioedit programme. The translation of nucleotide sequences to proteins (open reading frame) was done using Expasy analysis tools. The aminoacid sequences were then analysed with the Basic Local Alignment Search Tool (BLASTp) against *Glossina* GeneDB (ESTScan) database and other protein databases. SignalP and TMHMM bioinformatics softwares were used to identify signal peptides and transmembrane proteins respectively. Identification of sequence signatures was done using ScanProsite (Expasy).

CHAPTER THREE

RESULTS AND DISCUSSION

EFFICACY OF IMMUNIZATION ON TRYPANOSOME TRANSMISSION AND FECUNDITY OF THE FLY

3.0 RESULTS AND DISCUSSION

3.1 *Isolation of the midgut membrane proteins*

The isolation of the membrane proteins from the midgut of the tsetse *Glossina pallidipes* was achieved by the use of a non-ionic detergent Triton X-114. Under the conditions employed, two fractions were obtained **(Fig 4)**. These were: the detergent phase (DET fraction, amphiphilic intergral proteins) and the aqueous phase (AQ fraction, peripheral proteins).

3.2 Physical and chemical properties of the gut membrane proteins

3.2.1 *Electrophoretic profiles of the midgut proteins*

On gradient SDS-PAGE (5-20%), the polypeptide bands of the DET and the AQ fraction were found to be within M_r 10,000-200,000 **(Fig. 4)**. Polypeptide bands of M_r 11,500, 14,100, 28,200, 32,000, 40,000, 42,000, 48,000, 63,500, 64,500, 100,000 and 142,000 **(Fig 4, lane 2)** were the predominant ones in the DET fraction while polypeptides of M_r 12,500, 14,300, 26,000, 28,000, 31,000, 36,000, 46,000, 54,000, 58,000, 63,000, 66,000, 85,000 and 200,000 were predominant in AQ fraction **(Fig. 4, lane 3)**.

3.2.2 *Glycosylation of membrane proteins*

Lectin binding was used to assess glycosylation of the polypeptides. When the DET fraction was applied to Con A affinity column and the bound fractions analyzed by SDS-PAGE, proteins with M_r 32,000, 22,000, 20,000, 18200, and 11,500 were detected **(Fig. 5, lane 2).** Similarly, proteins of M_r

85,000, 66,000, 58,000, 33,000, 20,000, and 12,500 were detected in the AQ fraction (**Fig. 5, lane 4**).

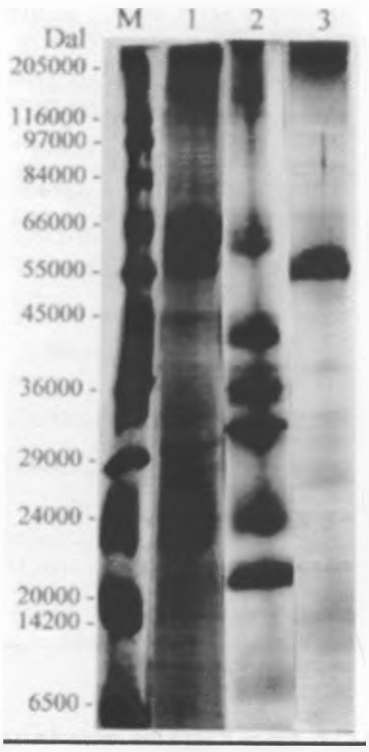


Fig.4. Denaturing gel electrophoresis of DET and AQ midgut protein fractions

Protein samples from the tsetse fly midgut were separated by SDS-PAGE (5-20%) and visualized by silver staining. Lane M- Wide range molecular weight standards (Sigma); Lane M, Wide range protein standard marker; 1- Crude midgut extract (10µg); 2-DET fraction (10µg); 3-AQ fraction (10µg)

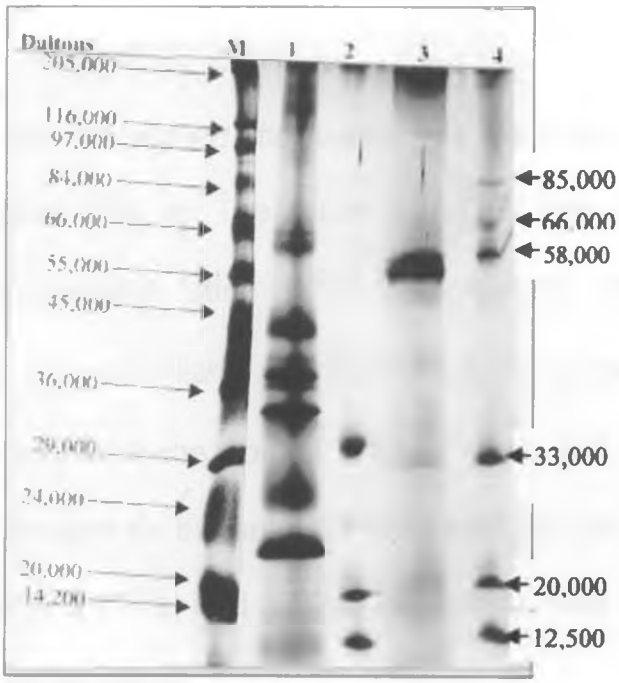


Fig. 5. SDS-PAGE analysis of DET and AQ fraction after lectin affinity chromatography.

The fractions were loaded on to a Con A-Sepharose column (1.0cm, 1.d x 10cm) and washed with Con A buffer (0.01M Tris-HCl, pH 7.5 containing 1mM $CaCl_2$). The bound proteins were eluted with 200 mM methyl α-D-mannopyranoside in Con-A buffer. The bound fractions (10 µg) were analyzed by SDS-PAGE (5-20%).

Lane M, Wide range protein standard marker (Sigma, UK); 1- DET fraction; 2-Con-A (DET); 3- AQ fraction; 4-Con-A (AQ)

3.3 *Effect of immunization on survival and fecundity of G. pallidipes*

3.3.1 *Mortality rate*

The mortality rates were assessed after tsetse flies were fed on serum obtained from immunized rabbits (**Fig. 6**). The serum from DET immunized rabbits gave significantly higher mortality rates (X^2=1.194, P<0.05) than the AQ one (X^2=0.735, P<0.05). The dissimilarity matrix between the two treatments was X^2 = 0.462, P<0.05. Serum from the control animals gave the lowest number of deaths. All (100%) the flies for the anti-DET treatment died by the 60th day while only fourty three percent (63%) and 33% of the anti-AQ and Control treatments had died within the same period.

3.3.2 *Fecundity*

Feeding tsetse flies on AQ immune serum caused a significant increase in abortions when compared to the control group (**Fig. 7**). The flies fed on DET serum had the highest number of abortions (X^2 =2.151, P<0.05) when compared to both the AQ and the control group. The flies fed on control serum had significantly higher (X^2 =2.117 (95%), P<0.05) larviposition. DET and AQ group of flies had a larviposition (AQ; X^2 =0.952, (63%) P<0.05 DET; X^2 =1.054, (69%) P<0.05) lower than the control (**Fig. 8**). The pupal weight (mean) for the control flies was slightly higher than either the AQ or DET, with a dissimilarity matrix of low significance (X^2 =0.325, P<0.05) (**Fig. 7**).

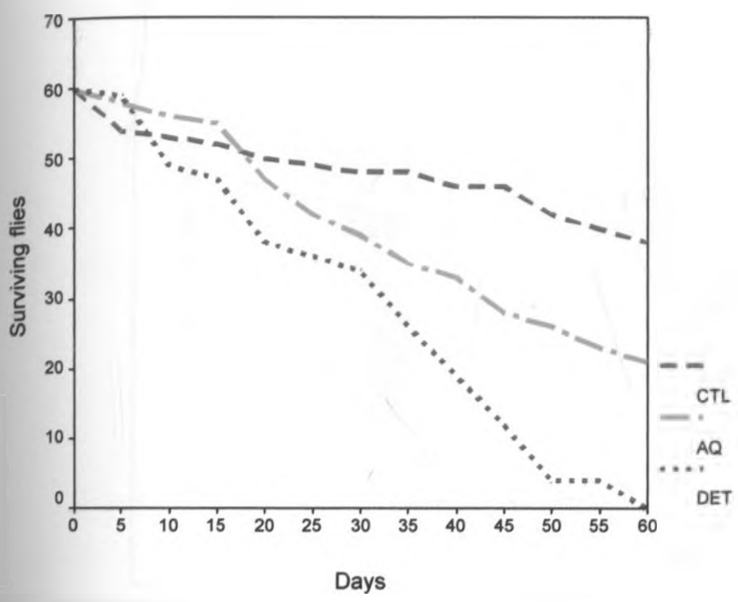
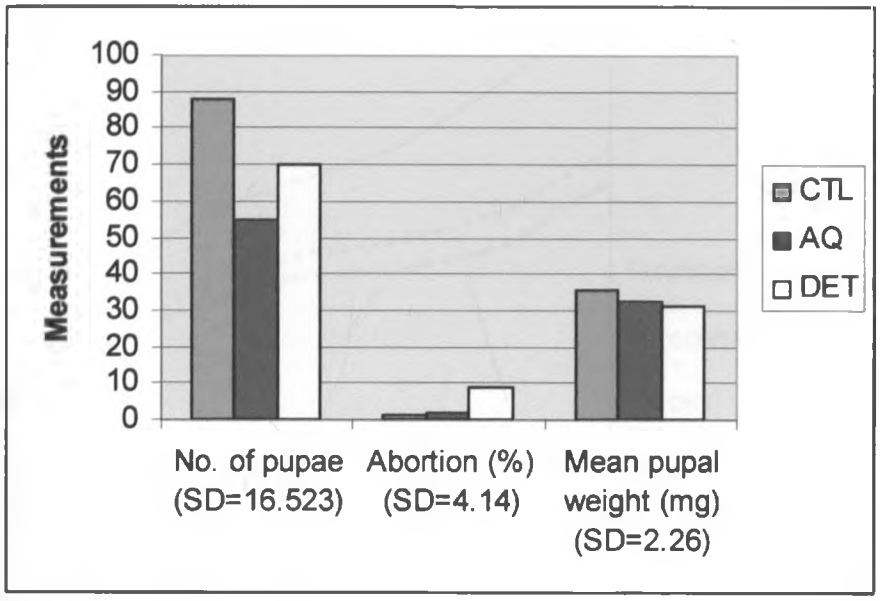


Fig. 6. The effect of immunization on mortality

Two groups of tsetse flies were maintained *in vitro* for 60 days on serum from rabbits immunized with DET and AQ proteins respectively. The third group of flies was the control. The effect of immunization on mortality was determined (SD=21.008)

Fig 7: Effects of immunization on fly fecundity

The mean pupal weight and pupal abortions were used to assess fecundity on three groups of female tsetse flies maintained on serum from DET, AQ and control immunized rabbits.



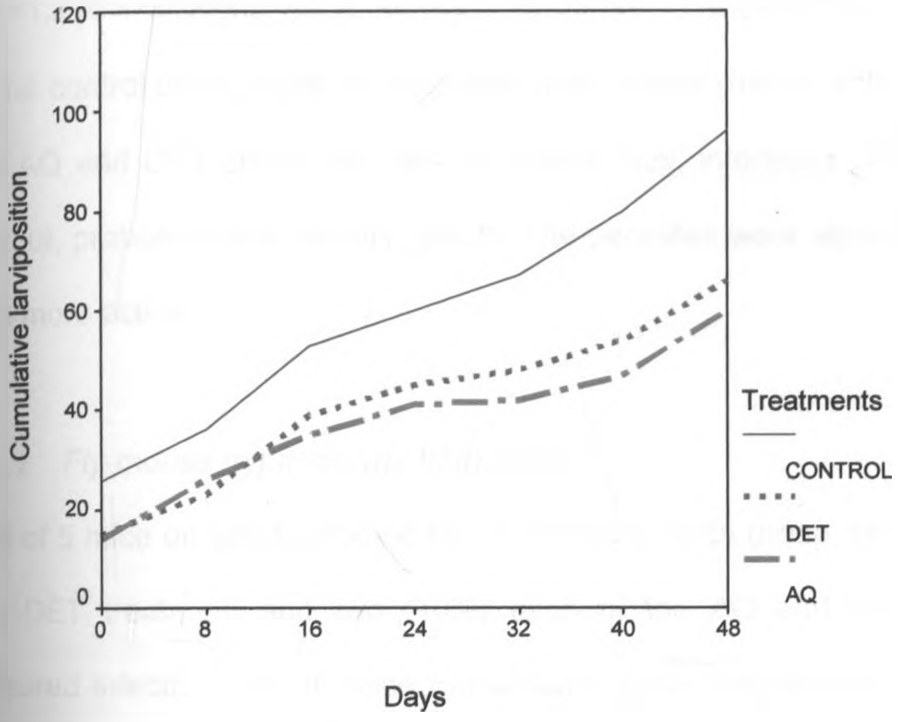


Fig. 8. Larviposition of flies fed on anti-DET and anti-AQ serum

Two groups of 60 mated female tsetse flies each were maintained on serum from DET and AQ immunized rabbits respectively. The third group was the control. The flies were fertilized in the first week after emergence and cumulative larviposition assessed among the three groups.

3.4 Effects of immunization on trypanosome transmission

3.4.1 Infection of the flies

Flies fed on anti-AQ and anti-DET serum had significantly lower infections (X^2 =1.210 P<0.05 (30%), X^2 =0.662, P<0.05 (20%) respectively) when compared to the control ones (Table 3). Parasites were predominantly within the midgut for the AQ and DET serum fed flies. In control flies, infections (45%) were in the midgut, proboscis and salivary glands. The parasites were also larger in number and more active.

3.4.2 Fly-mouse trypanosome transmission

Out of 5 mice on which infected flies were fed in each group, only one (20%) for the DET treatment and two (40%) each of the AQ and control treatments acquired infection. The fly-mice transmission gave insignificant difference since only one mouse for DET and two mice for each of AQ and Control acquired infection.

Table 3: Effects of immunization on trypanosome establishment within the tsetse fly

Three groups of tsetse flies were maintained on serum from rabbits immunized with DET, AQ and control respectively. The initial serum meal was inoculated with *T.b. rhodesiense* trypanosomes. Five flies from each group were dissected after every 7 days.

Immunizing fraction	No. of flies dissected	Infection status					
		Day 7	Day 14	Day 21	Day 28	% Infected SD=2.506	% SG infected
DET	20	None	None	None	4(M)	20	0
AQ	20	None	None	None	4(M) 2(P)	30	0
Control	20	None	1(M)	2(M)	M+P+S=6	45	30

Key

M-Number of flies infected in the midgut only

P-Number of flies infected in the proboscis only

S-Number of flies infected in the salivary grands only

M+P+S - Number of flies infected in the midgut, proboscis and salivary grands

3.5 DISCUSSION

The present study has indicated that feeding *G. pallidipes* on serum from rabbits immunized with detergent solubilized and aqueous midgut proteins increases their mortality rate. In anti-DET treated flies, a 100% of the flies had died by the 60th day while only 33% of the control flies had died within the same period. The anti-AQ had contributed to 63% deaths. The antibodies and other cellular factors in the bloodmeal could have interfered with digestion of the blood thereby denying the vector of vital nutrients. Such 'starvation' may have led to the early deaths of the flies. Immune factors could also have interfered with important biological functions of the tsetse flies. In other studies, *G.m. morsitans*, which were fed on rabbits immunized with crude tsetse midgut proteases, could not digest their bloodmeals properly and larviposited prematurely (Otieno, *et al.*, 1984). Albumin is essential for osmoregulation in *Glossina morsitans*, and antibodies absorbed through the gut by feeding tsetse on rabbits immunized with human albumin resulted in suppression of crop emptying, primary excretion and death (Nogge and Giannet, 1980).

