DOCTOR OF PHILOSOPHY

Vector trypanosome relationships

Malele, Imna Issa

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By

Imna Issa Malele

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To

YHWH, for His faithfulness;

And to

Elohim, for the magnificent beauty of Tarangire National Park;

And to

Stephen, my beloved son, who missed much of my attention at a tender age as I pursued this programme.

SUMMARY

Blood sucking insects have defence strategies against infection. In the stable fly, *Stomoxys calcitrans*, one of the defensive mechanisms is a lectin which is blood meal stimulated. While attempting to clone and sequence the lectin from the reservoir zone of the midgut tissue of *S. calcitrans,* a trypsin was obtained. The trypsin is blood meal induced, and is midgut tissue specific. The cDNA of the mature protein has 260 amino acid residues and has a structural similarity with a trypsin - like proteinase Try29F of the fruit fly *Drosophila melanogaster* (57% sequence identity); with trypsin 7 precursor of African malaria mosquito *Anopheles gambiae* and trypsin 3A1 precursor of yellow fever mosquito *Aedes aegypti* (both 55%). The protein has a relative molecular weight of 28 kDa. It has both the serine and the histidine active site signatures; with its catalytic domain between 60 to 240 amino acids. It has an N-glycosylation site (NES) at position 163 to 166 with a significant probability of occurrence (P = 0.005). The cloned trypsin was extracted from the midgut of 8 - 9 day old flies which have been starved for 24 hours prior to dissection. The extract from which the trypsin was purified, was capable of agglutinating rabbit red blood cells an activity that was strongly inhibited by N-acetyl-D-glucosamine. The molecule was purified by affinity chromatography designed to purify lectins and the subsequent isolation of lectin using SDS-PAGE. The trypsin molecule suggests the presence of a trypsin - lectin dimer in *S. calcitrans* midgut, and in the light of work on tsetse flies, this possibly helps to explain why *Stomoxys* is not a cyclical vector of trypanosomiasis despite being sympatric with *Glossina*. The study has also shown that parasites have adaptive evolutionary changes which help them to evade the defence mechanisms of vectors. The study presents three groups of newly isolated trypanosomes of *T. godfreyi* types similar to the known *T. godfreyi* by 96 & 95.6% sequence identity; the *T. simiae* Tsavo by 95% and *T. vivax* type by 86.2%. This work has also shown that different tsetse species respond differently to infection even if they share the same habitat and hosts. The patterns of infection in four different species investigated, (*Glossina swynnertoni* Austen, *G. morsitans morsitans* Westwood, *G. brevipalpis* Newstead and G. *pallidipes* Austen) varies from species to species and from one place to another and also are conserved within species.

CONTENTS

SELECTED LIST OF ABBREVIATIONS

RLO's	Rickettsia Like Organisms
RE	Reservoir Extract
RBC	Red Blood Cells
PM	Peritrophic Matrix
PCR	Polymerase Chain Reaction
TTRI	Tsetse & Trypanosomiasis Research Institute
SIT	Sterile Insect Technique
HAT	Human African Trypanosomiasis
VGS	Variable surface Glycoproteins
TANAPA	Tanzania National Parks
VAT	Variant Antigenic Type
VSA	Variant Specific Antigenic
Con A	*Concanavalin A*
GlcNac	N-acetyl-glucosamine
ssu rRNA	small subunit ribosomal RNA

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Chapter One

LITERATURE REVIEW

African trypanosomiasis is transmitted by various species of tsetse fly. These are medium to large brown flies with long, forwardly directed proboscis sheathed by equally long palps. Both sexes are haematophagous and can transmit infection when they feed on man and domestic animals. Tsetse flies are viviparous; the females produce fully grown larvae (Buxton, 1955). They have a low reproduction rate, classified as K-selected with a population which is relatively steady, changing only slowly with time; and they live in a habitat which is either constant or predictably seasonal in time (Kettle, 1995).

Tsetse flies belong to the genus *Glossina*, family Glossinidae, within the order Diptera; and are found in the afro tropical region although two species have been found in the south west of Saudi Arabia (Kettle, 1995). In this region, the species of *Glossina* are widely but not universally distributed, though one or more occur wherever the environment is suitable. Temperature plays an important role in determining the limit of tsetse distribution; usually temperatures below 7°C or above 39°C are taken as critical preventing the development of the puparia.

The genus *Glossina* contains 31 living taxa, 23 species and 8 subspecies (Jordan, 1993). The species are divided into three subgenera *Glossina*, *Nermohina* and *Austenina* which commonly are known as *Morsitans, Palpalis* and *Fusca* groups respectively (Kettle, 1995). However based on isozyme analysis, morphological, ecological criteria and nuclear DNA sequence analysis, the 31 taxa of *Glossina* have been placed into four groups: *Austenina* (= *fusca* group), *Nermohina* (= *palpalis*

group), *Glossina* sensu stricto (= *morsitans* group), and *Machadomyia* (= *austeni* group) (Chen *et al.*, 1999).

The subgenus *Morsitans* contains five species of which two, *Glossina morsitans* and *G. pallidipes,* are of major economic importance, whereas *G. swynnertoni* and *G. austeni* are of local significance. These species are found in savannah woodland and evergreen thickets, except for *G. austeni* which is restricted to the coastal forest and relict forest. The subspecies of *G. morsitans (morsitans, centralis, submorsitans)* range widely in western and central Africa; *G. longipalpis* is found in The Democratic Republic of Congo (DRC), formerly Zaire, and eastern and southern Africa. *G. swynnertoni* in found in eastern Africa and *G. austeni* in eastern and southern Africa (Jordan, 1993).

The subgenus *Palpalis* contains five species of which three, *G. palpalis, G. fuscipes* and *G. tachnoides,* occur in riverine and lakeside habitats and are particularly important as vectors of human trypanosomiasis. The subgenus occurs mainly in western and central Africa, with *G. tachnoides* extending into eastern Africa and *G. pallicera* occurring in Angola. *G. fuscipes* is mainly found in central and eastern Africa, but also in Chad, Angola and Zambia (Jordan, 1993).

The 13 species in the subgenus *Fusca* are forest dwellers found in western and central Africa with some representation in eastern Africa and Angola, but with the exception of *G. brevipalpis* and *G. longipennis*. The latter is only found in eastern Africa and *G. longipennis* in eastern and southern Africa. The species in this genus have little contact with humans or livestock, and so are of little economic importance, except when cattle are moved in forested areas in search of water and pastures during draught periods (Bikingi-Wataaka, 1975 and Jordan, 1993).

Tsetse flies infest approximately 10 million km^2 of Africa. However, the distribution of tsetse is rapidly being affected by the increasing human population. In already

densely populated countries this is causing the removal of tsetse habitats and hosts and thus the disappearance of tsetse over large areas. Examples are Nigeria, The Gambia, Burkina Faso, Rwanda, Malawi and parts of Tanzania and Zimbabwe. The main effects of man are on the *Morsitans* and *Fusca* species, where as the *Palpalis* is mainly affected in drier areas. In sparsely populated countries like Mali, southern Sudan, DRC, Angola, Mozambique and Zambia, tsetse are so far little affected by man and in the some places are even invading new land (FAO, 1992).

Tsetse transmitted diseases are one of the major factors retarding rural development in Africa. It is believed that if it was possible to eradicate trypanosomiasis from Africa, the predicted benefit to overall agricultural production would gradually rise to 4.5 billion US $ per year; the lives of over 40% of sub -Saharan Africa's 625 million population would benefit significantly; 55,000 deaths per year from sleeping sickness would be avoided (Budd, 1999).

The estimated costs of trypanosomiasis, accounting for social, medical and veterinary fractions, is 50 billion US$ per year. In addition an estimated revenue of the same figure, 50 billion US$, could be generated if 70% of the tsetse belt was utilised for cattle production (Murray *et al.*, 1991). Currently, Africa supports 173 million cattle with 44 million of these being produced within the tsetse belt (Murray *et al.*, 1991).

Trypanosomiasis causes a reduction in livestock productivity in the form of weight loss, fertility, capacity for work, growth rates, milk yields and an increase in abortion (d'Ieteren *et al.*, 1999). When considered in conjunction with the agricultural process as a whole, the impact of this disease can be more comprehensively appreciated. In absolute production terms the effect is compounded by the consequential reduction in traction power supplied by livestock and the loss of manure for fertiliser (d'Ieteren *et al.*, 1999). Meat and milk off-take is also reduced by at least 50% over the entire tsetse area (Swallow, 1999).

In addition to the direct effects of the disease on production, trypanosomiasis has social implication in the form of rural instability. The contraction of the human form of the disease is caused by *T. b. rhodesiense* and *T. b. gambiense*, which result in acute and chronic sleeping sickness respectively. In rural communities, the close proximity of both domestic and wild animals therefore the vectors result in relatively high incidence of infection, acting as disease reservoirs, and humans has been provided by Murray *et al.,* (1991). This clarification comes in the form of trials which have shown that animals show no clinical signs of infection but when treated with trypanocidal drugs, infection levels of the human trypanosome species are cleared and growth rates are increased (Murray *et al.*,, 1991).

The repercussions of sleeping sickness in these rural areas include not only a reduction in labour in terms of sufferers but also their associated carers (Swallow, 1999). Compounding this issue is increased migration by communities attempting to evade trypanosomiasis and settling in already inhabited areas. This can result in friction between communities due to the inevitable increased pressure on resources that are often already stretched (Swallow, 1999).

INSECT AND TRYPANOSOME INTERACTIONS

Trypanosomiases and Leishmaniases

Trypanosomiases are diseases of humans and livestock, and the leishmaniases largely diseases of humans. They are caused by parasitic flagellate protozoa of the order Kinetoplastida (Phylum Sarcomastigophora). Members of this order possess a single mitochondrion, Golgi; a relatively small and compact kinetoplast, a DNA containing particles, close to the flagellar pocket; and a single flagellum (Vickerman, 1976; Seed & Hall, 1992).

Species of two genera, *Trypanosoma* and *Leishmania*, are of economic importance, causing, respectively, trypanosomiasis and leishmaniasis. The trypanosome cell surface changes as it moves between vertebrate and invertebrate hosts; glycoconjugates on the surface often playing a crucial role in determining parasite survival and infectivity both to its mammalian and insect hosts (McCoville & Ferguson, 1993).

A characteristic feature of trypanosome infections in animal is that their abundance follows a wave like pattern with time. In each wave of parasitaemia one variant will dominate and then be cleared as the host develops the appropriate antibody to the surface coat protein (VSA, variant specific antigen) of the parasite. Research shows that the glycoprotein is of approximately 60,000 kDa. Following clearance, another variant will dominate but the switching from one another is not due to exposure to the antibody because it occurs in immunodepressed animals. More than 60 different antigenic types (VAT) have been detected in a cloned strain of *T. b. gambiense*. The VSA of metacyclic forms is similar and the injected population may contain as many as 27 different VATs (Turner *et al.*, 1988). The predominant VAT in the metacyclics will be the one that dominated in the infective blood meal. In *Glossina* the procyclic trypomastigotes have no VSA coating but are uniformly coated with a new antigen, procyclin (Seed & Hall, 1992).