Immunization also resulted in interference with the fertility of the tsetse flies. The number of pupae was significantly lower (by 32%) for the flies fed on anti-AQ and anti-DET (by 26%). Some difference was also observed with the weight of the pupae (< by 9% and 12% for AQ and DET respectively). Other workers have also reported increased mortality and slightly decreased fertility of the flies fed on animals immunized using whole *G. m. morsitans* (Nogge, 1978).

In a similar experiment, tsetse flies either died or their development was affected (Sclein and Lewis, 1976).

The number of mice infected for this study was few (one-20%, two-40% and two-40% for DET, AQ and control respectively). This is after feeding *Trypanosoma brucei rhodesiense* infected tsetse flies on mice. This could be explained by the fact that *T. b. rhodesiense* has significantly lower transmission index than the non-human infective *T.b. brucei*, (Welburn *et al.*, 1995) which may partially explain the focal nature of human sleeping sickness compared with trypanosomosis of livestock. However, the current work has indicated that the two fractions contain antigens that could be used to block transmission of *T. b. rhodesience* by tsetse flies. The control flies had 45% of them carrying the infection within the midgut, proboscis or salivary glands with 30% carrying mature infections.. The parasite's ability to infect the tsetse flies could be dependent on the immunizing antigen. As such the low infection rates observed for the AQ and DET treatments may be because antibodies or effector cells in immune sera against these antigens were reacting to some epitopes within the midgut, thereby interfering with parasite establishment. Interference with the midgut has been shown to curtail maturation of *T. congolense* in tsetse flies (Welburn and Maudlin, 1990). For example, when lectin activity in the midgut was inhibited, the trypanosomes remained as procyclic forms. The so-called anti-vector vaccines that inhibit transmission of disease causing organisms have been investigated mainly for malaria transmission by mosquitoes (Billingsley, 1994), and the same

approach has been examined for blocking transmission of trypanosomes by tsetse (Murray *et al.*, 1990; Nantulya and Moloo, 1988). Other studies have shown that ingestion by tsetse of monoclonal antibodies to a defined procyclic surface antigen significantly reduces cyclical development of *T.b. brucei.* This approach was suggested as a possible method for the control of sleeping sickness by immunization of livestock, which are hosts of peridomestic disease vectors (Nantulya and Moloo, 1988). Interference with certain molecules within the midgut may lead to reduced trypanosome establishment within the tsetse fly. Indirect evidence from sugar inhibition experiments suggests lectins play a role in determining the initial success of trypanosome infections in tsetse flies and stimulates the maturation of successful trypanosome infections (Welburn and Maudlin, 1999). The severance of lectins-trypanosome interaction would certainly interfere with the trypanosome establishment in the tsetse fly.

CHAPTER FOUR

RESULTS AND DISCUSSION

'BIOCHEMICAL AND IMMUNOLOGICAL CHACTERIZATION OF THE PROTECTIVE ANTIGENS'

4.0 RESULTS AND DISCUSSION

4.1 Immunochemistry of the tsetse midgut proteins

4.1.1 Double radial immunodifusion and immunoelectrophoresis analysis

Anti-AQ and anti-DET antibodies were detected by double immunodiffusion (Ouchterlony, 1968) and immunoelectrophoresis. No antibodies were detected after the first boost but the second boost gave weak precipitin bands. However, after the third boost strong precipitin lines were observed on the agarose gel for both the DET (several lines) (**Fig. 9a, i**) and AQ (**Fig. 9a, ii**) fractions. Similar observations were also made with immunoelectrophoresis analysis. Precipitin lines were observed towards the positive and the negative side of the electrophoregram for both the DET (**Fig. 9b, i**) and AQ (**Fig. 9b, ii**) fractions respectively.

4.1.2 Analysis of purified immunoglobulin G

Purified IgG antibodies were analyzed by SDS-PAGE (5-20%) and revealed by silver staining (**Fig. 10**). The IgG was run alongside the crude anti-sera. The usual M_r of 25 kDs for the light chain and >50 kDs for the heavy chain.

4.1.3 Immunoblot analysis

To identify the antigenic proteins in both the DET and AQ fractions, protein samples of both the AQ and DET fractions were separated on SDS-PAGE (5-20%) and then transferred electrophoretically to a nitrocellulose membrane. The blot was reacted with anti-DET and anti-AQ sera **(Fig. 11)**. Horseradish peroxidase (HRP) conjugated goat-anti-rabbit IgG (Sigma) was used to locate

the protein-antibody complex. Color development was done by incubating the blots with 4-chloro-1-naphthol and H_2O_2. Protein bands of M_r 64,500, 63,500, 28,200, 14,100 and 11,500 were enriched in the DET immunoblot while proteins of M_r 85,000, 66,000 and 14,300 were predominant for the AQ fraction.

4.1.4 Purification of the protective antigens by immunoaffinity chromatography

CNBR-activated Sepharose 4B was used in this procedure. The purified antibodies (IgGs) were coupled to separate columns for the DET and AQ fractions. The bound proteins were then eluted and analyzed by SDS-PAGE (5-20%) (**Fig 12**). Polypeptide bands of M_r 64,500, 63,500, 28,200, 14,100 and 11,500 (**Fig 12, lane 2**) were enriched in the bound DET fraction while polypeptides of M_r 85,000, 66,000, and 14,300 were enriched for AQ fraction **(Fig. 12, lane 3).**

4.1.5 Determination of molecular weights for the native immunoaffinity purified DET and AQ antigens

Non-denaturing gel electrophoresis was performed on both on AQ and DET antibody-affinity purified antigens. Three proteins (M_r 150,000, 66,000 and 53,000) were predominant for the DET fraction **(Fig. 13, lane 1)** and a similar number (M_r 160,000, 97,000 and 55, 000) for the AQ fraction **(Fig. 13, lane 2)**.

4.1.6 Determination of ionic properties of the antigenic proteins

The DET and AQ fractions were analyzed on 2-dimensional gel electrophoresis (2-DE) to find out their polypeptide distribution over a broad range of pI (3.5-10.00). The polypeptide bands in both the AQ and the DET fractions appeared as spots on 2-DE **(Fig. 14, a)**. Polypeptide bands of M_r 40,000, 38,000, 32,000 and 28,200 were predominant of DET fraction **(Fig. 14, a)** and polypeptides of M_r 85,000, 66,000, 28,000, 26,000, 7500 and 6,000 predominated the AQ fraction **(Fig 14, b)**. The polypeptides of both DET and AQ fractions were shown to range from basic, neutral and acidic in the IEF analysis.

4.1.7 Identification of antigenic glycoproteins

Concanavalin A isolated glycoproteins for both the DET and AQ fraction were introduced into CNBR-activated sepharose 4B column with purified IgGs as the ligand. The bound glycoproteins were eluted as in section 3.5.3. and SDS-PAGE (5-20%) used to analyze the eluent **(Fig. 15)**. Polypeptide bands of M_r 28,200, 14,100 and 11,500 were enriched for DET glycoprotein fraction **(Fig 15, lane 2)** while polypeptide bands of M_r 85,000, 66,000 and 12,500 were enriched for the AQ fraction **(Fig. 15, lane 4)**.

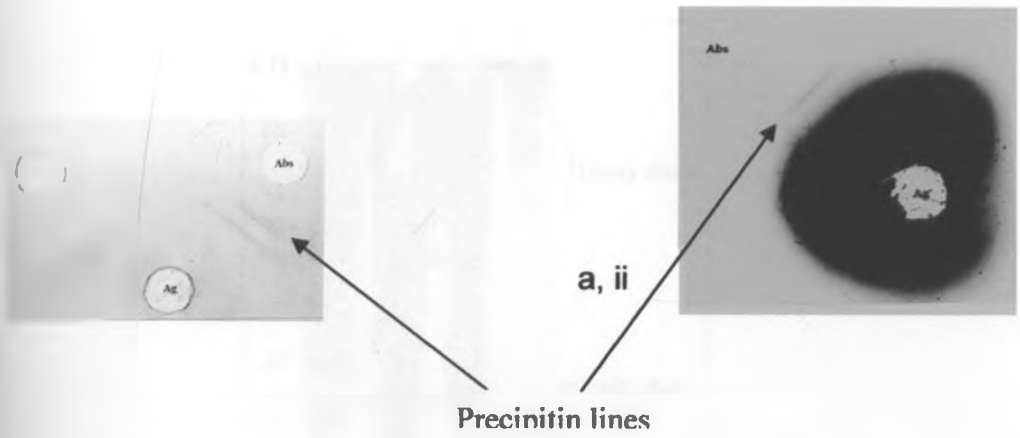


Fig. 9a. **Double radial immunodiffusion showing reactions for antibodies against the antigens**

The central well had 20µg antigen namely (i) DET, (ii) AQ while the peripheral well contained the respective antiserum. The precipitin bands were stained using Coommasie Brilliant Blue.

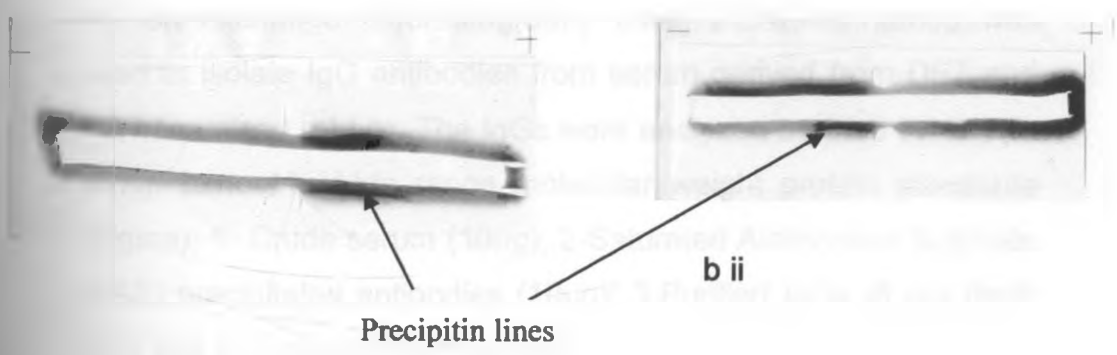


Fig. 9b. **Immunoelectrophoresis showing reactions for antibodies against the antigens**

The wells had 30µg antigen namely (i) DET (ii) AQ while the middle trough contained antiserum. The precipitin bands were stained using Coommasie Brilliant Blue.

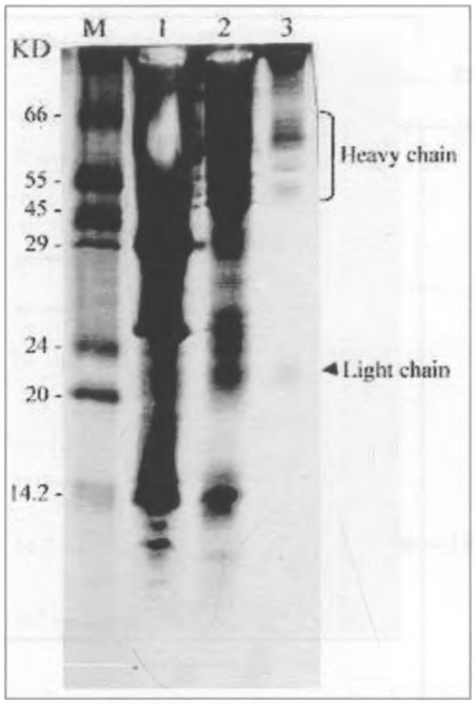


Fig.10. Gel electrophoresis profile of purified Immunoglobulin G

An ion exchange chromatography using DEAE-sepharose was used to isolate IgG antibodies from serum derived from DET and AQ immunized rabbits. The IgGs were analyzed by SDS-PAGE (5-20%). Lane M- Wide range molecular weight protein standards (Sigma); 1- Crude serum (10µg); 2-Saturated Ammonium Sulphate (SAS) precipitated antibodies (10µg); 3-Purified IgGs (6 µg) (both DET and AQ gave similar profiles)

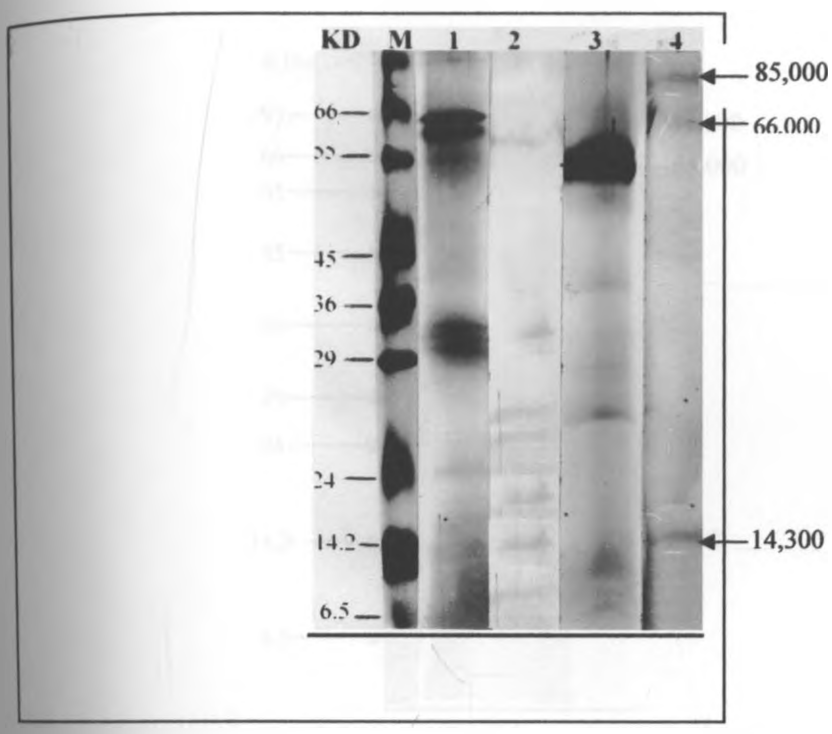


Fig. 11 Immunoblot analysis of the DET and AQ protein fractions (anti-DET and anti-AQ used as primary antibodies)

The proteins were separated on SDS-PAGE (5-20%) and then electrophoretically transferred onto a nitrocellulose membrane. The blots were incubated with antibodies against the DET antigens. Lane M- Molecular weight protein standards (Sigma); lane 1, DET fraction (10µg); lane 2, DET immunoblot; lane 3, AQ fraction (10µg); lane 4, AQ immunoblot (arrows).

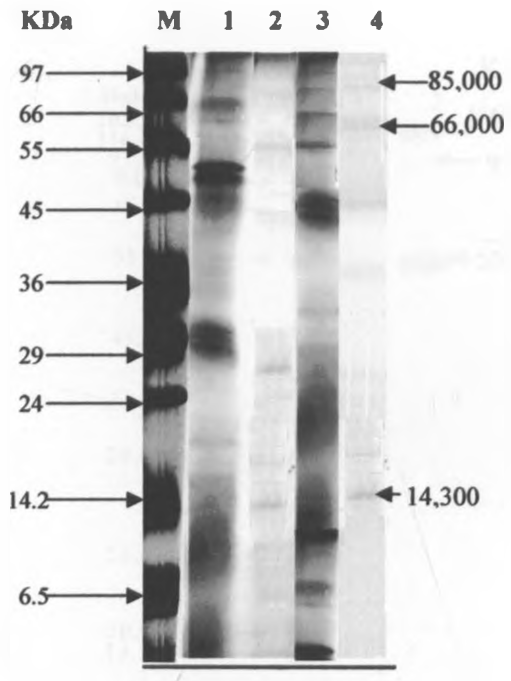


Fig.12. DET and AQ immunoaffinity purified antigens

Protein samples from the tsetse fly midgut were separated by SDS-PAGE (5-20%) and visualized by silver staining. Lane M- Molecular weight protein standards (Sigma); 1- DET fraction (10µg); 2-DET affinity purified antigens (7 µg); 3-AQ fraction (10µg); 4-AQ affinity purified antigens (5 µg).

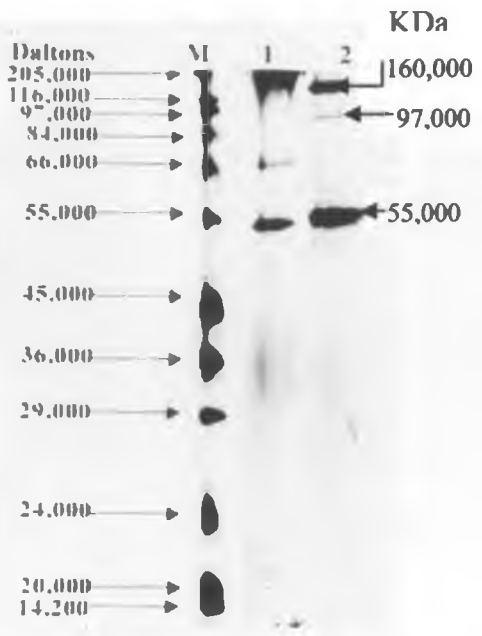


Fig. 13 Non-denaturing polyacrylamide gel electrophoresis of immunoaffinity purified DET and AQ antigens.

Affinity purified antigens from the tsetse fly midgut were separated by Native-PAGE (5-12%) and visualized by silver staining. Lane M- Wide range molecular weight protein standards (Sigma); 1- DET antigens (10µg); 2-AQ antigens (10µg).

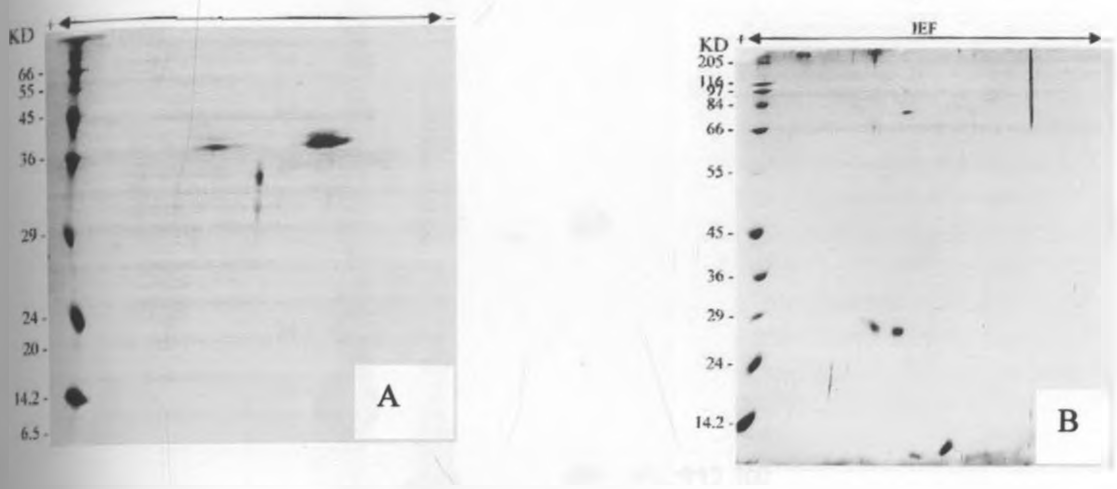


Fig. 14. 2-Dimensional gel electrophoresis of DET and AQ affinity chromatography purified antigens

The samples (10µg), DET (A) and AQ (B) were separated by IEF for the first dimension and after the run the rod gel was overlaid and separated onto a slab gel (SDS-PAGE, 5-20%).

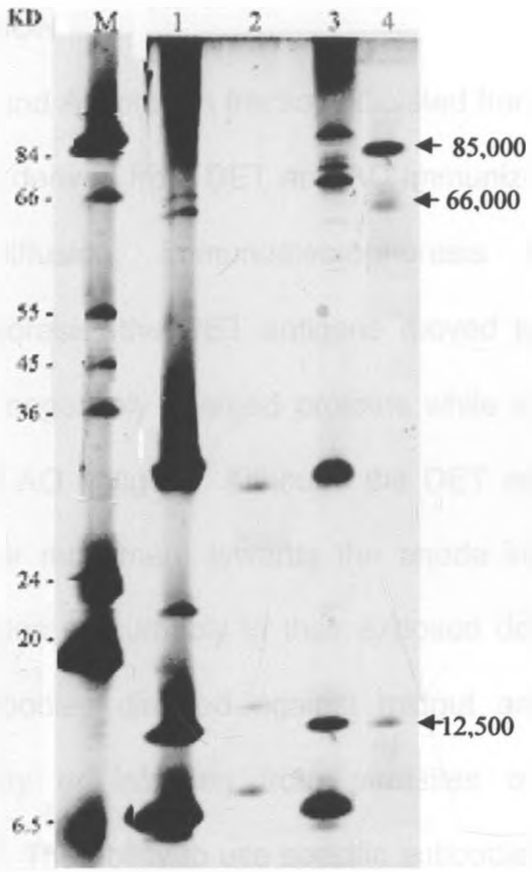


Fig. 15. Electrophoregram profiles of DET and AQ glycoprotein antigens

The isolated glycoproteins from the DET and AQ (previous section) were applied into CNBR-activated cellulose column with purified IgGs as the ligands. This was aimed at identifying the antigenic glycoproteins from the fractions. The eluted bound glycoproteins were analyzed by SDS-PAGE (5-20%) alongside the DET and AQ glycoproteins. Lane M-Molecular weight protein standards (Sigma); lane 1, DET glycoproteins (10 µg); lane 2, DET immunoaffinity eluent (7 µg); lane 3, AQ glycoproteins (10 µg); lane 4, AQ immunoaffinity eluent (7 µg).

4.2 DISCUSSION

The DET and AQ protein fractions isolated from the midgut were shown to react with serum derived from DET and AQ immunized rabbits by use of double radial immunodiffusion, immunoelecrophoresis and Western blot. On immunoelectrophoresis, the DET antigens moved towards the anode (+ve) a characteristic of negatively charged proteins while an opposite movement was observed for the AQ antigens. Although the DET antigens are expected to be hydrophobic, their movement towards the anode indicates presence of some hydrophilic moieties presumably in their exposed domains. Many studies have shown that antibodies directed against midgut antigens can reduce vector survival, fecundity or infection from parasites or viruses (Willadsen and Billingsley, 1996). The ability to use specific antibodies to inhibit important vector antigens will allow scientists to dissect and understand physiological pathways in which the antigen is involved.

The DEAE-sepharose ion exchange chromatography was successfully applied in isolating the immunoglobulin G from the crude serum (Fig. 10). The heavy chain and the light chain were resolved as polypeptide bands of M_r ~ 50,000-70,000 and 25,000 respectively. The purified IgGs were used in an affinity chromatography for the purification of antigenic proteins. The SDS-PAGE profile of the affinity purified antigens revealed antigens of M_r 64,500, 63,500, 28,200, 14,100 and 11,500 for the DET fraction while antigens of M_r. 85,000,

66,000 and 14,300 were enriched for AQ fraction. Similar results were observed with western blot analysis.

Polypeptides of similar molecular weight range have been used as candidates for vaccine development against ticks and mosquito. A commercially available anti-tick vaccine (TickGARD™) against *Boophilus microplus* (Willadsen *et al.*, 1995) is based on a midgut protein of molecular weight 86,000. Another protein of molecular weight 91,000 has also been identified as a possible anti-tick candidate. Further work has revealed antigenic proteins (M_r 50,000-95,000) within the midgut of the tick *Amblyomma variegatum* that causes reduction in reproductive capacity by 70% (Kinyua *et al.*, 2002).

Some of the antigens isolated by immunoaffinity chromatography had glycoprotein moieties. An SDS-PAGE of immunoaffinity purified glycoproteins revealed glycoprotein antigens of M_r 32,000, 14,100 and 11,500 for the DET fraction while glycoprotein antigens of M_r 85,000, 66,000 and 12,500 were revealed for the AQ fraction. The native Bm 86 antigen, a glycoprotein was found to induce immunity that reduced challenge to tick feeding success by 61% to 70 % and reduced reproduction capacity by 91 to 93% (Tellam *et al.*, 1992). The immunological role of glycoproteins is not yet certain. Willadsen and colleagues (1991) are of the opinion that the sugar moiety is not necessary for the protection while Lee and Opdebeeck (1991) think otherwise. Similarly, the role of carbohydrate moieties for the protection observed for this study is yet to be ascertained. Nevertheless, glycosylated lectin-like proteins have been shown to

stimulate transformation of blood-stream form trypanosomes into procyclic forms *in vitro* (Abubakar *et al.*, 2003). Therefore blocking activity of such molecules is crucial for the interference of trypanosome establishment in the vector. A midgut protein, *AgMucl*, an abundant, highly glycosylated protein on the luminal midgut epithelium isolated from *Anopheles gambiae* has been used to vaccinate mice. A significantly higher mortality from mosquitoes fed on immune mice as compared with control was observed (Lal *et al.*, 2002).. Moreover, since *Plasmodium* ookinetes bind to luminal midgut epithelium carbohydrate residues in order to penetrate the midgut (Zieler *et al.*, 1999), *AgMucl* has been suggested as a potential target antigen for transmission blocking vaccine (Lal *et al.*, 2002). The observed protective ability of the native Bm 91 antigen, in *B. microplus* ticks is thought to be shared between anti-polypeptide and anti-carbohydrate responses (Willadsen, 1997).

The 2-DE of the immunoaffinity purified antigens gave a more defined immunogenicity picture of the protein sub-units in both the DET and AQ fractions. Polypeptide spots of M_r 40,000, 38,000, 32,000 and 28,200 were predominant in the DET fraction while polypeptides of M_r 85,000, 66,000, 28,000, 26,000, 7500 and 6,000 were enriched for the AQ fraction. Some relatively low molecular weight proteins were also observed for the AQ fraction. The polypeptides could be subunits of a native protein. Similar profiles were observed for *Amblyomma variegatum* ticks after 2-DE analysis (Kinyua *et al.*, 2002). Notably, some vaccine candidates are shared among the haematophagous vectors. An example is

Angiotensin-converting enzyme (ACE) homologue found in *Drosophila melanogaster*, *Anopheles stephensi* and other insects where the enzyme seems to be involved in reproduction, and specifically enhancing male fertility (Wijffels *et al.*, 1996, Isaac *et al.*, 1999). An ACE homologue (Bm 91) has also been used as a vaccine candidate in *B. microplus* (Willadsen, 1997). As important homologues of ACE are discovered in insects and ticks it is likely that other immune targets will have homologues in many vector and non-vector insects.

An SDS-PAGE of the native DET and AQ immunoaffinity proteins revealed proteins of high molecular weight. Proteins of M_r 150,000, 66,000 and 53,000 were predominant for the DET fraction while proteins of M_r 160,000, 97,000 and 55,000 were predominant for the AQ fraction. The fact that numerous antigens were identified on denaturing SDS-PAGE suggests that the native proteins are composed of antigenic subunits. However, the relative contribution of the various subunits in protection could not be quantified in this study.

Controlling blood-sucking arthropods by immunization of hosts with antigens of arthropod tissues ("concealed" antigens) is gaining increasing attention (Willadsen and Kemp, 1988). The use of immunogens directed against internal antigens in the arthropod is appealing, since they are probably not introduced into the host during feeding and would avoid potential cutaneous hypersensitivity reactions in host tissue (Wikel, 1993). The most promising approaches to vaccine induction of anti-arthropod immunity may be digestive tract derived antigens because of their accessibility to antibodies in the

bloodmeal (Wong and Jackson, 1992; Wikel, 1993). Proteins which are essential to the life of the vectors include cell membrane receptors and components of regulatory system within the cells.

In ticks, molecules linked with receptor-mediated endocytosis of lysed blood-meal components have been used for an anti-tick vaccine. A recombinant vaccine against a gut protein has been developed (Tellam *et al.*, 1992). Antibodies to a membrane bound glycoprotein (Bm 86) bind to the gut digest cells and inhibit endocytosis.

Many researchers have reported on anti-mosquito immunity from immunization with midgut protein (Fall, 2002). Increases in mosquito mortality and decreases in mosquito fecundity were reported in some of these studies (Zieler *et al.*, 1999). When tsetse flies feed on immunized animals, a spectrum of immune factors (antibodies, lectins, complement, and immune effector cells, such as cytotoxic T lymphocytes, natural killer cells, eosinophils, and macrophages) are ingested, and all may act separately or synergistically to impair the insect homeostasis. Antibodies by themselves can bind to their antigen targets and could sterically hinder enzyme activity and/or the function of protein ion channels or could simply block pores, as is thought to be case with antibodies against peritrophins of the blowfly *Lucilia cuprina* (Wijffels *et al.*, 1999). There are numerous digestive enzymes within the tsetse midgut whose activity could have been interfered with in this study. In *Glossina* species, at least six proteolytic enzymes have been shown to be involved in the digestion of

bloodmeal (Cheeseman and Gooding, 1985). These include trypsin, trypsin-like enzymes, chymotrypsin-like enzymes, carboxypeptidases and aminopeptidases (Cheeseman and Gooding, 1985). Reducing the activity of these enzymes may interfere with the fly survival. In addition, it has been shown that antibody fragments can cross into the hemocoel and thereby have access to many other critical vector targets (Jacobs-Lorena and Lemos, 1995). Acting as a receptor for complement, antibodies can therefore direct membrane attack complexes to the surface of cells or they can direct lymphocytes toward the cell surfaces to which they are binding.