At different stages of the developmental cycle the parasites are known to take various forms, classified according to the arrangement of the flagellum, its starting point, its course either inside or outside the body of the parasite and its point of emergence to the exterior of the organism. Based on these characteristics; five stages occur in *Trypanosoma*. In the amastigote stage, the parasites are round, oval, or in elongated form devoid of flagellum. The promastigote are elongated form, the flagellar base is also anterior to the nucleus and there's a flagellum emerging from the anterior end of the body. In the epimastigote the flagellar base is anterior to the nucleus and the flagellum emerges laterally to form the undulating membrane which

runs along the body to the anterior end. The trypomastigote forms are elongated parasites with a postnuclear kinetoplast, the flagellum arising near it and emerging from the side of the body to run along the surface to form an undulating membrane. Lastly, the sphaeromastigote are rounded form with a free flagellum which represents a transitional stage between an amastigote and mastigote form (Molyneux & Ashford, 1983).

Species of *Trypanosoma* occur as blood parasites in a wide range of vertebrates, from fish to mammals. Trypomastigote and epimastigote stages are common to nearly all trypanosome life cycles. *Leishmania* species are characterised by intracellular amastigotes in the mammalian host and extracellular promastigotes in the gut lumen of phlebotomine sandflies (Vickerman, 1976).

Classification of *Trypanosoma* and *Leishmania*

Kingdom:	*Prostita*
Subkingdom:	*Sarcomastigophora*
Phylum:	*Sarcomastigophora*
Subphylum:	*Mastigophora*
Class:	*Zoomastigophorea*
Order:	*Kinetoplastida*
Suborder:	*Trypanosomatina*
Family:	*Trypanosomatidae*
Genus:	*Trypanosoma, Leishmania*

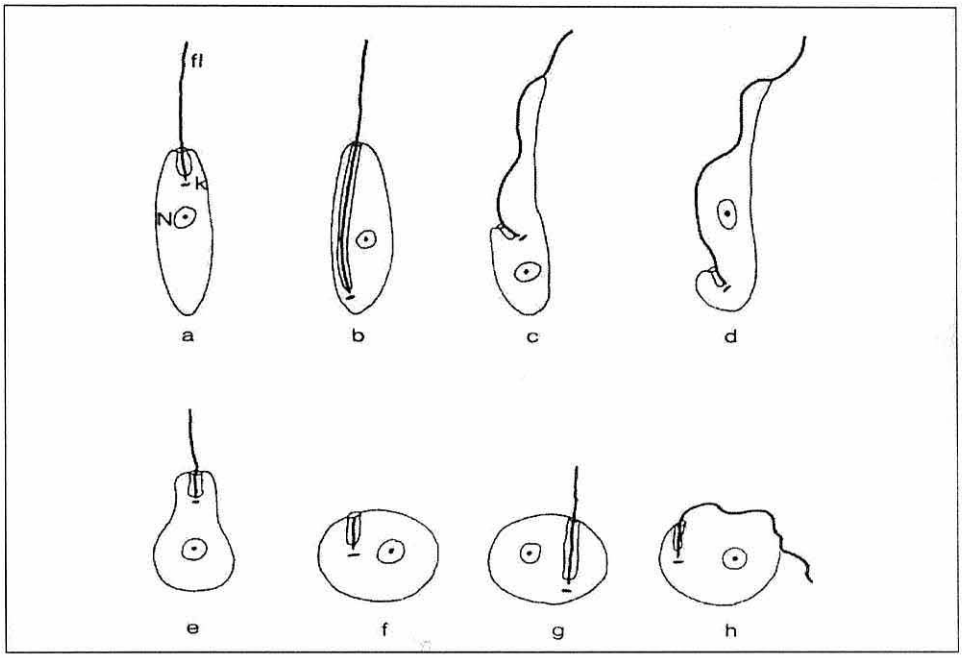

Figure 1:1. Diagramatic configuration of morphological types of Trypanosomatidae (x1300). a, Promastigote. b, Opisthomastigote. c, Epimastigote. d, Trypomastigote. e, Choanomastigote. f, Amastigote. g, Paramastigote. h, Sphaeromastigote. Abbreviation: fl – flagellum, N – Nucleus, k – kinetoplast.

Source: Molyneux & Ashford, (1983).

Leishmaniases

This is a disease of humans caused by infection with *Leishmania*, in which parasites are intracellular amastigotes in the reticuloendothelial cells. They are transmitted from host to host by bites of phlebotomine sandflies in which the parasites are motile and extracellular. The vector species are *Phlebotomus* and *Lutzomyia*. Clinical leishmaniasis have three main forms; visceral, cutaneous and mucocutaneous. The disease transmitted by *Lutzomyia* is zoonoses, involving reservoir hosts, while with *Phlebotomus,* the diseases are anthroponoses with no animal reservoir (Kettle, 1995). The disease is fatal if untreated and causes death. The mammalian stages of the genus *Leishmania* are intracellular amastigotes, the promastigote forms are found in the vector sandflies *Phlebotomus* and *Lutzomyia.*

Among vertebrates, only mammals and reptiles are infected with *Leishmania*, and sandflies are the only known effective vectors.

The amastigote stages are intracellular parasites of macrophages and it is only in more restricted and rare conditions that other cells are invaded. Visceral and cutaneous manifestations of leishmaniasis occur, but in wild reservoir hosts few symptoms are seen.

The amastigotes are taken up by sandflies and rapidly they transform through elongation into promastigotes, the typical form which normally occurs in the vector. Promastigotes are the infective form inoculated when infected sandflies bite hosts. Transmission of mammalian *Leishmania* is by the bite of an infected sand fly after development in the midgut, and occasionally hindgut, cibarium and pharynx (Molyneux & Ashford, 1983).

Transmission of leishmaniae from infected sand fly to a vertebrate host has been difficulty to prove. It has been long known that feeding sandflies with sugar, tend to enhance transmission due to the fact that sugar inhibits attachment and thereby increasing the number of free swimming promastigotes able to colonize the proboscis. Carbohydrates are inhibitors of lectin-mediated agglutination reactions of leishmanial promastigotes. The saliva of *Lu. longipalpis* substantially enhances the infectivity of *L. major*, a parasite with which it is not associated in nature. It has been established that two pathways operate; one involving the production of metacyclic promastigotes and the other involving the development of non-dividing promastigotes which attach to the cuticle of the stomodeal valve and foregut and block the intestine (Killick-Kendrick, 1990).

Infected phlebotomines have difficulty in engorging and tend to probe repeatedly. The reason postulated is being due to blockage caused by parasites on the cibarial sensilla, which are few in number. The absence of sensory information from the

cibarium would be beneficial to the parasites in ensuring that some were deposited in sites suitable for development (Molyneux & Killick-Kendrick, 1987). Infection rates in wild-caught phlebotomines have varied from 0 to 15.4%, these infections include both pathogenic and non-pathogenic leishmaniae and they vary from one species to the other. The focus of this review in on tsetse transmitted trypanosomes; hence *Leishmania* will not be discussed in details.

Trypanosomiases

Trypanosomiases are diseases of human and livestock. Species of the genera, *Trypanosoma* are of economic importance, causing African trypanosomiasis.

The species of *Trypanosoma* occur as blood parasites and sometimes in other tissues of vertebrates and the gut of blood sucking invertebrates. These are transmitted by arthropod vectors (except for *T. equiperdum* which causes a venereal disease in equines). Transmission is cyclical or non - cyclical. The non cyclical is essentially mechanical transmission in which the trypanosomes are transferred from an infected host to another by the interrupted feeding of biting insects, especially tabanids and *Stomoxys* (Urquart *et al.*, 1987 and Lehane, 1991).

In cyclical transmission the arthropod vector is a necessary intermediate host, in which the trypanosomes multiply, undergoing a series of morphological transformations before becoming infective. This is referred to as biological transmission and is divided into two groups, the *Stercoraria*, (the posterior station transmission); and *Salivaria*, (the anterior station transmission) (Hoare, 1964). In *Stercoraria*, developmental stages multiply in the gut and the metacyclic trypomastigote (the infective forms) accumulate in the hind gut and migrate to the rectum from where they are passed out with faeces for example *T. cruzi* which causes chagas' disease (Urquart *et al.*, 1987). In *Salivaria,* the developmental stage (e.g. epimastigotes) multiply in the digestive tract and proboscis and the infective stage accumulate in the mouthparts or salivary glands so that the infection is transmitted when the vector takes a blood meal. This is the inoculative method of

transmission. Salivarian trypanosomes, with the exception of two forms are transmitted by tsetse flies and these are divided into four subgenera (*Trypanozoon, Nannomonas*, *Duttonella* and *Pycnomonas*) as reviewed by Logan-Henfrey *et al.*, (1992). The two exceptions, *T. evansi* and *T. vivax viennei*, are mechanically transmitted by tabanids and *Stomoxys*.

(a) Stercorarian trypanosomiasis

T. cruzi, is the only economic important stercorarian trypanosome, a causative organism of Chagas' disease. The disease is most prevalent in South and Central America, and results in 70,000 deaths per annum (Dusanic, 1991). *T. cruzi* has a broad host range being found in more than 100 species of mammals of 24 families including marsupials and six orders of eutherian mammals. It is found in wild animals in the USA, but human cases are rare north of Mexico. The vectors of *T. cruzi* are haematophagous triatomine bugs (Dusanic, 1991).

In vertebrate host, slender and stumpy trypomastigotes are found in the circulating blood. It is believed that it is the slender forms which penetrate the host's cells where they develop into oval amastigotes and multiply forming pseudocysts which bursts after 4 to 5 days, releasing trypomastigotes into the blood stream from which they invade other cells. When triatomine bug ingests an infected blood meal the trypomastigotes differentiate into epimastigotes in the midgut and later adhere to the walls of the rectum where they develop into metacyclic trypomastigotes infective to a vertebrate host. The cycle in the bug takes about 20 days after which the bug remains infective for life and may transmit *T. cruzi* for several years. Transmission to the vertebrate is effected by metacyclics being deposited in excretory material on the skin of the host. The initial faecal deposit may contain fewer metacyclics than the subsequent drops of urine (Dussanic, 1991).