CHAPTER FIVE

RESULTS AND DISCUSSION

'FUNCTIONAL CHARACTERIZATION OF THE PROTECTIVE ANTIGENS'

5.0 RESULTS AND DISCUSSION

5.1 Expression of recombinant antigens

5.1.1 Total RNA isolation

A total RNA extraction kit (Qiagen, UK) was used according to the manufacturer's instructions. The extracted total RNA was resolved in a 1.2% agarose gel containing 0.3µg/ml ethidium bromide and visualized under UV. The RNA profile indicated two prominent bands representing 18S and 28S ribosomal RNA **(fig. 16, lanes 1 and 2).**

5.1.2 cDNA synthesis

A Creator™ Smart™ cDNA Construction kit (Clontech, BD Sciences) was used according to manufacturer's protocol. A smear of above 0.4 bp was observed **(Fig. 17, lane 2).**

5.1.3 Titer of plasmid libraries

The number of colonies **(Fig. 18)** was counted after incubating plates overnight at 37°C to determine the titer in colony forming units per milliliter (cfu/ml). The titer was calculated as follows:

Colony # Dilution A (1: 10^3) x 10^3 x 10^3 = cfu/ml

2000 x 10^3 x 10^3 = 2 x 10^9 cfu/ml

OR

Colony # Dilution B (1: 10^6)/plating volume x 10^3 x10^3 x 10^3 = cfu/ml

300/100 x 10^3 x10^3 x10^3 = 3 x 10^9 cfu/ml

The mean for the two calculations = 5 x 10^9/2 =2.5 x 10^9 cfu/ml

5.1.4 Immunoscreening of the transformants and Isolation of plasmid DNA from positive colonies.

A total of 23 and 17 colonies were initially identified as positive ones by anti-DET and anti-AQ respectively. Plasmid DNA was isolated from the positive colonies using FastPlasmid™ Mini kit (Eppendorf, Germany) according to manufacturer's instructions. The isolated plasmids were separated by 0.8% agarose gel electrophoresis containing 0.3 µg/ml ethidium bromide and visualized under UV **(Fig. 19)**. Out of the 23 colonies for anti-DET, 12 were distinct for anti-DET while 9 were distinct for the anti-AQ colonies.

5.1.5 PCR screening and selection of transformants for sequencing

The positive plasmids (3 for DET and 5 for AQ) were screened by PCR using M13 universal primers **(Fig. 20)**. Three and five plasmids for DET **(Fig. 20, lane 5, 6 and 12)** and AQ **(Fig. 20 lane 16, 18,19, 20 and 21)** which were 600 base pairs and above were selected for sequencing (see also **Fig. 21 and 22).**

5.2 Sequence analysis of the potential vaccine candidates

Only two and three cDNA sequences were considered for further analysis for both DET and AQ respectively. The rest as selected in the above section did not give meaningful sequences and were disregarded. The sequences **(Fig. 23)** were screened for vector sequence contamination with the VecScreen software (NCBI). The open reading frame (ORF) for each of the sequences was determined with ExPASY translation Tool. The ORF aminoacid sequences were analysed using the Basic Local Alignment Search Tool (BLAST) for similar

alignments within the *morsitans* GeneDB database. Global alignments (MultiAlign) were carried out for the ORFs of the cDNA sequences (Ks1-M13, Ks7-M13 and Ks2-M13, Ks4-M13, Ks8-M13 for DET and AQ respectively) with full length sequences (aminoacid) from *Glossina* GeneDB (best hits) and other protein databases **(Fig. 24-28)**. The ExPASY Scan Prosite was used to assign signatures to the ORF sequences **(Fig. 24, 25 and 27)** and SignalP **(Fig. 27b)** for signal peptides. The Transmembrane Hidden Markov Model (TMHMM) was used to reveal the transmembrane helices **(Fig. 24b).** Functions were assigned from signatures and similarity matches. Ks1-M13 was assigned a serine peptidase inhibitor signature, Ks2-M13, a trypsin signature, Ks7-M13 an aspartate-Glutamate racemace signature. The rest of the sequences (Ks4-M13, and Ks8-M13) were assigned functions from their similarity matches only. Ks1-M13 was predicted to have structural similarity with a putative protein Gmm 8438 in the Glossina geneDB. The putative protein is thought to be involved in oogenesis/larval development. Ks2-M13 was also predicted to have similarity with exocyst and golgi complex proteins (Fig. 26b,c). The recombinant protein Ks8-M13 was predicted to have a sulphotransferase/ribonuclease activity (**Table 4**).

Table 4: The table shows a summary of functional analysis and characterization of the predicted proteins from the sequenced cDNA

DET (2 antigens)	AQ (3 antigens)
1. **Ks1-M13 (GeneBank AC: EF 585239)** Inference: Serine peptidase inhibitor (antithrombin) Similarity: to Gmm 2766, putative protein E-value 7.4e-27 Mwt: 92,300 Daltons Function: Inactivates the complement and coagulation cascades in the bloodmeal Other attributes: Transmembrane with possible organic anion transport activity	1. **Ks2-M13 (GeneBank AC. EF585240)** Inference: Trypsin (lectizyme, proteolytic lectin, chymotrypsin-like precursor) Similarity: to Gmm-8438, putative protein, E-value 8.5e-26 Mwt: 58000 Daltons Function: Involved in bloodmeal digestion
2. **Ks7-M13 (GeneBank AC. EF 585242)** Inference: Asp-Glu racemase, E-value 0.3e Mwt 28, 300 Daltons Function: Asp-Glu racemase-Interconversion of L-glutamate /L-aspartate to D- Glu/Asp.	2. **Ks4-M13 (GeneBank AC. EF585241)** Inference: Exocyst/Golgi complexes Mwt 372000 Daltons Similarity: to Gmm 8438, putative protein Function: Involved in embryonic development/oogenesis and in regulation of epidermal growth factor receptor signaling, role on larval development. -Exocyst component section 10-affects secretory and basolateral plasma membrane proteins. -Golgi complex-Assembly and transportation of proteins to their destined locations
	3. **Ks8-M13 (GeneBank ACC. 585238)** Inference: Sulphotransferase and ribonuclease Mwt 34,000 Daltons Function: Sulphotransferase activity-sulfation of compounds e.g. amines, steroids, drugs. Sulfation is a detoxification pathway for many compounds. :Ribonuclease activity

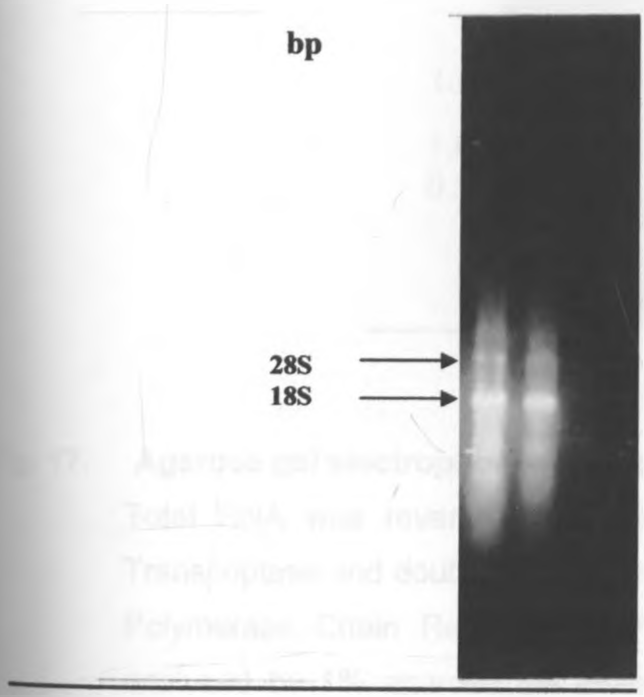


Fig. 16. Analysis of RNA from the midguts of *G. pallidipes*

Total RNA was extracted from twice-fed midguts of *G. Pallidipes* using RNeasy^{R} Mini kit according to manufacturer's instructions. The lanes shows the RNA (5µg) resolved on a 1.2% agarose gel containing 0.3 µg/ml ethidium bromide and visualized under UV.

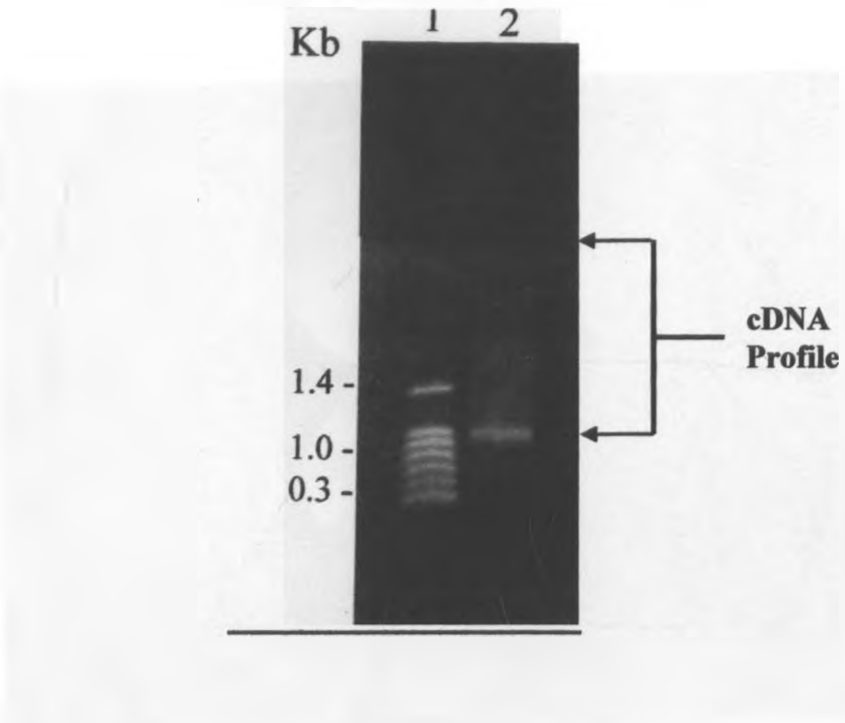


Fig. 17. Agarose gel electrophoresis of ds cDNA from the total RNA

Total RNA was reverse transcribed using PowerScript™ Reverse Transcriptase and double-stranded cDNA generated by Long-Distance Polymerase Chain Reaction (LD-PCR). Amplification reaction was analyzed by 1% agarose gel electrophoresis and ethidium bromide staining.

Lane M, Standard DNA marker

Lane 1– 1 kb DNA marker (Boehringer)

Lane 2– 5 µg cDNA

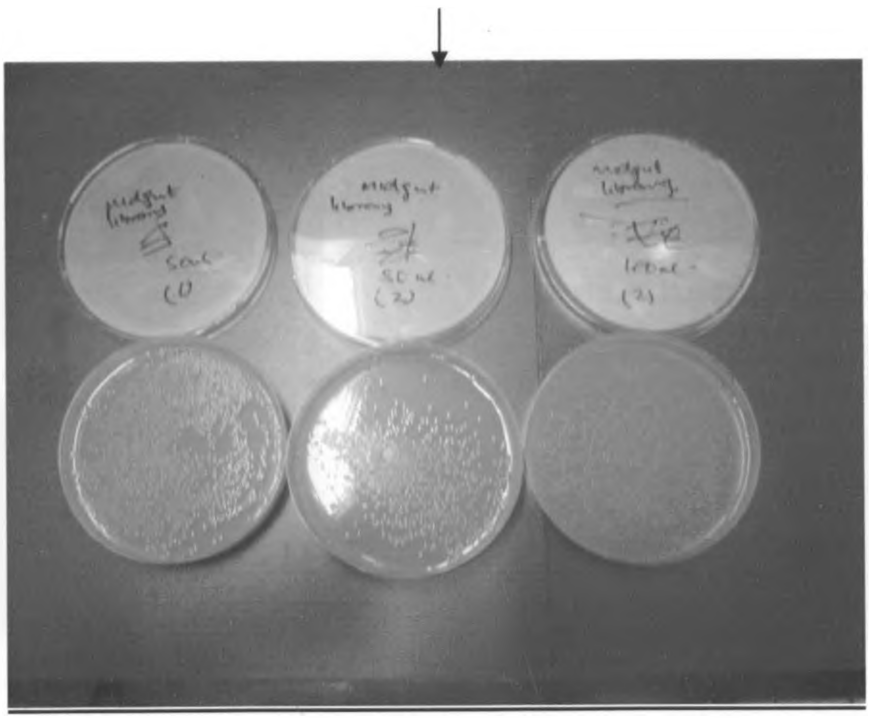


Fig.18. **Growth of transformed *E. coli* in LB/Cm medium**

Transformed *E.coli* were grown in LB medium having 30 µg/ml Chloramphenicol. The Plasmid free colonies did not grow in presence of the antibiotic. Various dilutions were made to get the titer of the plasmid library.

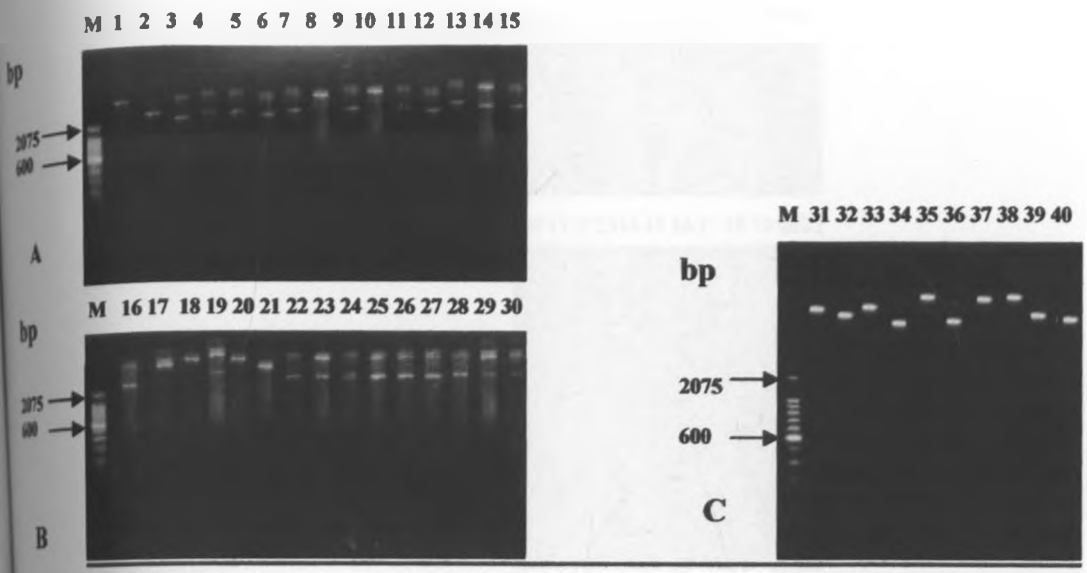


Fig. 19. Electrophoretic analysis of plasmid DNA

cDNA library was screened with the polyclonal antibodies against the DET and AQ fractions. 23 and 17 positive colonies were identified for DET and AQ respectively. Plasmid DNA was isolated from the colonies and analyzed by 1% agarose gel electrophoresis with 0.3 µg/ml ethidium bromide.

Lane M, Standard DNA marker

Lane 1-23 (A and B), 5 µg DET plasmid DNA

Lane 23-40 (B and C), 5 µg AQ plasmid DNA

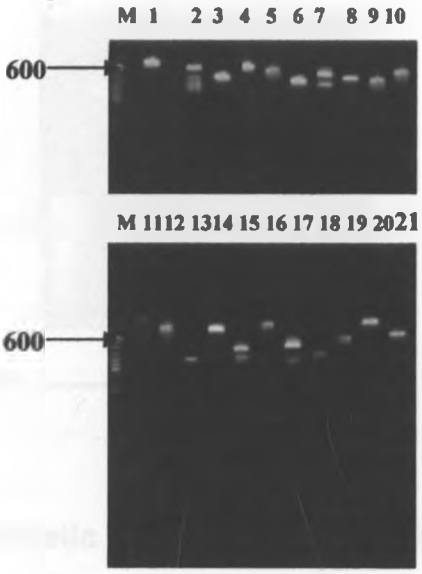


Fig. 20. PCR screening of the positive colonies using M13 universal screening primers

Twelve and 9 plasmid DNA positive on immunoscreening with anti-DET and anti-AQ respectively were screened by PCR using M13 primers to determine the size of inserts. The PCR products were analyzed by 1.2 % agarose gel electrophoresis with 0.3µg/ml ethidium bromide.

Lane M, Standard DNA marker

Lane 1-12, 5 µg DET plasmids PCR products

Lane 13-21, 5 µg AQ plasmids PCR products

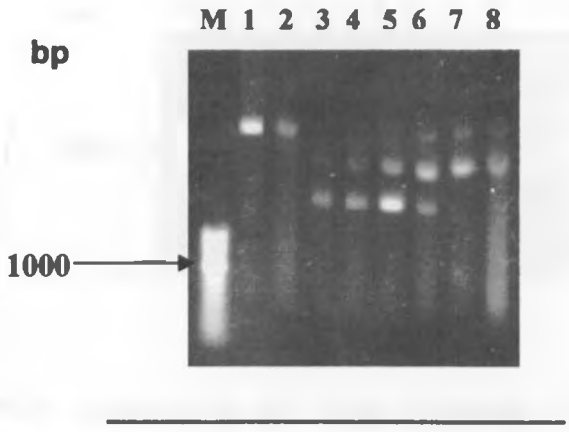


Fig. 21 **Electrophoretic analysis of plasmid DNA**

Eight plasmids were selected from the 21 plasmids screened by PCR (fig. 20). Three and five plasmids for DET and AQ respectively were analysed by 1% agarose gel electrophoresis with 0.3 µg/ml ethidium bromide.

Lane M, Standard DNA marker

Lane 2, 3 and 7- 5 µg DET plasmids

Lane 1, 4, 5, 6, and 8 -5 µg AQ plasmids

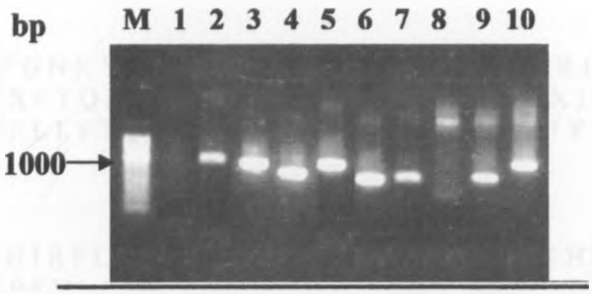


Fig. 22 PCR screening of nine plasmid DNA with M13 universal screening primers

The PCR was carried to determine the size of the inserts. Three and five plasmids were chosen for DET and AQ respectively. The PCR products were analyzed by 1.2 % agarose gel electrophoresis with 0.3µg/ml ethidium bromide.

Lane M, Standard DNA marker

Lane 1 Negative control

Lane 2 posive control

Lane 4, 5, 7-5 µg DET plasmids PCR products

Lane 3, 6, 8, 9, 10 - 5 µg AQ plasmids PCR products

Fig. 23 FASTA formats for the Nucleotide sequence translations of the candidate vaccine molecules

>Ks1-M13 (DET)
ASNSHDQDVFGNKYVHVHLLTGPARQTNGLERFFRAEGADRQXFFFF
FFFLFIKTLFIXFTQXFSLCVLQSFILVYIDFSAXLFNYHSLLVTFTVMS
HIDMLTVTIHFLLFTQKLARIGISVAIGTANRFIYLIAHTAISVGLAFFV
MGHPSQADQ

>Ks2-M13 (AQ)
XQSTVPDICPGIRPLRPGRNDESDVQIRKVTGKHQQIVHEKYGGDVGP
NDIGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYGWG
RDNSGFLPNILNTLDVNIIGYEECKKALPSDAPLDPVNICSYTPDATDG
ACNGDSGGPMVRVTPDGTELVGIVSWGYVPCASTTTPSIYSWTAAFG
KWIEESIENYVVPAHLLX

>Ks4-M13 (AQ)
VFQLFTYSRCRFFASSVSNFGSFSHFCXRTVTFIGGIGCPSATISSXTM
NEPSYXRDSGGPISEPARCSACITCIEFYGRCIPQSTYSRIRXAIXRVRT
EXMHDYSPTHARQIRTRAESGVYYSQERVNERPQYSKHAYFHAQSAV
YINAVGMTGFLTGNRSLCATQLLRVSSIIRLPRLYALSFPLMTSSRLTL
TKGTSLAGLKEFAINNTRFLLQPEEEEHHLDRRILSPHHDHVRRSVATV
HVPQMNDLKALSYLQRNQYKVVEWKDRXXHHXTSHLD

>Ks7-M13 (DET)
RDTRMTXARIFTLLGRVDHRRAFFRCGNPSTSRGHDLLGKSYDHVYL
HWGVANQTQGVEFFLRAQELDTPSAGPFFIXFQVVNSGAFYLGGTIM
FAGPFTIFHTGADRLFSVNFQFSFIFIHIDRHEPISDMLTESITFSFLRTK
IHKDRDKCCRRNRKPVHISDCAHSHIPRIRLIGDGPSPPRGSKVQXRK
XRKHRNSS

>Ks8-M13 (AQ)
WPFPGLALACACSVFSACVLRLAVVPPFALGAVVCPLPFALLPRVLGR
VFLRLPCLLSRFLRVLCASRLQLARHLSLRPAGLSARGSGSFFLSPLAR
WPFLSGFVLVRLASVLWCHFCLVALVTLRASGSRLSLACCRPRSRLCV
FPRLASLMVALPWVFFPLGLLAFPGLCLFSGLLWAFWAAAAIRAALFSV
RWCVLWVGGCFSCCCPFFPGPLPSMGSPPXVAEXGSPLWGRCPRCAFL
VPAVVWGGSFFLXPFPXXX

Fig. 24 a: Sequence alignments of Ks1-M13 (DET) with putative proteins Gmm 2766 from the *Glossina* geneDB (E. value= 7.4e-27)

Amino acid sequence alignments of Ks1-M13 (DET) cDNA was aligned with Gmm-2766 putative protein from the *morsitans* GeneDB. The ExPASY Scan prosite was used to determine the signature to Ks1-M13 protein. The protein was inferred to have a serine peptidase inhibitor activity (putative CG31758-PA) a function inferred from its signature (NQIYEPVCGTDGNTYPNPCXFLC, 77-101 aa) and also through GeneDB BLAST analysis. It was also predicted to be integral to membrane and having organic anion transport activity.

```
           1       10        20        30        40        50        60
           |--------+---------+---------+---------+---------+---------|
Ks1        XSRRYRTYARFFGHYGREFNLAVNFQPTSLKFGVHAYIXKVIMKYFCAFFIFFIVLLIRL
Gmm-2766
Consensus  ............................................................

           61      70        80        90       100       110       120
           |--------+---------+---------+---------+---------+---------|
Ks1        AGMAHHEEGQANRDGCVCNQIYEPVCGTDGNTYPNPCXFLCEQKKMYRHGQHIYMAHDGE
Gmm-2766     MAHHEEDEASGDGCVCNRIYEPVCGTDGNTYPNPCEFLCVQRKMYRIGQHIDMAHDGQ
Consensus  ..MAHHEEd#AnrDGCVCNrIYEPVCGTDGNTYPNPCeFLCeQrKMYRhGQHIdMAHDG#

           121     130       140       150       160       170       180
           |--------+---------+---------+---------+---------+---------|
Ks1        CYXQRMIIEQSRGKVYIHKYERLXNAQXKLLCKLNKKRLNKQKKKKKKKXLPVGPLRPKE
Gmm-2766   C
Consensus  C...........................................................

           1881
           ||
Ks1        SF
Gmm-2766
Consensus  ..
```

Fig. 24b. Transmembrane analysis for Ks1-M13

This graph, taken from the output of Transmembrane Hidden Markov Model (TMHMM) describes the location of predicted segments, their predicted topology and reliability of prediction. The pink line is the probability that a certain position is outside of the cell, the blue line is the probability it resides in the cytoplasmic side of the membrane and the red line is the probability that this residue is in the membrane. TMHMM was used to analyze the Ks1-M13 protein for trans-membrane helices. The protein was predicted to have transmembrane helix between amino acid 41-63 (IMKYFCAFFIFFIVLLIRLAGM), Hits by PS00282 (Kazal serine protease inhibitors family). The outside of the helix is between 64 and 187 aminoacids while the inside is between amino acid 1 to 40 as shown below

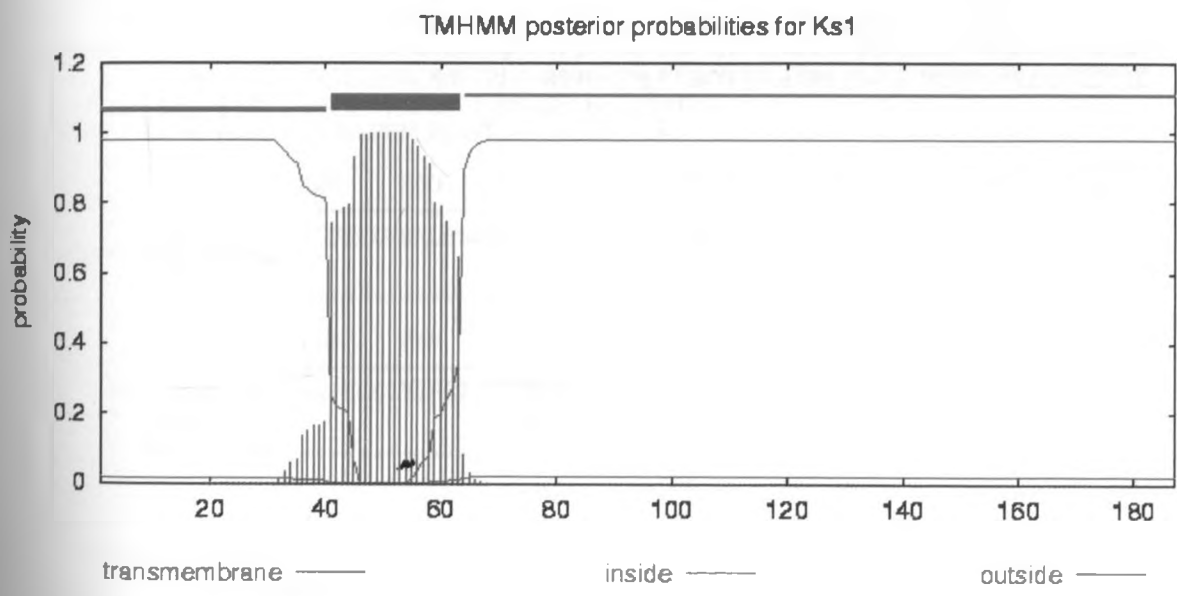


Fig. 25 (a) **Sequence alignments of Ks2-M13 (AQ) with putative proteins (Gmm 3330) from the *Glossina* geneDB** (E. value= **8.5e-26)**

(a) Amino acid sequence of Ks2-M13 cDNA was aligned with Gmm-3330 putative protein from the *morsitans* GeneDB. The ExPASY Scan prosite was used to determine the signature to Ks2-M13 protein. The trypsin domain (Hits by PS50240 **TRYPSIN_DOM)** active site is between 37-201aa.

```
                1       10        20        30        40        50        60
                |-------+---------+---------+---------+---------+---------|
Ks2-M13
Gmm-3330        RFLAEFALFVASVSAAWLRAFAKPSFPERRITNGHEAEKSEAPFIVSLKTTIHFCGGSII
Consensus       ............................................................

                61      70        80        90       100       110       120
                |-------+---------+---------+---------+---------+---------|
Ks2-M13           XQSTVPDICPGIRPLR-----PGRNDESDVQIRKVTGKHQQIVHEKYGGDVGPNDIGL
Gmm-3330        AENWVLTAGHCLGLDEFEIVAGLHSRNDESDVQIRKVTGKHQQIVHEKYGGDVGPNDIGL
Consensus       ..nqsltadhClGirelr.....hgRNDESDVQIRKVTGKHQQIVHEKYGGDVGPNDIGL

                121     130       140       150       160       170       180
                |-------+---------+---------+---------+---------+---------|
Ks2-M13         IYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYGWGRDNSGFLPNILNTLDVNI
Gmm-3330        IYVDKPFNLNALTRDGTSCSGQGQFANRQI
Consensus       IYVDKPFNLNALTRDGTaasaqg#lanrqi..............................

                181     190       200       210       220       230       240
                |-------+---------+---------+---------+---------+---------|
Ks2-M13         IGYEECKKALPSDAPLDPVNICSYTPDATDGACNGDSGGPMETVRVTPDGTELVGIVSWG
Gmm-3330
Consensus       ............................................................

                241     250       260       270     278
                |-------+---------+---------+-------|
Ks2-M13         YVPCASTTTPSIYSWTAAFGKWIEESIENYVVPAHLLX
Gmm-3330
Consensus       ........................................
```

Fig 25(b) A match of Ks2-M13 with proteolytic lectin, AC. AAY59001 (*Glossina austeni*) Length=274, Expect = 1e-52

```
                1        10        20        30        40        50        60
                |--------+---------+---------+---------+---------+---------|
     Ks2-M13
  prot_lectin   MKFFAVFALCVASVSAANLDAIAKPGFPAGRIINGHEAEKGEAPFIVSLKAGKGHFCGGS
    Consensus   ............................................................

                61        70        80        90       100       110       120
                |--------+---------+---------+---------+---------+---------|
     Ks2-M13              XQSTVPDICPGIRPLRPGRNDESDVQIRKVTGKHQQIVHEKYGGDVGPND
  prot_lectin   IIAENWVLTAGHCLIFDEFEIVAGLH-SRNDESDVQIRKVTGKHQQIVHEKYGGGVGPND
    Consensus   ..........gqcl!fDeceg!agLr.gRNDESDVQIRKVTGKHQQIVHEKYGGdVGPND

                121     130       140       150       160       170       180
                |--------+---------+---------+---------+---------+---------|
     Ks2-M13    IGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYGWGRDNSGFLPNILNTLD
  prot_lectin   IGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYGWGLDNSGFSPNILNTLD
    Consensus   IGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYGWGrDNSGFlPNILNTLD

                181     190       200       210       220       230       240
                |--------+---------+---------+---------+---------+---------|
     Ks2-M13    VNIIGYEECKKALPSDAPLDPVNICSYTPDATDGACNGDSGGPMETVRVTPDGTELVGIV
  prot_lectin   VNIIGYEECKNALNSDAPLDPVNICSYTAGAIDGACNGDSGGPM--VRITPDGTELVGIV
    Consensus   VNIIGYEECKnALnSDAPLDPVNICSYTadAiDGACNGDSGGPM..VR!TPDGTELVGIV

                241     250       260       270       281
                |--------+---------+---------+---------+|
     Ks2-M13    SWGYVPCASTTTPSIYSWTAAFGKWIEESIENYVVPAHLLX
  prot_lectin   SWGYQPCASTTMPSVYTWTSAFDKWIEDSIENYAQLL
    Consensus   SWGYqPCASTTmPS!YsWTaAFdKWIE#SIENYaqla....
```