Another stercorarian Trypanosoma, is *T. rangeli*. It resembles *T. cruzi* in the fact that it is transmitted by triatomine bugs, particularly *Rhodnius prolixus*. It occurs in

Central and South America where it has been recovered from humans and 23 species of marsupials, carnivores, edentates, primates and rodents. *T. rangeli* is not pathogenic to vertebrates but in humans it must be differentiated from *T. cruzi*. Flagellates ingested in the blood meal multiply in the midgut of the bug and penetrate between the cells lining the posterior midgut to enter the haemocoele and multiply and form epimastigotes. These pass through the haemocoele and penetrate the salivary glands, in which they produce metacyclic forms, which are passed with saliva when the bug feeds. In the haemocoele, some flagellates parasitize haemocytes and form dividing amastigotes which give rise to trypomastigotes. They are released when the infected cell bursts but their subsequent fate is not known. Some flagellates will proceed down the midgut to the rectum and be deposited with the faeces but there is no attachment to the wall of the rectum. Posterior station transmission is considered to occur very rarely. Six species of *Rhodnius* have been found with salivary infections in nature and two more *Rhodnius* species and five species of *Triatoma* have produced salivary gland infections experimentally. *T. rangeli* infections are pathogenic to *R. prolixus* causing the formation of excessive haemolymph and adversely affecting moulting (D'Alessandro-Bacigalupo & Saravia, 1992). The focus of this review is salivarian trypanosomes transmitted by tsetse flies, hence more detailed information and discussion will concentrate on salivarian transmitted trypanosomes and the disease they transmit.

(b) Salivarian trypanosomes (anterior station development)

When tsetse flies feed on an infected host they ingest trypanosomes with blood or lymph. Thereafter the parasites lose their glycoprotein surface coat. In the case of *T. brucei* and *T. congolense* the parasites become elongated and multiply in the midgut to the trypomastigote stage which lasts for 10 days. Initially, the dividing forms in the midgut are broad with a kinetoplast midway between the nucleus and the posterior end. The long slender forms are produced by days 10-11 and migrate backwards, entering the space around the peritrophic matrix and then penetrate the proventriculus, within 12-20 days after infection (Soulsby, 1986). The parasites then

migrate interiorly to the oesophagus and pharynx, onwards to the hypopharynx, salivary glands (*T. brucei*) and proboscis (*T. congolense*). There they undergo a transformation in which they lose their typical trypomastigote form. The latter is characterised by the kinetoplast being located just in front of the nucleus (Soulsby, 1986; Urquart *et al.,* 1987) (Fig. 1:1). Further multiplication of the epimastigote forms take place within 2-5 days where they are transformed again into small, typical forms of trypomastigote with a glycoprotein surface coat. These are the infective forms for the next hosts and are called metacyclic trypanosomes. They are small and stumpy, resembling the stumpy forms found in the blood. The entire process takes at least 2-3 weeks after which the metacyclic trypanosomes are inoculated into the new host with saliva when the tsetse fly feeds (Soulsby, 1986; Urquart *et al.*, 1987; Vickerman *et al.*, 1993). Several thousand trypanosomes may be injected into the host with each bite (Soulsby, 1986).

Life cycle of Salivarian Trypanosomes

(a) In mammalian hosts.

When tsetse feed on a host, the metacyclic trypanosomes are deposited (along with the discharged saliva) in the dermal connective tissue. This leads to the development of a "chancre" which is a local inflammatory reaction. From the chancre the parasites enter the draining lymphatic and then the bloodstream. In the case of *T. congolense* the parasites multiply in the tissue of the chancre as a morphologically distinct phase before invading the bloodstream (Roberts *et al.*, 1969). Usually all three cyclically transmitted species of trypanosome undergo a change in form at this stage to emerge with the characteristic morphology of the dividing blood stream stages in the life cycle.

When the chancre phase is completed, *T. congolense* and *T. vivax* remain largely as intravascular parasites, with *T. congolense* occupying/residing in small blood vessels where it attaches to endothelium. They can also be found in the lymphatic tissues, where as *T. brucei, T. evansi* and *T. equiperdum* may secondarily escape

from the blood stream into the soft connective tissues and multiply in the tissue fluid. *T. equiperdum* is found principally in such tissues in its natural host. Invasion of the brain and cerebrospinal fluid occurs in chronic *T. brucei* group infections, such that the brain becomes the source of relapse infections after chemotherapy (Jennings *et al.*, 1979, Poltera, 1985).

In the case of *T. vivax*, the central nervous system and aqueous humor of the eye become the extra vascular foci of *T. vivax* in goats and a potential source of relapse infections. Intracellular stages are absent from the life cycles of African trypanosomes but present in the life cycle of *T. cruzi* (Vickerman, 1985). In salivarian trypanosomes, infections in the natural hosts are characterised by an undulating parasitaemia. Each fall in trypanosome numbers is equivalent to a destruction of a major antigenic type by the host's immune response and each subsequent increase in numbers corresponds to a subsequent increase in numbers corresponding to a proliferation of trypanosomes of a different antigenic type (Soulby, 1986 & Vickerman, 1976).

T. brucei is pleomorphic in the blood and multiplies by binary fission in the ascending parasitaemia as a long, slender flagellate. It transforms into a non-dividing, short and stumpy parasite when the parasitaemia passes through crisis into remission. The stumpy forms are known to be more capable of surviving in the tsetse fly's blood meal and they initiate the cycle of development in the fly (Soulby, 1986, Vickerman, 1993). *T. evansi* rarely produces stumpy forms although morphologically resembles *T. brucei* (Hoare, 1972). In *T. brucei*, pleomorphism is absent especially in stocks which have been mechanically passaged through laboratory rodents. *T. congolense* and *T. vivax* are less markedly pleomorphic than *T. brucei* and show differences from the latter species in their life cycle in the vector.

(b) In the insect vector

The gut of blood sucking insects is the first organ that is exposed to viruses and parasites. Some of these microorganisms undergo maturation and replication in the gut environment in contact with the secreted products of the intestinal cells. Increasing experimental evidence indicates that both blood components and dipterans digestive enzymes can determine how efficiently a blood sucking insects (which in most cases they are vectors as well) will function as a vector for parasites like trypanosomes and *Plasmodium* etc.

When the infective metacyclic forms of *T. brucei* are injected by a feeding tsetse into a vertebrate host, they develop into rapidly dividing slender trypomastigotes. At a later stage they switch to an intermediate form, which is rarely seen to divide and then to a non dividing short stumpy form which lacks a free flagellum. The short stumpy forms have been suggested to be not infective to the mammalian host and that they and the intermediate forms are infective for tsetse (Kettle, 1995) (Figure 1:1). Trypanosomes pass with the ingested blood into the crop and enter the midgut (via the proventriculus) where they are contained within the peritrophic matrix (in the endoperitrophic space).

In the midgut the intermediate and short stumpy forms differentiate into procyclic trypomastigotes which develop and divide extensively. It has been shown that a single ingested trypanosome can infect a tsetse fly (Maudlin & Welburn, 1989a). The trypanosomes move into the ectoperitrophic space via the open free end of the membrane or by penetrating the membrane. This process takes about 2 weeks after an infective feed. In the ectoperitrophic space the trypanosomes move forward to the proventriculus where they pass through the soft newly secreted peritrophic matrix to become free in the oesophagus and move down the food channel to the opening of the salivary duct in the hypopharynx. They pass along the salivary duct to reach the salivary glands where they develop into epimastigotes which attach to the epithelial cells of the microvilli. Extensive division takes place and finally

metacyclic forms detach from the microvilli and develop a surface coat similar to that found in trypanosomes in the vertebrate host. If metacyclic forms are injected into a susceptible vertebrate host again, they develop into rapidly multiplying long slender trypomastigotes and the process/cycle of events continues.

T. congolense

The development cycle of *T. congolense* in tsetse is similar in part to that of *T. brucei*. In the midgut, *T. congolense* procyclic trypomastigotes develop with a surface coat of procyclin. They pass through the same stages and move forward through the proventriculus to the pharynx and food channel, where they differentiate into epimastigotes and attach themselves to the walls of those structures. Here they divide and eventually differentiate into metacyclic trypomastigotes. Uncoated premetacyclics attach to the wall of the hypopharynx before becoming free, coated, metacyclic trypomastigotes capable of infecting a susceptible host. In *G. m. morsitans; T. congolense* penetrates the peritrophic matrix in the central region of the midgut and 7 days after an infective feed heavy infections can be found in both the endo and ectoperitrophic spaces; by 21 days epimastigotes can be found attached to the food canal by their flagella and a week latter they appear in the hypopharynx as free forms. *T. congolense* was found to penetrate the midgut folds between the midgut cells and not by penetrating the cells (Evans *et al.*, 1979*). T. congolense* forms large clusters of organisms on the labrum of tsetse and it has been suggested that they interfere with the sensory receptors of the fly, favouring increased probing and thus facilitating transmission of the parasite. It has been observed that tsetse infected with *T. brucei* or *T. congolense* probe more than uninfected flies (Molyneux *et al.*, 1979b).

T. vivax

Ingested trypomastigotes of *T. vivax* attach to the walls of the food channel, pharynx and oesophagus by means of a junction (hemidesmosomes) formed between their flagella and the chitinous walls of the surrounding structures. Attached

trypomastigotes multiply and differentiate into epimastigotes and finally into premetacyclic forms which detach and migrate to the hypopharynx where they mature into coated infective metacyclics. Trypomastigotes of *T. vivax* that pass into the midgut of the tsetse fly perish.

Factors affecting infection rate in tsetse and the implication of trypanosome cycles in tsetse flies

The rate of infection of tsetse flies by trypanosomes varies from species to species and from one place to another. In the *Fusca* and *Palpalis* groups, the rate of infection is usually much lower than in the *Morsitans* in which at least 10% of individuals in the population might be infected with trypanosomes (Buxton, 1955 and Jordan, 1974).

Various factors do influence the establishment of infections of salivarian trypanosomes in *Glossina* as reviewed by Jordan (1974) and Molyneux, (1980). Leak (1999) sums them up in three groups as:-Endogenous factors, Ecological factors and Parasite and host factors as presented in Table 1:1. Extensive work has been going on for the past 20 years to verify these factors. For example age and tenerality (Welburn & Maudlin, 1992); the peritrophic matrix as a potential barrier to infection in non teneral flies when inhibitor sugars are not used (Lehane & Msangi, 1991); physiological and biochemical states (Turner *et al.,* 1988); number of parasites which are infective (Maudlin & Welburn, 1989); concurrent infections with rickettsia like organisms, RLO's (Welburn & Maudlin, 1991) and availability of preferred hosts (Hide *et al.,* 1996 & Lefrancois *et al.,* 1998). The role of the immune system and lectins in particular has been a subject of wider interest. Lectins have a range of properties, especially that of binding specific carbohydrates, by acting as agglutinins. The lectin binding properties play a major role in the interactions between parasites and host cells, and are important in invertebrate immunity (Rudin *et al.*, 1989). Endogenous lectins are believed to be involved in cell - cell recognition mechanisms. Cellular and non - cellular immune reactions

may occur against pathogenic organisms infecting insects and agglutinins, of which lectins are the commonest identified.