Fig 25(c) A match of Ks2 with lectizyme, AC AAM82602 [*Glossina fuscipes fuscipes*] Length=274, Expect = 3e-51

```
                1       10        20        30        40        50        60
                |--------+---------+---------+---------+---------+---------|
     Ks2-M13
   lectizyme    MKFFAVFALCVASVSAANLDAIAKPGFPAGRIINGHEADKGEAPFIVSLKAGKGHFCGGS
   Consensus    ............................................................

                61       70        80        90       100       110       120
                |--------+---------+---------+---------+---------+---------|
     Ks2-M13              XQSTVPDICPGIRPLRPGRNDESDVQIRKVTGKHQQIVHEKYGGDVGPND
   lectizyne    IIAENWVLTAGHCLIFDEFEIVAGLH-SRNDESDVQIRKVTGKHQQIVHEKYGGGVGPND
   Consensus    ..........gqcl!fDeceg!agLr.gRNDESDVQIRKVTGKHQQIVHEKYGGdVGPND

                121     130       140       150       160       170       180
                |--------+---------+---------+---------+---------+---------|
     Ks2-M13    IGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYGWGRDNSGFLPNILNTLD
   lectizyne    IGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGKGKLYGWGLDNSGFSPNILNTLD
   Consensus    IGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGeGKLYGWGrDNSGF1PNILNTLD

                181     190       200       210       220       230       240
                |--------+---------+---------+---------+---------+---------|
     Ks2-M13    VNIIGYEECKKALPSDAPLDPVNICSYTPDATDGACNGDSGGPMETVRVTPDGTELVGIV
   lectizyne    VDIIGYEECKNALNSDDPLDPVNICSYTAGAIDGACNGDSGGPM--VRITPDGTELVGIV
   Consensus    V#IIGYEECKnALnSDaPLDPVNICSYTadAiDGACNGDSGGPM..VR!TPDGTELVGIV

                241     250       260       270       281
                |--------+---------+---------+---------+|
     Ks2-M13    SWGYVPCASTTTPSIYSWTAAFGKWIEESIENYVVPAHLLX
   lectizyne    SWGYQPCASTTMPSVYTWTSAFDKWIEDSIKNYAQLL
   Consensus    SWGYqPCASTTmPS!YsWTaAFdKWIE#SIeNYaqla....
```

Fig 25(d) A match with chymotrypsin-like serine protease precursor AC AAF91345 [*Glossina morsitans morsitans*] Length=276, Expect = 7e-61

```
                1       10        20        30        40        50        60
                |-------+---------+---------+---------+---------+---------|
          Ks2-M13
nsser_protease  MKLFVAIAALVIACASAASLDGIARPGFPEGRIINGLPATKGQAPFIVSLKSGSHFCGGS
      Consensus ............................................................

               61       70        80        90       100       110       120
                |-------+---------+---------+---------+---------+---------|
          Ks2-M13       XQSTVPDICPGIRPLRPG---RNDESDVQIRKVTGKHQQIVHEKYGGDVGPNDI
nsser_protease  IIDEHWVLTAAHCLTKSQFQLVAGLYERSDESDVQIRNVNGKQFIFTHEIYGGNVGPHDI
      Consensus ......vqsaahdickgqrqLraG...RnDESDVQIRnVnGKqqqitHEiYGG#VGPnDI

               121      130       140       150       160       170       180
                |-------+---------+---------+---------+---------+---------|
          Ks2-M13  GLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYGWGRDNSGFLPNILNTLDV
nsser_protease  GLSIVEEAFDLNACLVMDLLPLPALTCLLANMKALAVVNS-GWGRDNSGSLPNTLQTLEV
      Consensus GLiiV#eaF#LNAclrddlaalaalncllanmealaegnl.GWGRDNSGfLPNiL#TL#V

               181      190       200       210       220       230       240
                |-------+---------+---------+---------+---------+---------|
          Ks2-M13  NIIGYEECKKALPSDAPLDPVNICSYTPDATDGACNGDSGGPMETVRVTPDGTELVGIVS
nsser_protease  DIIGYTECKAAVPLDAPLADVNICSYTAGTKDGACNGDSGGPL--VKNTKGGYELVGLVS
      Consensus #IIGYeECKaAlPlDAPLadVNICSYTadakDGACNGDSGGP$..VrnTkdGtELVGiVS

               241      250       260       270       280
                |-------+---------+---------+---------|
          Ks2-M13  WGYVPCASTTTPSIYSWTAAFGKWIEESIENYVVPAHLLX
nsser_protease  WGYVGCASTQMPSIYTSVASYKQWIADTIAAYKN
      Consensus WGYVgCASTqmPSIYsstAa%gqWIa#sIaaYkn......
```

Fig. 26(a) Sequence alignments of Ks4-M13 (AQ) with putative protein, CG11101 (Gmm 8438) (E. value 0.9996) from the *Glossina* geneDB and a AJ547811 protein derived from TrEMBL (AC P42519)

Amino acid sequence of Ks4-M13 cDNA was aligned with Gmm-8438 putative protein from the *morsitans* GeneDB. The molecular function is predicted to use protein binding and the biological processes are: branched duct epithelial cell fate determination (sensu Insecta), embryonic development, epidermal growth factor ligand processing, mesoderm development, oogenesis (sensu Insecta) peripheral nervous system development (MultAlign alignment not shown)

Fig. 26(b) Ks4 sequence match with Exocyst component section 10 GA20365-PA (*Drosophila pseudoobscura*).

This family contains the Sec10 component (approximately 650 residues long) of the eukaryotic exocyst complex, which specifically affects the synthesis and delivery of secretory and basolateral plasma membrane proteins.

```
                1       10        20        30        40        50        60
                |-------+---------+---------+---------+---------+---------|
  Ks4-M13       NADMSTEYIETLWLTMEQEIVGTFPAMSTVERQIIDSCVAELKSVRDTLKACVDFGMHQL
  Ecosyst       NADMSTEYIETLCQTMEQEIAGTFPCTSSVERQLLDSCLTELKTVRDALKATVDFGMQQL
Consensus       NADMSTEYIETLcqTMEQEIaGTFPamSsVERQiiDSClaELKsVRDaLKAcVDFGMqQL

                61      70        80        90       100       110       120
                |-------+---------+---------+---------+---------+---------|
  Ks4-M13       RSSAIKPRLHPWVDQYLNYKHHLSEEELAAYEAGETFVQYLIVQIDGLLSSFKLILTVRN
  Ecosyst       RSSAIKPRLNPWINKFLSYSHNLTEEELAIYEAEETFVQFFIVHLDGLLNSFKTALSPRN
Consensus       RSSAIKPRLnPW!#q%LnYkHnLsEEELAaYEAeETFVQ%lIVqiDGLLnSFKlaLspRN

                121     130       140       150       160       170       180
                |-------+---------+---------+---------+---------+---------|
  Ks4-M13       YDALVSILATELTSRLERAIKKCTFNRLGGLVLDQEIRALGAYLASVTSWSVRDKMTRLT
  Ecosyst       YDALVSILATEVTIQLERAIKKCSFNRLGGLVLDQEVRALGTYLTGATSWSVRDKMTRIS
Consensus       YDALVSILATElTirLERAIKKCsFNRLGGLVLDQE!RALGaYLagaTSWSVRDKMTRis

                181     190       200       210       220       230   236
                |-------+---------+---------+---------+---------+-----|
  Ks4-M13       QIATILNLEKVSELNEYWDPENSSESPSWRITPNEVRAILTLRTDFRMEEIKRLRF
  Ecosyst       QIATVLNLDKVSELSEFWSPDNNKEMPSWHLTPNEVRAILALRIDFRIEDIKRLQL
Consensus       QIAT!LNL#KVSELnE%WdP#NnkEmPSWriTPNEVRAILaLRiDFRiE#IKRLrl
```

Fig. 26(c) A match with Golgi complex component 4 Length=776
Score = 358 bits (919), Expect = 1e-97

```
                1       10        20        30        40        50        60
                |-------+---------+---------+---------+---------+---------|
Ks4-M13         NADMSTEYIETLWLTMEQEIVGTFPAMSTVERQIIDSCVAELKSVRDTLKACVDFGMHQL
Golgi           NADISTEYIETLCQTMEQEIAGTFPQTTQVERQMLDSCLTELKAVRDALKATVDFGMQQL
Consensus       NADiSTEYIETLcqTMEQEIaGTFPamsqVERQiiDSClaELKaVRDaLKAcVDFGMqQL

                61      70        80        90        100       110       120
                |-------+---------+---------+---------+---------+---------|
Ks4-M13         RSSAIKPRLHPWVDQYLNYKHHLSEEELAAYEAGETFVQYLIVQIDGLLSSFKLILTVRN
Golgi           RSSVIKPRLNPWINQFLNYSHNLNEEELAAYEAGETFVQFFIVQLDGLLNSFKNSLSPRN
Consensus       RSSaIKPRLnPW!#Q%LNYkHnLnEEELAAYEAGETFVQ%lIVQiDGLLnSFKniLspRN

                121     130       140       150       160       170       180
                |-------+---------+---------+---------+---------+---------|
Ks4-M13         YDALVSILATELTSRLERAIKKCTFNRLGGLVLDQEIRALGAYLASVTSWSVRDKMTRLT
Golgi           YDALVSILATEVTIQLERAIKKISFNRLGGLVLDQEVRALGSYLTGATSWSVRDKMTRIS
Consensus       YDALVSILATElTirLERAIKKcsFNRLGGLVLDQE!RALGaYLagaTSWSVRDKMTRis

                181     190       200       210       220       230   236
                |-------+---------+---------+---------+---------+-----|
Ks4-M13         QIATILNLEKVSELNEYWDPENSSESPSWRITPNEVRAILTLRTDFRMEEIKRLRF
Golgi           QIATLLNLDKITELSEYWNPENNKEMSSWHLTPNEVRTFLTLRNDFRIEDIKRLQL
Consensus       QIATiLNL#K!sELnEYW#PENnkEmpSWriTPNEVRaiLTLRnDFRiE#IKRLrl
```

Fig. 27 (a) Sequence alignments of Ks7-M13 (DET) with an Aspartate Glutamate racemase protein (YP_613117) from the SwissProt Database

Amino acid sequence alignments of Ks7 cDNA was aligned with a Glutamate racemace, YP_613117 from the SwissProt database. The ExPASY Scan prosite was used to determine the signature (Hits by PS00923, (**VANQTQG**) aa 67-75) **ASP_GLU_RACEMASE_1.** Expect = 0.3

```
                1       10        20        30        40        50        60
                |--------+---------+---------+---------+---------+---------|
  Ks7-M13                          RDTRMTXARIFTLLGRVDHRRAFFRCGNPSTSRGHDLLGKSYD
YP_613117  MAVGIFDSGLGGLTVLDAAQKRLPDVDFLYYGDNSHAPYGVRDAEDIYELTHKAVHDMWD
Consensus  ..................aaqrrlpardFlllGrndHaragfRda#ditergHdalgdnwD

                61       70        80        90       100       110       120
                |--------+---------+---------+---------+---------+---------|
  Ks7-M13   HVYLHWGVANQTQGVEFFLRAQELDTPSA----GPFFIXFQVVNSGAFYLGG-----TIM
YP_613117   RGCNLVILACNTASAAALRRMQEAGVPEGKRVLGVFVPLIEALTERQWGDNSPPREVAVK
Consensus   rgcnhwglAn#TagaaalrRaQEadtPea....GpFfili#alnerafgdng.....a!k

                121     130       140       150       160       170       180
                |--------+---------+---------+---------+---------+---------|
  Ks7-M13   FAGPFTIFHTGADRLFSVNFQFSFIFIHIDRHEPISDMLTESITFSFLRTKIHKDRDKCC
YP_613117   HVALFATPATVASRAFQRELAFRAIGVDVEAQACGGVVDAIEEGDMILAEALVKSHVDAL
Consensus   haalFaifaTgAdRaFqr#laFraIg!d!#aqacggdmdaeeegdmiLaeaihKdrddac

                181     190       200       210       220       230       240
                |--------+---------+---------+---------+---------+---------|
  Ks7-M13   RRNRKPVHISDCAHSHIPRIRLIGDGPSPPRGSKVQXRKXRKHRNSSX
YP_613117   LRKMPRPEAAILGCTHYPLMEDVFQKALGPDVQVFSQGRLVADSLAHYLERRPEMIGGGN
Consensus   rRnrkrpeaadcacsHiPrird!g#galgPrgqkfqqrrlradrnahy.............

                241     250       260       269
                |--------+---------+--------|
  Ks7-M13
YP_613117   AGYVTTGMGNRVSSRATQFLRREITFEAA
Consensus   ............................
```

Fig. 27 (b) Analysis for signal peptides from the Ks7-M13 aminoacid sequence

This graph taken from the output of SignalP signal prediction tool, describes the possibility of a signal peptide. These predictions were made for the Ks7-M13 midgut cDNA sequence.