Table 1:1 Factors influencing trypanosome infection rates in tsetse.

Endogenus factors associated with tsetse	Ecological factors	Parasite and host factors
Tsetse species Sex Age at infective feed Age structure of tsetse population Genetic differences/variation between & within species Behaviour i.e. host preference Concurrent infections (viruses, bacteria, fungi) Interactions between lectins and RLOs Physiological/biochemical state	Climatic factors Availability of infected hosts Hosts available for subsequent feeds	Parasite numbers available to tsetse Parasite species and its infectivity to tsetse Immune state of host Susceptibility Intercurrent infection Behaviour and attractiveness to tsetse

Source: Leak, (1999).

Lectins play a part in the transformation of trypanosomes in their vectors. This was demonstrated in the crop, midgut and haemolymph of *Rhodnius prolixus,* the vector of *Trypanosoma cruzi* in South America. The lectins of *R. prolixus* agglutinated epimastigotes of *T. cruzi* but not the trypomastigote stage. It is believed that the sugar residues exposed to the interacting surfaces enable the trypanosomes to pass

through the gut epithelium. Lectins show specificity for carbohydrates such as sugars for carbohydrates of erythrocyte membranes and for the protozoan parasites such s trypanosomes. The lectin like agglutinins, have also been identified from sandflies, *Phlebotomus papatasi*, in which they are responsible for agglutination of erythrocytes and *Leishmania* parasites (Wallbanks *et al.*, 1986).

Also, lectins are responsible for increasing resistance with age to infection with both the *brucei* and *congolense* group of trypanosomes. Maudlin & Welburn, (1987 & 1988), showed that the absence of lectins or when they are blocked in teneral flies, the infection tend to develop; where as, in older flies, lectin production was stimulated by the blood meal, which in turn prevented the subsequent infection. There is some evidence also that older flies and non teneral flies can be infected if starved (Gingrich *et al*, 1982; Makumyaviri *et al.,* 1984). Welburn *et al.,* (1989) suggested that starving flies would lower the level of lectins in the tsetse gut, thus allowing infection to take place.

Further research indicates that agglutinins from *G. austeni* midgut were blocked by D-(+)-glucosamine *in vitro* (Ibrahim *et al.*, 1984). Production of D-(+)-glucosamine resulted from activity of endochitinases generated by the presence of rickettsia like organisms (RLO) in tsetse midguts. Blocking midgut agglutinins by feeding tsetse with D-(+)- glucosamine with an infective blood meal resulted in a significant increase in the midgut infection rates of *T. congolense* and *T. b. rhodesiense* (Maudlin & Welburn, 1987). Susceptibility to trypanosomal infection in tsetse is therefore facilitated / mediated through midgut lectins responsible for killing of trypanosomes as they enter the fly midgut. In susceptible tsetse, it is assumed that the action of endochitinase produced by RLOs leads to an accumulation of glucosamine in the fly midgut, which blocks the lectin-mediated trypanocidal activity.

The developmental cycles of trypanosomes in tsetse differs from one species to the other. The cycle of *T. vivax* is completed in a much shorter time (5-13 days) than those of *T. congolense* (2 - 3 weeks) and *T. brucei* (3 to 5 weeks). The cycle of *T.* (*Pycnomonas*) *suis* in tsetse takes a similar time to that of *Trypanozoon* i.e. about 4 weeks (Kettle, 1995). This is a long time in consistent with the longevity of tsetse, which is about 6 weeks for males and 15 weeks for females. Infections with *Trypanozoon* and *Nannomonas* in tsetse last for the life of the fly, while those of *Duttonella* persist in the proboscis for up to 8 weeks (Raadt & Seed, 1977). As mentioned earlier, infection rates of *Trypanosoma* in natural tsetse varies between species and from one location to the other. For example, the infection rate in *G. fuscipes martinii* in Zambia was recorded to be 0.2%, while in *G. m. morsitans* in Nigeria, the rate was 76.6%. The highest infection rates were of *T. vivax* and the lowest of *T. brucei*, with infections of *T. congolense* being intermediate in frequency. These can be arranged in the order of magnitude difference in the percentage of three subgenera of *Trypanosoma* as follows: 20% for *Duttonella*, 2% for *Nannomonas* and 0.2% for *Trypanozoon* (Ford, 1971, Hoare, 1972, Jordan, 1974).

Susceptibility to infection with *T. brucei* is a function of the age of the vector. Unless a *Glossina* feeds on an infected host very soon after eclosion it is unlikely to become infective. Highest percentages of infection are established in flies that feed on an infected host within 48 h of emergence and preferably within 24h; that is at the first feed. Even at this stage, infectivity is unlikely to exceed 10%. Early feeding and infectivity lead to postulation that flies reared from puparia kept at high temperatures were more readily infected. At eclosion such flies would be deficient in food reserves and feed early.

The dependence of infectivity on age was related to the development of the peritrophic matrix, which is not secreted until after eclosion. It was postulated that flies that feed early in adult life have shorter membranes, and the trypomastigotes

would more easily reach the ectoperitrophic space. When the peritrophic matrix is fully developed the trypomastigotes would have to travel further down the midgut to find the end of the membrane, and this could carry them into zone where the pH is lethal (Freeman, 1973) and account for resistance to infection. However, now its accepted that infectivity is somehow resisted if an insect have had a blood meal. This is because the blood meal induces the production of lectin which is thought to play a part in delaying the establishment and maturation of trypanosomes. The peritrophic matrix is secreted continuously, but particularly after a blood meal.

Three stages (initial, established, mature) have been recognised in the development of an infection of *T. brucei* in tsetse. When *G. morsitans* was fed on infected blood, 50% of the flies developed initial infections in the midgut 3 days later but only 9% developed established infections in the ectoperitrophic space and infections in the ectoperitrophic space and foregut. Established infections were detectable 5 to 30 days after the infective feed. However, the number of mature infections, i.e. flies able to transmit metacyclics, was very low. Only 39 flies out of many hundreds examined successfully transmitted *T. brucei* by bite and only 15 of those had infections observed in the salivary glands (Dipeolu & Adam, 1974).

The probability of transmission occurring is enhanced by the fact that the first drop of saliva produced by the feeding tsetse, carry with it most metacyclics. If feeding is interrupted another large drop of saliva is produced when the tsetse attempts to feed again (Youdeowei, 1975). This implies that a probing fly is almost as likely to transmit as one that feeds to repletion.

Factors influencing the establishment and maturation of trypanosomes in invertebrate hosts

Insect - transmitted pathogens may have the greatest capability to spread rapidly through a susceptible host population, especially when the animal reservoir is involved. For successful transmission, most blood-borne insect-transmitted

pathogens face the inhospitable environment of the insect gut which can be critically important to pathogen transmission. Pathogens face drastic changes in their life cycle which forces them to make major biochemical, physiological and or morphological adaptation when they make transition from vertebrate bloodstream to the insect midgut. For example a drop in temperature from 37°C to 25°C, a battery of digestive enzymes and a much more primitive, non specific, immune system. Glucose rapidly disappears from the blood meal so trypanosomes must develop the ability to oxidise alternative substrates. As a result, blood form trypanosomes differentiate into procyclic forms, followed by ultra structural changes in mitochondrion and activation of oxidase systems and a cytochrome electron transport system. However, for many pathogens the difficult transition is from the insect back to the vertebrate host (Kaslow & Welburn, 1996). Trypanosomatidae also face the same challenge especially when they are transmitted from invertebrate host to vertebrate and then (sometimes back) to the invertebrate host. The whole process of interaction can be divided into two major pathways. The first pathway involves the establishment of the trypanosome itself as a dividing procyclic population in the midgut, and the second pathway is when the trypanosome leave the midgut and are forced to make its way e.g. to the salivary glands (this applies to the human infective trypanosomes) (Welburn & Maudlin, 1999).

(a) Trypanosome establishment in the vector midgut

Establishment of infection in tsetse flies/ sandflies, by gut adapted Trypanosomatidae is determined by complex factors intrinsic to the host, the parasite, the vector e.g. tsetse and its symbionts (Maudlin, 1991; Mihok *et al.*, 1993). These factors often affect interactions between parasites and lectins (Ingram & Molyneaux, 1991) and processes underlying blood meal digestion in the vector like tsetse. For example, midgut lectins (Maudlin, 1991), lysins (Stiles *et al.*, 1990) and proteases (Imbuga *et al.*, 1992) appear to be involved in clearing infection. Usually even if there's a high percentage of the host which are infected, it doesn't mean that flies in that area will have high infection rate because they feed on

infected host. Research has shown that most trypanosome fail to establish themselves in the new host. Dissection results on *G. f. fuscipes* Newst. in Busoga area showed that only 1% of flies in the area had salivary gland infection, despite the fact that there was an epidemic of sleeping sickness in the area (Okoth & Kapaata, 1986).The tendency of most infection failing to mature is being confirmed with PCR based determination of infection rate in tsetse where by a high prevalence of immature infection in tsetse population is found to be high, an indication that although flies are exposed to multiple infections, a high proportional of refractory tsetse is present in the population hindering the maturation of infection (Woolhouse *et al.*, 1996). Many factors are postulated as being behind the refractoriness of flies to infection. These include environment and intrinsic fly variables. For example susceptibility to midgut infection is thought to be a maternally inherited character in tsetse and in the lab; lines of refractory and susceptible to infection can be selected (Maudlin, 1982, Moloo *et al.*, 1998b). Susceptibility is thought to be caused by maternally inherited symbionts (Maudlin, 1991). Midgut symbionts have chitinolytic activity; the chitinase enzyme degrades chitin to produce the requisite lectin-inhibitory sugars. The inhibition of lectin enables the trypanosome to mature without hindrances from lectin (Welburn *et al.*, 1993).

Another factor behind refractoriness is the presence of midgut lectins (Maudlin, 1991). It has been found that lectin could influence the development of *T. cruzi* in Triatomine bugs (Pereira *et al.*, 1981). Further research found that tsetse flies have midgut lectin activity with molecular masses of 26 and 29 kDa from different tsetse species (Grubhoffer *et al.*, 1997). These molecules were found to play a key role in determining susceptibility when flies were fed with an infective feed containing specific lectin inhibitory sugars. 100% of infection rates could be obtained with the correct inhibitory sugar (Maudlin & Welburn, 1987). Also feeding pure procyclin (the predominant procyclin surface glycoprotein (Roditi & Pearson, 1990) to flies with infective feed has the same effect as feeding lectin-inhibitory sugars, resulting in significant increases in midgut infection rates and suggesting that the tsetse

midgut lectin normally acts via the procyclin coat of the trypanosome (Welburn & Maudlin, 1999) A similar lectin based system appears to operate in other vector-parasite systems such as sand fly vectors of *Leishmania major*, in which specific inhibitory sugars increase infection rates (Volf *et al.*, 1998), and purified lipophosphoglycan, the major surface glycoprotein of *Leishmania* promastigotes, strongly inhibits midgut lectin activity (Palanova & Volf, 1997); and the migration of *Brugia pahangi* microfilariae in *Aedes aegypti* (Ham *et al.*, 1991). *In vivo* experiments with tsetse have shown that the role of midgut lectin is to kill incoming trypanosomes. However this is effective in non teneral flies. Welburn & Maudlin (1992), demonstrated that teneral flies have low lectin activity, because midgut lectin is produced in response to the blood meal. The high levels of lectin activity in non-teneral flies could not be inhibited sufficiently by normal symbiont activity, resulting in refractoriness (Welburn *et al.*, 1994).