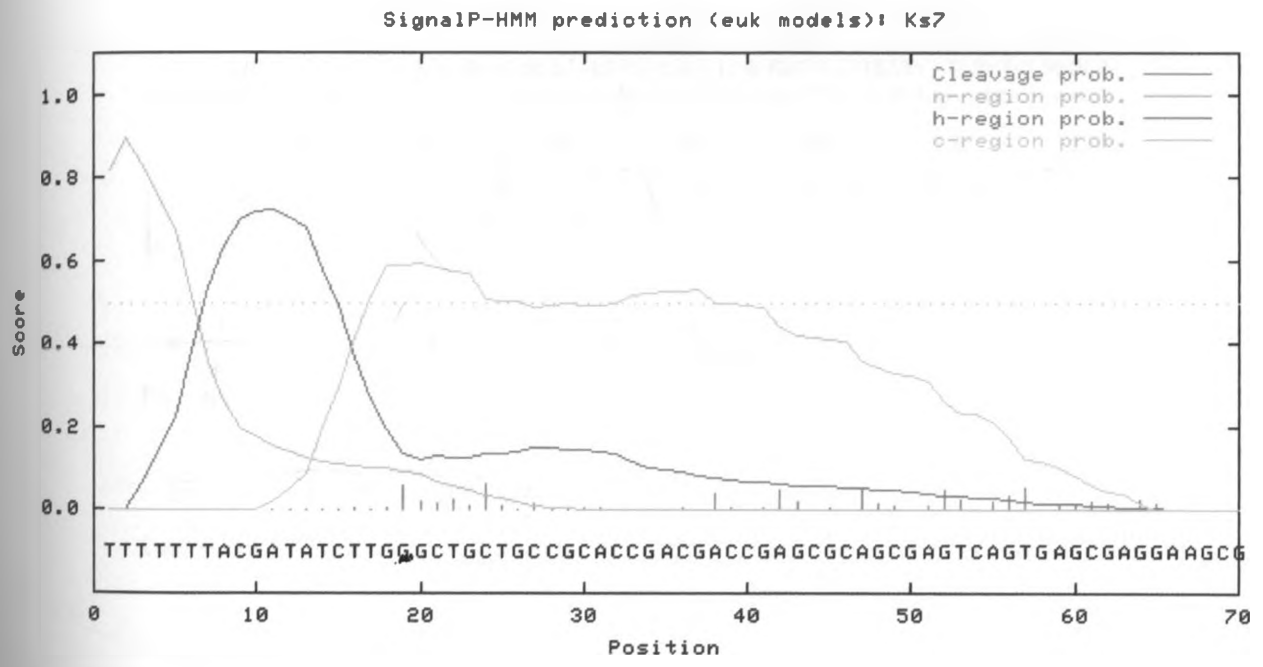


Fig. 28 (a) Sequence alignments of Ks8-M13 (AQ) with a putative protein, CG1135 (Gmm 0400) from the *Glossina* GeneDB database (E. value= 0.9).

The function of Gmm 400 is not yet known (Glossina geneDB). The analysis of this study did not ascribe any function to the protein. However, 'b' and 'c' matches below are suggestive of the possible functions of Ks8-M13 and as well as Gmm-400

```
             1       10        20        30        40        50        60
             |--------+---------+---------+---------+---------+---------|
Ks8-M13      WPFPGLALACACSVFSACVLRLAVVPPFALGAVVCPLPFALLPRVLGRVFLRLPCLLSRF
Gmm400                        SITGVGIAPEFD-GQTLAVLRGRLVRYLMRSKEISFGRDAKDC
Consensus    ..................c!lrla!aPeFa.GatlapLrgaLlrrl$rrkeirlgrdakrc

             61      70        80        90       100       110       120
             |--------+---------+---------+---------+---------+---------|
Ks8-M13      LRVLCASRLQLARHLSLRPAGLSARGSGSFFLSPLARWP-FLSGFVLVRLASVLWCHFCL
Gmm400       IVDVDLSLEGPATKISRRQGTIKLRSNGDFFIANEGRRPLFIDGVPLLTGNKTRLANNCV
Consensus    irdldaSreqlArhiSrRqagikaRgnGdFFianeaRrP.FidGfpLlrgaktrlannCl

             121     130       140       150       160       170       180
             |--------+---------+---------+---------+---------+---------|
Ks8-M13      VALVTLRASG--SRLSLACCRPRSRLCVFPRLASLMVALPWVFFPLGLLAFPGLCLFSGL
Gmm400       VEIAGLRFVFLVNYELINAIRHESAKTISPLN
Consensus    VaiagLRasg..nreliaacRhrSalc!fPrn..........................

             181     190       200       210       220       230       240
             |--------+---------+---------+---------+---------+---------|
Ks8-M13      LWAFWAAAIRAALFSVRWCVLWVGGCFSCCCPFPGPLPSMGSPPXVAEXGSPLWGRCPRC
Gmm400
Consensus    ............................................................

             241     250       259
             |--------+---------|
Ks8-M13      AFLVPAVVWGGSFFLXPFP
Gmm400
Consensus    .........-.........
```

Fig 28(b) **A match with sulphotransferace (E. value 0.05) Sulphotransferases (STs) exist as a family of functionally related forms which are involved in the sulphation of a diverse range of compounds including biogenic amines, steroids, hormones, bile acids**

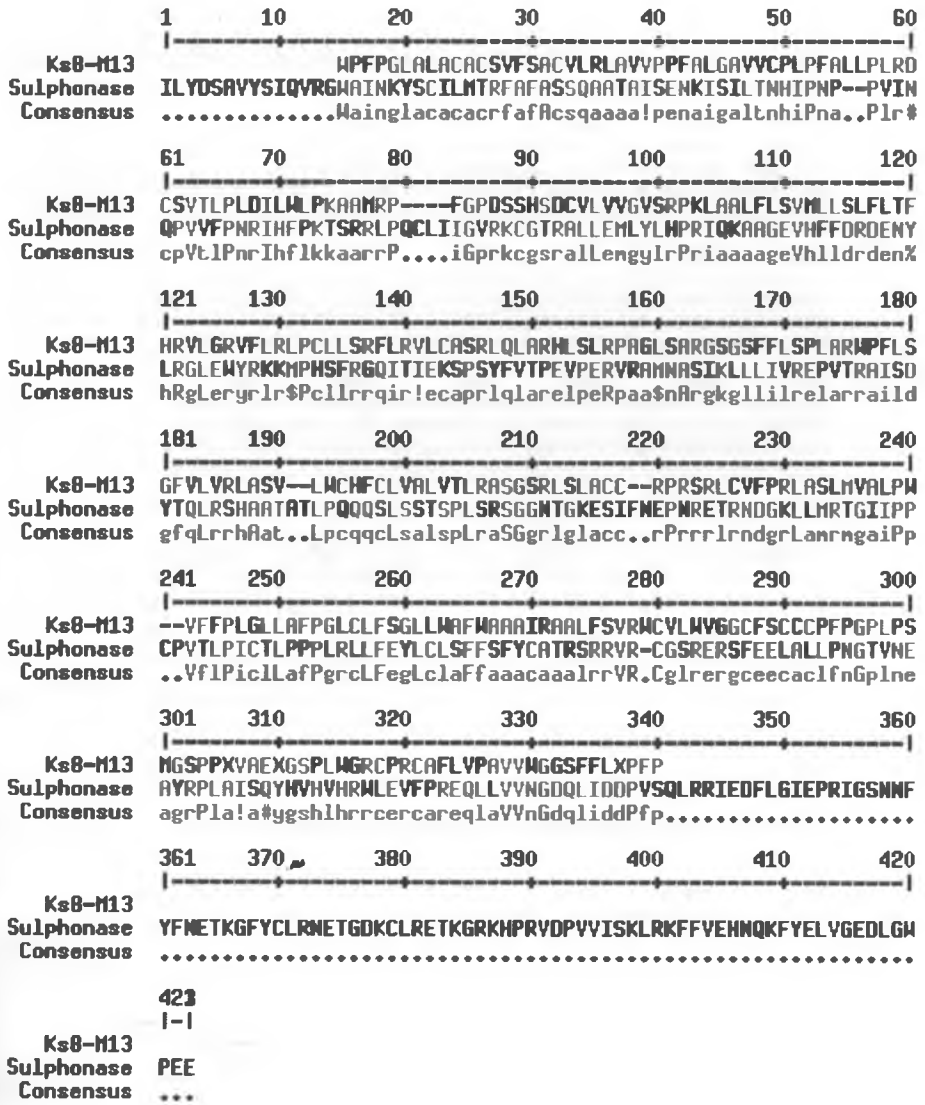
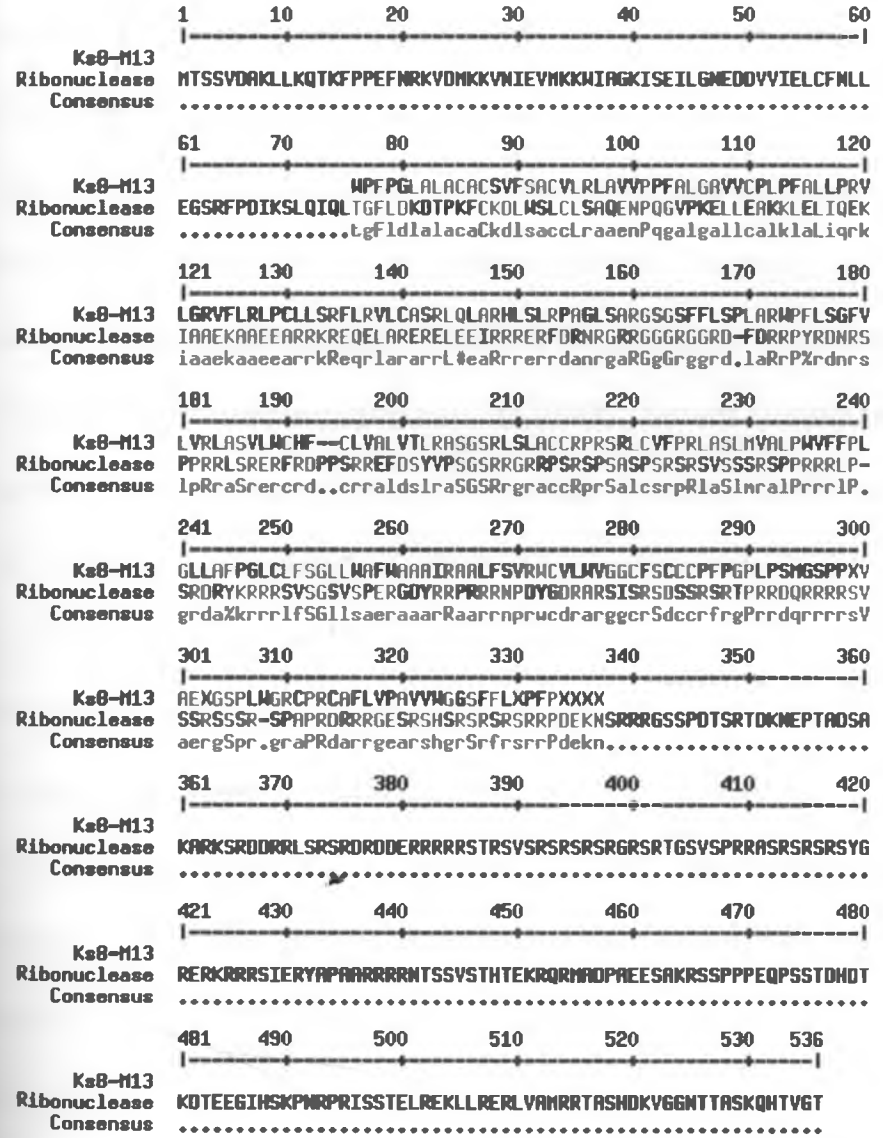


Fig. 28 (c) A match with a "Ribonucleases G and E [Translation, ribosomal structure and biogenesis]; COG1530" E- value 2e-04 involved in biological processes which include, endonucleolytic mRNA decay and mRNA breakdown.



5.3 DISCUSSION

In this study, molecules that could be critical to the biology of the tsetse fly have been identified. The putative functions of the vaccine candidates were inferred from DNA and protein databases. An example is the Sanger *Glossina* GeneDB database which is an invaluable resource of information on *Glossina* sequence analysis. The databank has information from a concerted effort of the International *Glossina* Genomics Initiative (IGGI). Currently, a total of 21,427 Expressed Sequence Tags (ESTs) have been produced from the midgut of the adult *Glossina morsitans* and grouped into 8876 clusters or singletons potentially representing unique genes (Aksoy *et al.*, 2006). Putative functions were ascribed to 4,035 of these by homologies. Of these, a remarkable 3,884 had their most significant matches in the *Drosophila* protein database. The sequences are available from the Sanger *Glossina morsitans* GeneDB (*http:www2.genedb.org geneb glossina index.jsp*).

While trypanosome biology is widely studied, knowledge of tsetse flies is very limited, particularly at the molecular level. This is a serious impediment to investigations of tsetse-trypanosome interactions. This study has undertaken to characterize some proteins identified as potential candidates for transmission blocking and anti-tsetse fly vaccines. In addition to characterization of the protein molecules some insight on their role in *Glossina* biology has been discussed. Ks1-M13 (DET) was revealed as a homologue of serine protease inhibitor with the conventional active site **NQIYEPVCGTDGNYPNPCXFLC**. The BLAST

analysis of Ks1-M13 inferred its structural similarity to Gmm 2766 (putative protein) from the *Glossina* GeneDB. The protease inhibitor (serpins) found here may reflect the need to inactivate the complement and coagulation cascades in the blood meal to retain the meal in a physical state suitable for digestion. Blocking the activity of such molecules would likely lead to coagulated bloodmeal and hence inaccessible to digestive enzymes. Consequently the vector fecundity is affected with a torpid reproductive capacity. Normal growth of the larva is a function of optimum feeding throughout the pregnancy cycle (Moloo, 1976a). In addition, many serine proteases involved in the bloodmeal digestion exist in fine balance with serine protease inhibitors to ensure that the impact of protease-activated cascades remains localized in time and space (Jiang and Kanost, 2000). Gmm-2766 is a homolog of Infestin which is reported in the SWISSPROT protein database (AAK57342) as a novel thrombin inhibitor present in the midgut of the blood-sucking hemipteran *Triatoma infestans.* Ks1-M13 was predicted to be a homologue of transmembrane protein with a possible organic anion transport activity. This implies that not only does it block coagulation but involved in the uptake of possible nutritive derivatives and/or detoxification. This could be a new family of protease inhibitors yet to be deciphered. Past studies have shown 11 putative proteinase inhibitors in the gut (Lehane *et al.*, 2003). The workers proposed that the protease inhibitors may be involved in inactivating serine proteases and regulating disease since proteolytic enzymes are important virulence factors in many pathogens (Amstrong, 2001).

Ks2-M13 was predicted to be a proteolytic enzyme trypsin. It was found to have the TRYPSIN_DOC active site with 164 aminoacids. Proteolytic enzymes are important virulence factors (Amstrong, 2001) and interfering with their activity may curtail virulence of trypanosomes within the tsetse fly midgut. Optimal trypsin activity in the midgut has been implicated in transformation of bloodstream form trypanosomes to procyclic forms (Imbuga *et al.*, 1992a). This process is crucial for successful establishment of infection in tsetse flies. However, the high levels of midgut trypsin activity 48-72 hours post-bloodmeal (Onyango, 1993) have been shown to lyse bloodstream form trypanosomes (Imbuga *et al.*,1992b; Nguu *et al.*, 1996). Therefore the use of trypsin for transmission blocking vaccines may require a prior analysis of benefits vis-à-vis a possible enhancement of infection. The protein, Ks2-M13 was revealed by functional genomics to have lectizyme and proteolytic lectin activity. Previous work (Abubakar, 2003), suggested *Glossina* proteolytic lectin as a physiological trigger for transformation of bloodstream form trypanosomes into procyclic forms in the midgut. Inhibition of these molecules with anti-midgut antigens may have severed the trypanosome-tsetse fly interaction at the midgut thereby affecting successfull transformation of the trypanosomes to infective forms.