Blood meal of a host is another important factor which play part in establishment of gut adapted trypanosomes in tsetse flies (Vickerman, 1985). Research shows that host blood meal at the first infective feed is critical, with subsequent meals of less importance. The initial host effect acts with a time lag, modifying parasite survival well after large procyclic populations have established (Olubayo *et al.*, 1994). The nature and source of blood affects both the differentiation and multiplication of parasites during adaptation to a new metabolic environment (Nguu *et al.*, 1996; Masaninga & Mihok, 1999). Infective feeds from goat facilitated infection, whereas feeds from wildlife species, such as eland and buffalo, inhibit infection. However, the factors in host blood especially wildlife species, are related to the presence of trypanotoxins in the blood of wildlife (Muranjam *et al.*, 1997), of which preliminary results suggest that many effects are related to the inhibition of midgut lectin-mediated killing of trypanosomes by serum and/or erythrocyte factors, depending on the host blood and the tsetse species (Mihok *et al.*, 1995)

Another factor behind fly refractoriness is the presence of peritrophic matrix, gut enzymes and vector competence (Weburn & Maudlin, 1999). It has been established that peritrophic matrix (PM) plays a central role in the vector competence of disease vectors and particularly in the transmission of diseases like malaria. The malaria parasite is enveloped in a chitinious sac, Type I PM (Miller & Lehane, 1993), produced by the mosquito midgut in response to midgut disintetion, and finds its way through the PM (Billingsley & Sinden, 1997). In tsetse, the PM differs from that of the mosquito in that it is continuously secreted by the proventriculus at the anterior end of the midgut, and this is a Type II PM. Formerly, the PM was thought to be a barrier to infection especially in older flies. However, that is not the case, because it is now clear that older flies with formed PM could be infected as easily as teneral flies simply by feeding them with sugars (Welburn *et al.*, 1994). It appears that trypanosomes are able to enter the ectoperitrophic space via the free end of the PM (Welburn & Maudlin, 1999).

In mosquitoes, the midgut protease activity is responsible for malaria parasite penetration through the mosquito midgut (Shahabuddin *et al.*, 1998); but in tsetse, proteases have been implicated as being or acting as a barrier to infection. It is suggested that trypanosomes can inhibit the activity of trypsin, which if active, cause a hostile environment to the parasites. It is thought that the addition of sugars to fly blood meal not only inhibits lectin but also inactivates the midgut proteases. This suggests that trypsin may play a part in determining refractoriness to infection in tsetse (Imbuga *et al.*, 1992). However other findings indicate that trypsin activity is normal at doses of lectin inhibitor that give 95% midgut infection rates (Maudlin & Welburn, 1987), and infected flies and uninfected flies show no differences in levels of midgut proteases (Mihok *et al.*, 1995). Feeding either trypsin, trypsin inhibitors or anti-trypsin antibody to tsetse has no effect on infection rates, and neither is there any difference in trypsin levels between lines of tsetse selected for susceptibility and refractoriness to trypanosome infection. However, midgut

proteases are invoked as being important in transformation of bloodstream form trypanosomes to procyclic forms (Hunt *et al.*, 1994).

Cells in multicellular organisms have evolved mechanisms whereby they tend to kill themselves (apoptosis) in the interest of the organism as a whole, so as to facilitate development and homeostasis and minimize the risk of mutagenesis (Vaux, 1993). African trypanosomes, *Leishmania, Trypanosoma cruzi* and *Plasmodium falciparum* (Welburn *et al.*, 1997 & Picot *et al.*, 1997) have all shown to be capable of apoptosis under certain conditions. *In vitro* experiments have shown that when midgut form of trypanosomes (procyclic forms) were treated with *Concanavalin A* (ConA), a commercial lectin, *T. b. rhodesiense* parasites showed many of the characteristics of apoptotic cells like nuclear condensation, surface membrane vesiculation and fragmentation of nuclear DNA (Welburn *et al.*, 1996). The apoptotic process is controlled genetically (Tibayrenc *et al.*, 1991), and when individual trypanosomes die, they do so in order to promote the welfare of that clone. Work by Reifenberg *et al.*, (1997a) using PCR to differentiate multiple infections of two clones of *T. congolense*, has shown that trypanosome clones do compete in the midgut of tsetse flies for resources. Both clones were able to establish midgut infections when fed singly to four species of tsetse, but when tsetse were infected simultaneously with two different clones, one of the clones (E325), out competed the other (CRTA/3); and only 4% of infected flies were able to establish infections of both types of clones (E325 & CRTA/3) in the midgut. Low mixed infection rates were scored in the species used in this experiment compared to the infection obtained when flies were fed with E325 alone. In this case it shows that clones do compete for survival and establishment in tsetse flies. Once an infection is established in a susceptible fly, procyclic trypanosomes will reside in the ectotrophic space for the life of the fly, which may be several month in a female tsetse. During this period, the parasite population density remains remarkably constant. Such that a state of equilibrium is advantageous to the parasite which is in competition with the vector (its host) for proline as an energy source, and it is thought that the parasite multiplication balance

is brought about by the parasites programmed cell death (Welburn & Maudlin, 1997 &1999).

(b) Trypanosome maturation.

Maturation of trypanosomes in tsetse is another important factor in the progression of the procyclic population through epimastigotes to mammalian infective forms (metacyclics) in the salivary glands (in the case of *T. brucei s.l*). Only a proportion of established midgut infections mature and factors responsible for regulating trypanosome maturation include the interaction with the midgut lectin, fly sex and trypanosome stock. It is clear that when midgut lectin activity is inhibited, the trypanosomes survive and are able to establish themselves in the midgut. However it is also clear that activity of the same lectin appears essential for successful maturation of the parasites. Maturation of midgut infections can be prevented simply by continuous addition of lectin inhibitory sugar to the fly feed (Maudlin, 1991). Removal of serum from the tsetse diet also prevents maturation of trypanosomes because midgut lectins are secreted in response to serum in the blood meal (Grubhoffer *et al.*, 1997). Feeding lectin inhibitory sugar for as little as five days after infection can affect maturation of both *T. congolense* and *T. brucei s. l.* (Welburn *et al.*, 1989 & 1993), suggesting that some parasites may receive the stimulus for subsequent differentiation before establishment in the ectoperitrophic space. The process of maturation is observed during a specific time period after establishment in the midgut, and this time varies between stocks (Dale *et al.*, 1995). The proportion of midgut infections that eventually mature decreases with increasing maturation time, i.e. higher rates of maturation are achieved in stocks that are able to mature earlier in tsetse (Milligan *et al.*, 1995). This shows that there is an opportunity for established midgut infections to mature, and this period covers between 8 to 11 days after infection for most *T. brucei s.l.* stocks. This is an indication that parasites have evolved a strategy to mature fast in order to avoid the consequence associated with fly mortality (Welburn & Maudlin, 1999).

Sex, is another factor affecting the maturation of trypanosomes like *T. brucei s.l.* in male fly. Male tsetse produces significantly more mature trypanosome infections than do females. The mechanism behind appears to be due to the product(s) of an X-linked gene that kills or prevents migrating parasites from maturing (Milligan *et al.*, 1995). Comparison of the survival distributions of infected and uninfected flies has shown that, while midgut infections have little or no effect on fly survival, salivary gland infections significantly increase tsetse mortality (Maudlin *et al.*, 1998). Parasite induced mortality became noticeable only after ~50 days in these experimental conditions, with life expectancy of 77-105 days, in relation to life expectancy of flies in the field which is difficult to estimate. Work by Hargrove & Williams (1998), has shown that tsetse population can't increase if there is a constant /sustained adult female mortality greater than ~0.03 per day, which give the approximate mean life span of 33 days. In their study they found that mortality rates for females and males showed well-marked seasonal fluctuations, and for females the mean loss rate was about 0.2 per week, which gives a mean life span of five weeks. Males have a higher death rate both in the field and in the lab. Mean death rates for males on their study area (island) was about 0.3 per week, implying that a mean life span is 3.3 weeks. However with this mean life span it shows that wild flies would not live long enough for the parasite induced mortality to be significant. Msangi *et al.*, (1998) reported that 41% of female tsetse analysed were aged more than 71 days compared to only 18% of males. In this case, fly survival was more than 60 days. In the natural field situations female tsetse usually have higher infection rates than males simply because females live longer than males and therefore have greater likelihood of feeding on an infected host and picking up and maturing an infection. When age is taken into account, infection rates of males and females are relatively equal (Leak, 1999). All the same, even the premature deaths of a fraction of older flies may have demographic consequences. These factors may to some extent explain why very low levels of salivary gland infection are observed in wild flies, even when there is an abundance of infected hosts as it was the case in

sleeping sickness epidemic in Uganda and Burkina Faso (Okoth & Kapaata, 1986; Solano *et al.*, 1996).

The importance of understanding how interactions of the parasite-vector work/exist is essential due to the fact that it can lead to the development of novel control strategies to interrupt disease transmission. Genetic manipulation of tsetse midgut symbionts (*S. glossidinius*) can be used to interrupt parasite transmission within the insect vector (Dale & Welbun, 2001), as most obligate haematophagous insect, depend on symbionts to provide essential nutrients (Lehane, 1994).

Effects of lectins on trypanosome development and survival in tsetse flies

African trypanosomes evade immune destruction in the mammalian host by continually changing the structure of a glycoprotein that covers the external surface of the parasite, the variant surface glycoprotein (VSG). Each trypanosome has many VSG genes and may occur in partially homologous genes families. However, the homology is very little among members of different gene families except at the 3' ends of the genes. Antigenic variation is one of the unique evolutionary mechanisms of trypanosomes. Within the nuclear genome, genes coding for variable surface glycoprotein (VSGs) exhibit gene switching. These codes for surface proteins that protect the parasite from phagocytosis by macrophage and lysis by alternative complement pathway (Vickerman, 1994). Despite the VSGs in trypanosomes, tsetse flies contain glucosyl and galactosyl lectins which are lytic to trypanosomes, presumably through interaction with the procyclic surface coat (Welburn *et al.*, 1993 & 1994), a successful development of the protective effect achieved by the release of N - acetyl - glucosamine (GlcNAc) by the endochitinase activity of the rickettsia - like organisms present in the midgut of some tsetse fly species. So the binding of lectins to the surface of procyclic forms appears to be of key importance in determining signal transduction events implicated in establishment, maturation & cell death of trypanosomes in the midgut of the insect vectors. In other research, it has been proposed that the binding of midgut lectins to terminal carbohydrate

residues of procyclin may lead to cell death, while binding to other parts of the molecule might stimulate proliferation (Maudlin & Welburn, 1994)

INSECT IMMUNITY

Insects have been particularly successful in evolution. It is currently estimated that 90% of all known species with the animal kingdom belong to this class. The evolutionary success of the insects has been their ability to invade and exploit a diverse range of ecological niches. Insects have evolved and, in many situations, thrived in environments replete with potentially parasitic and pathogenic competitors. Disease in insects may reduce vigour, productivity, and ultimately survival of individuals and thus may contribute to the regulation of population levels. The presence of both infectious organisms and metazoan parasites in ecosystems occupied by insects has exerted a strong selection pressure for insects resistant to infection (Dunn, 1986; Labandeira & Sepkoski, 1993). In this case, the modern species of insects may represent the most extensive summary of successful defensive strategies against infection in the animal kingdom.

Primary defence systems include the rigid exoskeleton (cuticle), which protects the insect from pathogens and endoparasites. No epithelia are directly exposed to invaders since the cuticle completely covers the outside as well as the foregut, hindgut, and tracheal tubes. Even the intestinal epithelium of the midgut is protected in most insects by the peritrophic matrix, a structure that encloses the gut content. The peritrophic matrix surround the food bolus and protect the midgut epithelium is very important especially for blood sucking insects which in most cases come in direct contact with live parasites present in the blood of the host. The peritrophic matrix lines the gut of most insects at one or more stages of life cycle. It also facilitates the digestive processes in the gut and the protection of the insect from invasion by micro-organisms and parasites (Lehane, 1991 & 1997).

The secondary defence system includes the cellular and humoral defensive responses. The initial haemolymph response of insects to foreign particles is mediated by circulating haemocytes. Insect haemocytes are extremely efficient at removing foreign particles such as bacteria, fungi, nematodes, and eggs of hymenoptrean endoparasites from the hemocoel, by either phagocytosis, nodule formation, or encapsulation (Ratcliffe, 1993).

Phagocytosis occurs when particulate material like bacteria are engulfed and taken into haemocytes in large vesicles. Phagocytized bacteria are then killed and their remains are digested. This process is effective as a primary defence mechanism against a certain number of bacteria below a certain threshold level, which may vary for different insect species. When the number of bacteria is very high i.e. above a threshold, phagocytosis is augmented by nodule formation (Ratcliffe & Walters, 1983). Both the bacteria and haemocytes become trapped to the tissues and are melanized. The speed and size of nodule formation is direct proportional to the pathogenicity of the bacterium. It is believed that the more pathogenic the bacterium is, the larger the nodules and the more rapid they are formed (Ratcliffe & Walters, 1983).

Encapsulation is another form of cellular defensive response, and involves the formation of multilayered cellular envelopes around the foreign objects (including mature nodules) in the insect hemocoel. Melanin deposits form in the inner layers of the capsule near the surface of the foreign object. The formation of this capsule initially restricts growth and movements of the invader and may result in its death (Dunn, 1986). The two processes above are effected by two classes of cells namely plasmatocytes and granulocytes. Granulocytes and plasmatocytes are the primary phagocytes and plasmatocytes are the predominant cells in the capsules. It is postulated that granulocytes initiate both nodule formation and encapsulation by discharging granular content on the surface of the foreign particles. It is in the

interest of the insect to get rid of all encapsulated and melanized material out of its body to avoid being intoxicated by accumulating wastes.

Humoral responses occur naturally and are non specific. In some cases they are inducible, for example if viable non-pathogenic bacteria and of sublethal doses or vaccines of pathogenic bacteria are injected into individuals of several insect species elicits an acquired humoral immunity to subsequent bacterial infection that may persist for several days depending on species (Dunn, 1986).The development of the acquired protected state corresponds temporally with the synthesis of several proteins that appear in the haemolymph. This group of proteins includes the enzyme lysozyme (which hydrolyses bacterial cell walls), several families of bactericidal proteins that kill gram-negative bacteria, and many additional proteins with different biological activities (Hetru *et al*, 1994). The synthesis of these haemolymph proteins requires new RNA synthesis and occurs within hours following a septic injury or invasion. The synthesis of new haemolymph proteins in response to infection has been demonstrated in both larval and pupal stages of several species. These proteins may differ between developmental stages within a single species (Meister *et al*, 1997), an indication that insect developmental hormones are sometimes involved in modification of humoral defensive responses.

Apart from lysozyme, other known antibacterial compounds include Drosocin from *Drosophila melanogaster*, cecropins from Lepidoptera (*Hyalophora cecropia*) and some Diptera, attacins from *Manduca sexta* (Meister *et al*, 1997). In some cases these compounds have been found to work together in response to infection. For example, it has been demonstrated that attacin facilitates the action of cecropin and lysozyme, therefore enabling the three immune proteins to work in consonance (Boman & Hultmark, 1987) against *E. coli* in the gut of *Antherae* larva. Other inducible antibacterial proteins were detected in the larvae of the fly *Phormia terranovae*, called diptericin. Amino composition and sequence data show that

diptericin constitute a novel family of antibacterial proteins different from cecropins and attacins (Boman & Hultmark, 1987).

In order for the invading particle to be recognised, insects have a mechanism which enables them to recognise the foreign bodies. The invader is either known as a result of physiochemical parameters such as differences in surface charge and hydrophobicity between its own tissues and those of the foreign body, and or by a direct interaction between molecules on the surface of the invader and receptors on the haemocytes, and or intermediary molecules which act like a bridge between the invader and the hemocyte (Hoffman & Reichhart, 1997). The first candidate is prophenoloxidase, this is widely distributed in both plants and in invertebrates. The enzyme is very reactive and is always stored in the form of an inactive prophenoloxidase. The end product of the prephonoloxidase reaction is melanin, a dark insoluble material deposited around micro-organisms or parasites in nodules and capsules. A similar reaction is also seen after wounding, and may have a protective function. Melanin is also postulated to be responsible in forming a mechanical barrier, preventing growth of entrapped parasites. It is also believed that the intermediates in melanin formation could be toxic to invading organisms. Detailed studies of prophenoloxidase from the silkworm *Bombyx mori*, indicated that the purified proenzyme was a dimer, and was activated by a specific serine protease (Lee *et al.*, 1996). The activation of this enzyme is started by bacterial peptidoglycan but not lipopolysaccharide. The activating enzyme removes a 5-kd peptide from each subunit of the prophenoloxidase. The activated one then binds strongly to various substrates and during the reaction it is found as aggregates of progressively higher molecular weight.

Secondary, agglutinin (lectin) present in the haemolymph plasma of some insects is one of the candidates for this recognition function. These carbohydrate-binding proteins are wide spread among prokaryotes and eukaryotes. Among the eukaryotes, lectins mediate many specific biological functions including cell-cell interactions,

protein trafficking, and primitive defence reactions (Drickamer & Taylor, 1993). It is believed that insect haemocytes possess cell surface lectins that bind foreign substances bearing appropriate carbohydrate moieties. They are known for their capacity to agglutinate red blood cells. The agglutination of red blood cells has in many cases been attributed to lectins, proteins with a highly specific multivalent capacity to bind to certain sugar residues on erythrocyte membranes. Apart from multiple binding sites specific for different carbohydrates, there is aggregation of different specific subunits in a single isomer and the occurence of multiple lectins. Multiple lectins are thought to be important in increasing the recognition capabilities of that individual, also for enhancing the activation of prophenoloxidase in haemocyte lysates of insects like *Blaberus discoidalis* (Chen *et al.*, 1993). The protein is inducible by injury in larvae of insects like *Sarcophaga peregrina*, but is made constitutively in pupae. In *S. peregrina*, the protein is composed of six subunits (four α-subunits and two β-subunits), and has shown to have specificity to galactose. The lectin is synthesised during embrogenesis and pupation (Komano *et al.*, 1980). The main possible function of lectins could be to agglutinate invading micro-organisms that carry surface components containing the corresponding sugar residues. The agglutinated foreign cells would then be more easily phagocytosed or encapsulated, and melanized.

TSETSE AND TRYPANOSOMIASIS IN TANZANIA

Tanzania lies between latitudes 1° and 12° South and longitude 30° and 40° East and has an area of 945,090 square kilometre. 70% of the land is tsetse infested by 10 species of subgenera *Glossina*. Climate plays a major role in affecting their distribution. As a result, trypanosomiasis is ranked third after rinderpest and tickborne diseases in affecting and killing livestock. *T. congolense* has the highest incidence (57%) and is the most pathogenic trypanosome. The human form of the

disease is Rhodesian sleeping sickness which is a zoonosis with bushbuck as a major reservoir host (Msangi, 1992).

The recorded history of human diseases in the country dates back to 1903 when an epidemic occurred as a result of inter-territorial movements of people in the infected areas and particularly movement of infected people. This is the case because during the early Arabian traders (695-1550 AD) the disease was not reported despite the movement of traders from coastal areas to the mainland of Tanzania (Farbrain, 1948 & Apted, 1970).

Initial outbreaks of trypanosomiasis in Tanzania were caused by *Trypanosoma gambiense* which originated from West Africa and reached Tanzania via Zaire around 1902. *T. rhodesiense* which is currently responsible for human trypanosomiasis in Tanzania was introduced from Mozambique around 1910 and quickly spread to many parts of the country. Transmission of the disease is by game-fly-man contact. The spread of Rhodesian sleeping sickness is also influenced by temperature. Burtt (1946) found that the infection rate was high in flies which had emerged from puparia maintained at high temperatures. Places where the mean annual temperature is high and the reservoir host available e.g. bushbuck and the vector present (*G. pallidipes*), the infection has generally been higher than places where the mean temperature is low (Heisch *et al.,* 1958). The disease is currently prevalent in the western, north and north-western parts, the southern highlands and southern regions. Over 6000 cases have been reported since 1979. Other report indicate that in the past 30 years, the number of new cases reported annually rarely rose above 500 (annual sleeping sickness reports for Tanzania, 1965 - 1995), although this is likely to be an underestimate (Komba *et al.,* 1997).

Tsetse distribution in Tanzania

Tsetse distribution in Tanzania has been published by Ford & Katondo (1977) and by Moloo (1985). The distribution is influenced by vegetation type, temperature, relative humidity, human habitation, host availability i.e. feeding preferences as

indicated in Table I & II (Appendix) However, remapping is needed to have the true picture of how tsetse is distributed today and their economic importance specifically to the growing livestock sector in the country. Tsetse flies found in the country are as follows:

Austenina (Fusca): *G. longipennis, G. brevipalpis, G. fuscipleuris.*

Nermohina (Palpalis): *G. fuscipes fuscipes, G. f. martinii* Zumpt 1933.