The sequence analysis of Ks4-M13 protein showed its similarity with Gmm-8438 putative protein submitted to *morsitans* GeneDB (NCBI). Gmm-8438 is thought to be involved in embryonic development/oogenesis and in regulation of epidermal growth factor receptor signaling pathway. This information if pursued

could lead to unprecedented insights of its role on larval development. The roles present the protein as a critical and potential protein for use in vaccine development. The flies fed on immunized rabbits showed reduced larviposition and therefore this protein singly or in combination may have interfered with the flies' reproductive capacity. Ks4-M13 was also found to match with exocyst complex component Sec10 (SWISSPROT. GA20365-PA, Expect = 2e-98), and Golgi complex component 4 (E = 1e-97). Exocyst complex component Sec10 family contains the Sec10 component (approximately 650 residues long) of the eukaryotic exocyst complex, which specifically affects the synthesis and delivery of secretory and basolateral plasma membrane proteins. Ks4-M13 was also found to be similar to golgi complex which is involved in the assembly and transportation of proteins to their destined locations. The exocyst and the golgi complexes could be involved in the assembly of the midgut proteins and in transversing secretory proteins across the midgut membrane. The results are interesting to learn that the complexes could have some direct association with peripheral proteins of the midgut structure or may have structural similarity with a midgut protein. Whatever the case antibodies cross the membrane therefore these proteins remain ideal as vaccine targets. Some of the secretory proteins could be involved in bloodmeal digestion or trypanosome establishment. Thus blocking the functions of the complexes and the exocysts with antibodies may interfere with the integrity of the membrane and the ability to transverse secretory proteins.

Ks7-M13 was inferred to be an aspartate-Glutamate racemase. Racemaces are enzymes that cause a racemization of the chirality center of a molecule interconverting L-glutamate/Aspartate to D-glutamate/Aspartate. D-glutamate is important in the protection of the peptidoglycan layer of bacterial cell walls from cellular proteases. Since D-glutamic acid is one of the essential amino acids present in peptidoglycan, glutamate racemase has been considered to be an attractive target for the design of new antibacterial drugs. However in tsetse flies this enzyme could be involved in the conversion of the L-aspartate and L-Glutamate to the D forms of carbohydrate intermediates for metabolism. Interference with the racemace activity may lead to deleterious effects to the metabolic biological processes of the vector. In addition racemaces could be involved in anti-microbial activity by converting the D-glutarnate of peptidoglycan to L-form. This would make the bacteria vulnerable to the midgut proteases. Interference on the racemaces of the host vector would aid in the pathogenicity of the microbes.

Ks8-M13 was analysed to have a sulphotransferase activity from the SwissProt database (Q7Q5W3). Sulphotransferases (STs) exist as a family of functionally related forms which are involved in the sulphation of a diverse range of compounds including biogenic amines, steroids, hormones, bile acids and a variety of xenobiotics including drugs and carcinogens (Mulder and Jakoby, 1990). A common feature of all reactions catalysed by the STs is the utilization of 3'-phosphoadenosine 5'-phosphosulphate (PAPS) as the sulphate donor. The

sulphation process is thought to be a detoxification pathway for many compounds. It would be un-doubtfully intriguing to elucidate this process within the genus *Glossina*. The pathway would possibly be used by the tsetse fly to eliminate the would be lethal compounds within the bloodmeal. By blocking this pathway the tsetse fly could become vulnerable to a battery of compounds found within the host bloodmeal. Due to the potential importance of the sulphation pathway to the tsetse fly, this study considers sulphotransferases as potential anti-tsetse vaccine candidates. Nevertheless, complete elucidation of this pathway would give unprecedented insight to its operation. Work is ongoing to find out whether tsetse flies can be exposed to trypanocide samorin infused bloodmeal before release during the SIT operations. The revelation of the sulphation pathway would put the samorin treatment into question. The tsetse fly would have the ability to 'decontaminate' the bloodmeal thereby making the samorin treatment futile. Ks8-M13 was also found to be expressed in all Open Reading frames (ORFs). Analysis of some ORFs predicted the protein to have a ribonuclease activity (degradation of mRNA). These could be a membrane bound enzyme involved in degradation of host ribonucleic acids. Anti-ribonucleases would interfere with RNA metabolism and the eventual turnover of nucleotides for RNA synthesis. This would affect protein synthesis since there will be diminishing amounts of mRNA (transcription) for the required proteins. Secondly since the mRNA produced is not degraded proteins whose use may be localized in time

and space will continuosly be produced. Therefore blocking this enzyme would have a dual effect; the sulphotransferase activity and the ribonuclease one.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSION

6.0 GENERAL DISCUSSION AND CONCLUSION

In tsetse flies, some factors e.g secretion of proteases (Imbuga *et al.*, 1992a), lectins (trypanoagglutinins) (Maudlin, 1991), trypanolysins (Osir *et al.*, 1999) create a hostile environment for trypanosomes ingested during an infective bloodmeal. This study has shown immature forms of *T. b. rhodesiense* when flies were fed on anti-DET with the trypanosomes confined to the midgut. Previous work has indicated that vector insects display both humoral (non-cellular) and cell mediated (hemocytes) immunity in order to counteract environmental pathogens (Azzolina *et al.*, 1985). In this study, the flies without antigen treatment had up to 30% of mature infection in Salivary glands. Therefore the use of transmission blocking vaccines against trypanosomes (in tsetse) could certainly augment the innate immunity of tsetse flies thereby reducing disease incidences.

The Transmission Blocking Vaccines (TBVs), if deployed in combination with other types of trypanosomosis control methods such as drugs could be effective in preventing the escape and spread of mutants resistant to those drugs. This is especially in areas with demonstrated drug resiatance (Anene *et al.*, 2001). This study has indicated that the flies fed on anti-midgut proteins had trypanosomes with curtailed mortility within the midgut and had lower trypanosome load. Mature metacyclic forms of trypanosomes were not evident in the salivary glands for both the AQ and DET treatments. Previous studies have made similar observations with *Plasmodium gallinaceum* (Duffy *et al.*, 1993).

In addition to blocking transmission, this study has shown a posibility of developing an anti-vector vaccine (AVv). In the absence of vaccines and effective and affordable drugs, control of vector-transmitted diseases relies heavily on vector control. However, the vector control methods are difficult to sustain since they require well coordinated programs (Hursey, 2001). Therefore the use of well sustaining AVvs would circumvent or considerably reduce some of the problems associated with vector control. The AVvs and TBVs- induced immunity could completely abort a potential epidemic or prevent it from reaching a high level. The fecundity of flies in this study was reduced by more than 50% by anti-DET and anti-AQ antibodies.

This study not only gives some insight on the potential vaccine candidates, but also a glimpse of tsetse flies' biology. With the onset of trypanosome and tsetse fly genome sequencing, the parasite-vector interaction will be better understood and possibly unravel the mystery of trypanosome transmission. Characterization of vaccine candidates will be made easier especially with the enormous genome profile information (Glossina and trypanosomes) and the advent of new and more robust Bioinformatics tools (Aksoy, *et al.*, 2006; El-Sayed *et al.*, 2003).

The sequence analysis of Ks1-M13, Ks2-M13, Ks4-M13, Ks7-M13 and Ks8-M13 are by the work of this study potential vaccine candidates. Ks1-M13 would, make the tsetse fly susceptible to microbial infection and have reduced longevity, Ks2-M13 would, interfere with trypanosome establishment; Ks4-M13

would interfere with the tsetse flies' fecundity (larviposition) and in assembly/transportation of proteins by the the golgi body complex, Ks7-M13 would, lead to susceptibility to bacterial infection/carbohydrate metabolism and Ks8-M13; inability to synthesize proteins and an effect on ribonucleic acid biosynthesis (reduced longevity/fecundity). The combination of all these antigens theoretically would lead to a superb vaccine formulation. However, it would be more feasible to have fewer molecules with a matched efficacy in order to avoid immune-masking.

This study therefore proposes the use of one or a combination of two (e.g. Ks1-M13 and Ks4-M13 or Ks7-M13) molecular vaccine targets: a formulation with dual functions (Transmission Blocking Vaccine and Anti-Vector Vaccine). The vaccine would hence be referred to as "TsetseGARD". The proposal on "tsetseGARD" not withstanding, recommendations for further developmental research are; firstly, investigate formulation, including ways of minimizing the risk of hypersensitity reactions. Secondly, develop ways of improving longevity of antibody response possibly by testing alternative delivery and adjuvant systems.

Lessons learned on target identification

Bloodfeeding vectors, both insects and ticks, ingest host blood for energy, larviposition or egg production. The bloodmeal contains red blood cells, serum, and immune factors that are normally present in the host bloodstream. If the host is vaccinated with antigens from vector tissue (e.g. midgut proteins), the host

immune response will be directed toward these antigens and the bloodstream immune factors can attack the native antigens (e.g the vector midgut) when ingested with vector bloodmeal. Subsequently, this immune attack can lead to the death or impairment of the vector. Therefore, the lynchpin for success in anti-vector immunization is the identification of molecular targets within the vector tissues. The question is, how easily can one identify the molecular targets? In 1939, William Trager first demonstrated anti-vector immunity when he immunized guinea pigs and rabbits with tick extracts and produced a lethal immune response against ticks bloodfeeding on the same immune animals (Trager 1939). Over the 67 years since, scientists have performed many similar experiments with most major vectors, yet have only identified a handful of specific anti-vector molecular targets (Foy *et al.*, 2002). In brief, this research has been extremely difficult, painstakingly slow and notoriously irreproducible. The difficulty most often lies in the primary experiments, which typically rely on immunization using homogenized vector tissue such as the midgut. Experimental animals produce polyclonal and multifactorial immune response to these heterogenous mix of vector antigens, and it becomes difficult to match an immune effector, such as antibody, to any particular antigen. Further complicating this is antigen immunodominance, immune-masking and antibody cross-reactivity. A possible partial remedy for the seemingly enormous difficulties in identification of vaccine targets (as illustrated in the study) is use of novel molecular and immunological techniques e.g. construction of cDNA libraries, screening methods, functional

genomics and proteomics. This study has successfully utilized these techniques. What would have possibly taken another two or three years (e.g. functional roles of native antigens) by use of conventional biochemical techniques has been reported here.

Post-scription of the study

1) The use of TBV and AVvs to reduce trypanosomosis transmission

In low and high endemic situations, reducing trypanosomosis inoculation rates with a TBV and AVv would be clearly beneficial, because it would reduce incidence of disease and mortality in proportion to effective TBV and AVv coverage. This would reduce the burden on the health services to a corresponding extent. TBV-induced immunity could, even at relatively low coverage, significantly retard the build up of trypanosomosis epidemic. Since the vectorial capacity (the power of the prevailing tsetse fly populations to transmit trypanosomosis) that drives an epidemic is usually time-limited, this could completely abort a potential epidemic or prevent it from reaching a high level.

2) TBVs/AVvs, when deployed in combination with trypanocidal drugs, could be effective in preventing the escape and spread of mutants resistant to those drugs. However, mathematical simulations are needed in order to quantify the protective effects of TBVs/TBVs on drugs and other control methods.

3) Studies to estimate the cost-effectiveness of TBVs/AVvs should be conducted. Nevertheless one general point can be made; in spite of the poverty

of the countries affected by trypanosomosis or indeed, because of it, the economic gains from reducing or eliminating trypanosomosis from the populations affected by use of TBVs/AVvs is enormous.

4) The design for testing of the vaccine is obviously crucial. This study proposes the following:

a. ***Phase I:*** A test of safety and immunogenicity in naïve or target populations. As part of the immunogenicity assessment, the ability of volunteers' antibodies to block infections of tsetse flies in a membrane feed, or other surrogate measures of efficacy, would be used.

b. ***Phase II***: A test of the ability of the vaccine to block the transmission of sleeping sickness from naturally infected vaccinees to tsetse flies by direct feed. The measures of transmission from person to tsetse fly may include:

    i. Investigation of infection rates in wild caught tsetse flies on individual vaccinees e.g tsetse flies caught near homesteads.

    ii. Community wide measures of tsetse flies' infection rates following vaccination of high proportion of community members

c. ***Phase III:*** A test of the ability of the vaccination to prevent people from becoming infected, i.e. to break the human- tsetse fly- human

infection cycle. A phase III trial may have several end-points, including:

i. The rate at which new infections are detected in humans by active case detection.

ii. The proportion of people who seroconvert for anti-trypanosome antibodies over a course of vaccine trial.

iii. The number of people presenting with disease symptoms (passive case detection).

This study recommends that the efficacy of the vaccine candidate(s) be demonstrated in *T. b. rhodesiense* primate model before the clical trials as highlighted above. This will help in determining the right formulation, the carrier adjuvants and accurate vaccine amounts for immunization. Future studies may consider use of a larger number of tsetse flies and evaluation against other tsetse species.

Chapter Seven

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7.0 References

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8.0 APPENDICES

Appendix 1

Silver staining method (Morrisey, 1981) modified by Kinyua *et al.*, 2004

After PAGE remove and fix in the following:

1st cycle

1. 50% methanol and 10% acetic acid for 2 min
2. Wash with 50% methanol for 20 min

2nd cycle

1. Wash with water for 5 min
2. Wash with 50% methanol for 20 min
3. Add 10% of 25% aqueous glutaraldehyde for 30 min
4. Rinse briefly with distilled water

3rd cycle

1. Wash with water for 5 min
2. Wash with 50% methanol for 20 min

Silver staining solution

Solution A 0.8g $AgNO_3$ in 2.5 ml water

Solution B In 1.0ml of 2M NaOH in 20ml water, Add 1.6ml concentrated ammonia

While shaking, add slowly (carefully not to form precipitate) solution A to solution B.

Add water to 100 ml

Stain gel in silver solution for 15 min

Wash in water for 5 min.

Develop the gel in cold (10°C) developing solution (2.5% Na_2CO_3, 100µl 37% Fomalaldehyde per 100ml, $Na_2S_2O_3$-$5H_2O$ 2mg/liter).

Color develops in 1-2 min

Ammonium Sulphate Precipitation

Reagents:

1. Ammonium sulphate (BDH 10033), saturated solution, pH 7.0. Make up 550 g to 950 ml with water, warm to dissolve and filter, adjust pH with ammonium hydroxide, make up to 1 liter and refrigerate. Crystals should form confirming that the solution is saturated.
2. Saline (0.85% NaCl)
3. Dialysis membrane which retain molecules of > 100 kDa.

Procedure:

1. For each ml of serum or ascites at 0° C, add 1 ml of cold saline pH 7.0.
2. Add 2 ml of cold saturated ammonium sulphate dropwise while stirring.
3. Stir for 30 min on ice while a white precipitate (globulin) forms.
4. Centrifuge 30 min at top speed in refrigerated (0° C) benchtop centrifuge.
5. Discard supernatant and take up precipitate in 2 ml of saline per ml of original serum.
6. Repeat steps 2-5 twice more: the final supernatant should be colorless. The final precipitate should be taken up in 1 ml or less.
7. Dialyze extensively against PBS to remove all ammonium sulphate.

Note:

Sodium sulphate may be used to precipitate immunoglobulin at room temperature (ideally 25° C). Follow the same procedure adding 36% sodium sulphate to a 50% dilution of serum in PBS. The third precipitation may be carried out bin 12-14% final concentration sodium sulphate.