Glossina (Morsitans): *G. morsitans morsitans, G. m. centralis* Machado 1970
G. swynnertoni, G. pallidipes, G. austeni.

G. brevipalpis

Occupy the residual and secondary forest communities of Tanzania. It is also reported in the forested areas of Mtwara, Lindi, Pwani, Dar Es Salaam and Tanga. It is also found in forests along the lake shores of Mara, Shinyanga and Kagera regions. The presence of the species in Mafia Island has been confirmed recently by sticky panels (TTRI, 1998 unpublish.). Its host preference includes bushpigs, buffalo and Hippopotamus.

G. longipennis

Found in semi arid parts of Northern Tanzania, in Arusha, Mara and Shinyanga. Host preferences include elephant and buffalo.

G. fuscipleuris

Inhabit forest of the savannah mosaic and one the edge of tropical moist forests. Occur in the isolated fly belts near Mara region and in Ruvuma region, near Tanzania - Malawi boarder. Feed on suid like hog and bushpig.

G. fuscipes fuscipes

A riverine species, restricted to gallery forests of savannah climate especially around lake shores of L. Victoria. Feed on bovid, reptiles and man.

G. f. martinii

Found along the lake shores and the rivers draining into L. Tanganyika. Feed on bovids, reptiles and man.

G. morsitans

Infest large areas of Tanzania mostly in miombo woodland, in vegetation dominated by *Brachystegia* trees. The western part of the country is dominated by *G. m. centralis* covering an area from Uganda to the bottom of L. Tanganyika. Infest the eastern part of the country, including lower areas of the Usambara Mountains and the southern part of the country. The distribution is not continuous as often it is disrupted by cities, human settlement and natural barriers e.g. high altitudes. Feed on bovids mainly kudu and suids.

G. swynnertoni

Found in the drier and more open type of woodland, characterised by trees of *Acacia* and *Commiphora* often with presence of small thickets. Found in the south east corner of L. Victoria and SW of Arusha region. It commonly co-exists with *G. longipennis*. It feeds on suids (warthog), giraffe and buffalo.

G. pallidipes

Found in thickets and forest edge areas but also can be found in open areas such as the *G. morsitans* habitats. Like *G. brevipalpis* it is widely distributed in the eastern part of Tanzania, but also in isolated pockets of dense thickets in Arusha and Kilimanjaro. Feed on bovids such as bushbuck and suids mainly warthog and bushpigs.

G. austeni

Found in dense thickets but can seek food in open areas. Occur along the coastal plains of the country between Pangani and Bagamoyo, extending to Lindi. In

Unguja Island of Zanzibar, it used to inhabit the Jozani forest before the recent, successful SIT campaign (Bailey, 1998). Feed on suids such as bushpigs.

The economic importance of tsetse in Tanzania

Tsetse flies are the vectors of trypanosomes, the causative organisms of trypanosomiasis, called nagana, in animals and sleeping sickness in man. Many of the wild mammals in tsetse infested areas are infected with trypanosomes without suffering disease symptoms, and they act as reservoirs of trypanosomes, hence wherever tsetse bites on them, there is a probability that tsetse flies will pick the trypanosomes.

In Tanzania, tsetse preclude a large part of the fertile land from being utilised for farming and grazing, thus leading to overgrazing in smaller areas which are tsetse free. Apart from that the presence of tsetse leads to under-exploitation of other natural resources (Finelle, 1974). As indicated in Fig 1.2, areas infested with tsetse are under utilized both in terms of mixed farming. The direct consequences of the vector and the disease include mortality for man and cattle, disease leading to emaciation, retarded growth, abortions and temporary sterility. It is a cost to the country in terms of expenses incurred on control operations for the vector and for chemoprophylaxis and trypanocidal drugs. Indirect consequences include shortages of meat and milk causing protein deficiency, effects on agricultural output through limiting tillage, livestock diversity and in general the vector and the disease limits the expansion of the agricultural sector (Finelle, 1974).

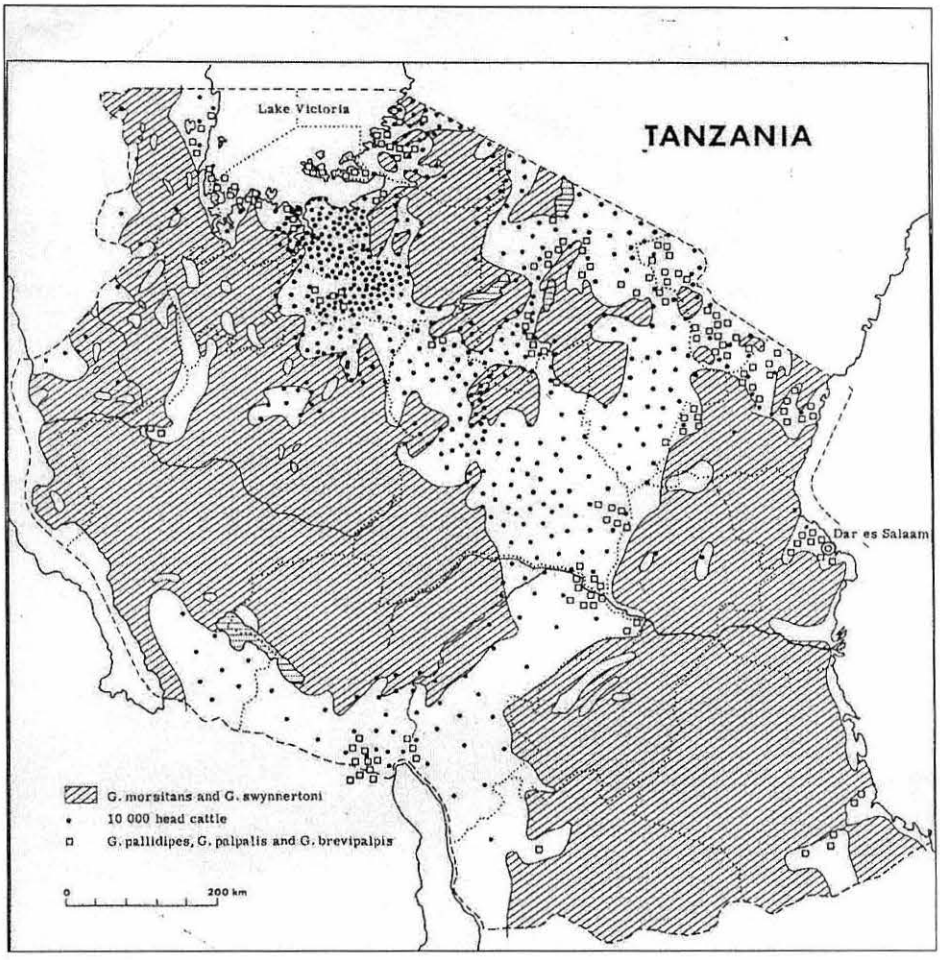

Figure 1:2. The influence of the tsetse fly distribution on animal production in Tanzania

Source: Finelle, (1974)

Four subgenera of trypanosomes occur, as reviewed by Logan-Henfrey *et al.*, (1992). In subgenus *Duttonella*, the development occurs only in the proboscis of the vector. These include species like *Trypanosoma vivax* (which cause diseases in ruminant livestock) and *T. uniforme. T. congolense* and *T. simiae* (*Nannomonas*), develop in the proboscis and midgut of the vector. The *Trypanozoon* are found in salivary glands and midguts and include *T. brucei brucei, T. b. rhodesiense* and *T. b.*

gambiense. Pycnomonas which include *T. suis* and is found in salivary glands and midguts (Table 1:2).

Table 1:2 Trypanosomes of medical and veterinary importance in Africa

Subgenus	**Species**
Pycnomonas	*T. suis*
Duttonella	*T. vivax* Ziemann, 1905 *T. uniforme* Bruce *et al.*, 1911
Nannomonas	*T. congolense* Broden, 1904 *T. simiae* Bruce *et al.*, 1912
Trypanozoon	*T. brucei gambiense* Dutton, 1902 *T. b. rhodesiense* Stephan & Tantham, 1910 *T. b. brucei* Plimmer & Bradford, 1899 *T. evansi* Steel, 1885 *T. equiperdum* Doflein, 1901

Source: Logan Henfrey *et al.*, (1992)

Animal Trypanosomiasis and control

The Animal trypanosomiasis is a disease of veterinary importance. It is a major constraint to livestock production in areas that are infested by tsetse fly. The cause of the disease in individual animals is affected by a number of factors. Not all species of animals are susceptible to all species of *Trypanosoma* and even within each species of *Trypanosoma* there is a wide range of strains of widely differing degrees of virulence, and some result in a long drawn out chronic disease. The type of husbandry practised has a major effect on the course of the disease in an infected animal. An animal which has a low plane of nutrition and which has to work or trek long distances for grazing and /or water is much more likely to suffer severely. The exotic cattle are at great risk and succumb rapidly to the disease, zebu cattle

are in general susceptible to the disease, but usually take a chronic form, whereas certain breeds, including the N'Dama and certain small West African shorthorn breeds are much more resistant to the disease unless exposed to high density of tsetse populations (Jordan, 1986).

T. congolense* and *T. vivax

T. vivax and *T. congolense* are the most important parasites for cattle and it is important but difficult to distinguish most infections clinically, although acute haemorrhage infections of *T. vivax* are a distinct syndrome. The form of the disease can vary from acute, with fulminating parasitaemia and death within two weeks of infection, to chronic, with no clinical symptoms. Soon after infection there, is an acute phase of the disease with temperature rising to 39 – 41°C, and with numerous trypanosomes in the blood and sometimes in other tissues. If the animal does not die at this stage, with no obvious signs, other than fever, it may deteriorate rapidly and die within a few weeks (Jordan, 1986). With the indigenous African cattle the disease will enter a chronic stage which can persist for months or even years. As a result the animal becomes emaciated and anaemic and in the terminal stages is recumbent and comatose. Trypanosomes may only infrequently be detectable in the blood. Pregnant animals suffering from chronic trypanosomiasis are liable to abort. *T. congolense* is the commonest cause of the infection and anaemia is one of the most important symptoms of the disease.

Infection of *T. congolense* and *T. vivax* in donkeys and horses can be fatal and they will often give rise to a chronic disease from which the animal recovers spontaneously. However pigs are completely refractory to *T. vivax,* but *T. b. brucei* and *T. congolense* will affect them with relatively low pathogenicity (Stephen, 1966).

T. brucei

In *T. b. brucei* infections, trypanosomes are generally scanty in the peripheral

blood and are rarely detected by examination of thick or thin blood films, except by inoculation of blood from cattle into laboratory rodents which are extremely susceptible to this parasite (Godfrey & Killick-Kendrick, 1961). The parasite cause acute disease in dogs. Many African indigenous cattle though readily infected do not show any clinical signs of the disease. Horses, donkeys and mules are highly susceptible to infection with *T. brucei* and death of untreated animals usually occurs within 3 months. The clinical symptoms are similar to those of the cattle disease with chronic cases showing increasingly severe emaciation and anaemia (Jordan, 1986).

T. godfreyi* and *T. simiae

Almost all trypanosomes are infective to cattle except *T. simiae* which is the most dangerous parasite of domestic pigs, causing a massive parasitaemia with death after a prevalent period of 4 – 6 days following a feed by infected tsetse flies (Stephen, 1966). T. *godfreyi* is also known to cause a fatal disease in pigs (McNamara *et al.*, 1994).

T. evansi, is a parasite transmitted by biting flies other than tsetse and is a causative organism of a form of camel trypanosomiasis known as surra, in the Middle East, Asia as well as in Africa (Jordan, 1986). *T. suis*, is of limited distribution and importance.

The distribution of *Glossina* species and the location of cattle in Tanzania are the converse of each other, where there is no cattle there are tsetse flies and vice versa (Finelle, 1974) (Fig. 1:2). The causative agents of animal sleeping sickness *are T. vivax, T. congolense, T. simiae* and *T. b. brucei*. The disease is widely distributed, thus very much affecting the livestock sector. However due to the high degree of resistance exhibited by cattle, *T. congolense* is the most pathogenic parasite for livestock (Msangi, 1992).

According to Willett (1970), *G. pallidipes* is a good vector for *T. congolense* even when it is present in very low densities. During dry periods animals are forced into deep bushes and forests in search of pastures and water, as a result the tsetse- cattle contact is very much increased, leading to an increase in the disease. The importance of the disease is also accelerated by poor availability of food especially if it is of poor quality, plus the stress imposed on animals by other diseases like tick-borne and East Coast Fever (Fox, 1991).

Control strategies against tsetse borne diseases in Tanzania include chemical control of vectors, treatment of animals. However, trypanocides alone do not provide a permanent solution to trypanosomiasis, though they eliminate suffering to cattle with the disease. Recent findings by Geerts & Holmes (1998) shows that all drugs currently in use throughout the tsetse belts, have shown various resistance against all trypanosomes species i.e. *T. vivax, T. brucei* and *T. congolense* (Table 1:3). This problem was long reported in Tanzania against Berenil and Samorin by Silayo *et al.*, (1992) and Fox *et al.*, (1993) respectively.

Isometamidium (Samorin), Ethidium (Homidium bromide) and Diminazene (Berenil) have been in use for more than 35 years and it is estimated that about 35 million doses per year are currently used in Africa. Since there is no indication that new products will become available in the near future, it is of utmost importance that measures are taken to avoid or delay the development of resistance and to maintain the efficacy of the currently available drugs (Geerts & Holmes, 1998). This can only be done through proper diagnosis of the diseased animal and proper treatment.

Table 1:3 Survey for drug resistance in trypanosomes

Country	Trypanosome Species	Number of Isolates Exam.	resist	% of Resistant isolates	Resistant To	Reference
Burkina	Tc	12	9	75	I	Pinder & Authie, 1984
Ethiopia	Tc	12	12	100	D	Codjia *et al.*, 1993
			11	92	I	
Ethiopia	Tc	10	10	100	D, H ,I	Mulugeta *et al.*, 1997
Kenya	Tc	7	2	29	I	Gray *et al.*, 1993
Kenya/ Somalia	Tv	7	6	86	I	Schonefeld *et al.*, 1987
			3	43	H	Ainanshe *et al.*, 1992
			5	71	Q	
Nigeria	Tv	19	12	63	D, H, I	Ilemobade, 1979
Nigeria	Tb	12	2	17	D, I	Kalu, 1995
			1	8	I	
Sudan	Tc, Tv, Tb	12	5	42	H	Abdel Gadir *et al.*, 1981
Uganda	Tb	36	1	3	D, I	Matovu *et al.*, 1997
Zimbabwe	Tc	14	6	43	D	Joshua *et al.*, 1995

D = diminazene; H – homidium bromide (ethidium); I – isometamidium; *Tc – T. congolense; Tv – T.* vivax; *Tb – T. brucei*

Source: Geerts & Holmes, (1998)

Human Trypanosomiasis, epidemiology and control

Two trypanosomes, *T. b. gambiense* and *T. b. rhodesiense* are the causative organisms of human trypanosomiasis. These are commonly referred to as the Gambian and Rhodesian sleeping sicknesses respectively. Both forms of sleeping sickness are diseases of the central nervous system and other tissues. There is an initial period of blood infection, which is followed by invasion of the cerebrospinal fluid and of the brain. The time course of these events varies. For the disease caused by *T. b. gambiense*, invasion of the central nervous system is delayed and death of

an untreated person may not occur for several years. Many months may be spent in an emaciated condition, with the patient displaying the typical "sleeping " symptoms.

In the typical disease caused by *T. b. rhodesiense*, there is a rapid development of severe toxaemia and if untreated the victim can die within a few weeks or months of infection. Typical features of the diseases are invasion by trypanosomes and consequent enlargement of the lymph glands especially the posterior cervical glands, and oedema of the face.

The diseases occur within the respective distributions of the two causative organisms (Scott, 1970 & Apted, 1970). In general, the approximate distribution of known foci of sleeping sickness in Africa can be described as Western and Central Africa for *T. b. gambiense* and Eastern foci for *T. b. rhodesiense* (Seed &, Hall, 1992). The vectors of *T. b. rhodesiense* are *G. morsitans* and its subspecies, *G. pallidipes*, *G. swynnertoni* and *G. fuscipes* of the palpalis. The *T. b. gambiense* is transmitted by tsetse fly species of the palpalis subgroup.
In Tanzania, the most important vector of the human disease is *G. morsitans* (*s. l*) and *G. swynnertoni. G. pallidipes* is encountered and *G. fuscipes* keeps reappearing in Kigoma (Kilama *et al.*, 1981).

The epidemiology of human sleeping sickness is far from being fully understood. Unlike animal trypanosomiasis, the distribution of both forms of human trypanosomiasis in Africa has been discontinuous both in space and time. It has long been recognised that the incidence of the disease is not directly related to the density of tsetse populations and epidemics can occur in regions where tsetse are relatively scarce. However, there are extensive areas where the vector species are widespread and abundant, but where sleeping sickness has never been recorded. Referring to all outbreaks of trypanosomiasis the epidemiologists tend to refer to periodicity (Baker *et al.,* 1990), epidemic outbreaks alternating with periods of low level endemicity.

In the Lake Chad basin and Northern Nigeria epidemics have occurred with a period of 25-30 years, and 20 year period in Zimbabwe. A 10-year periodicity is evident in Uganda and Tanzania (Baker *et al.,* 1990).

Currently between 4 and 5 million people in Tanzania are thought to be at risk of infection, but only 1% of these are under regular surveillance. At present, only the Rhodesian sleeping sickness occurs in the country. However the influx of refugees from the Gambian endemic countries on the western part of Tanzania increases the risk of forming new foci of the disease, especially when bearing in mind that the vector, *G. f. fuscipes* infests most of the western part (Komba *et al.,* 1997).

Control strategies against tsetse borne diseases in Tanzania include chemical control of vectors, treatment of patients with trypanocides and avoidance of human - tsetse contact (Kilama *et al.*, 1981). Treatment of human disease focuses on two stages; the early stage when the central nervous system is not yet infected by the trypanosomes and the late stage when the central nervous system is infected. Suramin and Pentamidine, are used in treating early stages of *T. b. rhodesiense* but not the late stages of the *T. b. rhodesiense* and resistant stages of *T. b. gambiense.* Melarsoprol is effective against late stages of human trypanosomiasis and as a chemo prophylactic against *T. b. gambisiense* (Jordan, 1986). However as a chemo prophylactic, Melarsoprol is not recommended in *T. b. rhodesiense* areas (WHO/FAO, 1979). The drug is not recommended to treat early stages of Gambian and Rhodesian sleeping sickness because early infections can be treated by Suramin. These drugs however, have severe side effects by affecting the optic nerves. Another drug is Efloinithine (α-Difluoromethylornthine, DFMO: Eflornithine; OrnidylR). This is licensed for treatment of late stage of Gambian sleeping sickness (Kuzoe, 1991 & Pepin *et al.*, 2000).

Tsetse borne trypanosomiasis occurs only in sub-Saharan Africa, thus the market is restricted to areas of uncertain economic stability, thus, no pharmaceutical firm is

likely to be prepared to meet the high development and production costs of a new drug for treatment from one economically poor region only. Hence new drugs are unlikely to be forthcoming in the near future (Jordan, 1986 & Geerts & Holmes, 1998). In Tanzania, Melarsoprol and Suramin have been drugs of choice and have been used extensively and effectively for both the early and late stages of the disease. Their side effects account for 1- 5% fatality of patients after injection (Veeken *et al.,* 1988).

Control of tsetse flies

Various methods have been applied to control tsetse flies especially in the past. These include methods like vegetation clearing. It well known that different types of vegetation serve as habitat for different species of tsetse. When the habitat is removed, tsetse flies disappear. This method was applied either by total clearance or by removing only the vegetation that was vital to the support of tsetse flies (Ford *et al.,* 1970). Another method was destruction of hosts. Tsetse feed on wild animals which act as a reservoir of trypanosomes infective to domestic animals and man. Removal of wild animals leads to the decline in fly infestation and reduction in the availability of trypanosomes. Control of tsetse by this method was widely used in East, Central and Southern Africa (Jordan, 1986). Game elimination was also practised in Tanzania, though on a small scale and led to the disappearance of *G. swynnertoni*, in areas like Shinyanga (Ford *et al.,* 1970). Biological control is another method which has been used though in small scale to control tsetse flies in Tanzania. This has been carried out using the Sterile Insect Technique (SIT). The method involve the control of insect populations by suppression of the fecundity of wild females following the release of sterile males into a natural population in a ratio that exceeds that of a natural population; thus increasing the chances of mating wild females by the sterilised males that produce nonviable offspring. Males are reared and sterilised before being released to mate the wild females. Insects are either sterilised by using ionising radiation or by mutagenic chemicals. So tsetse are either sterilised as adults or as puparia (Curtis & Langley, 1972; Van Der Vloedt *et*

al, 1978). Temporary significant SIT operations have been reported in Burkina Faso against *G. p. gambiensis* (Cuisanse *et al.*, 1980; Politzar & Cuisance, 1982), in Nigeria against *G. palpalis palpalis* (Takken *et al.*, 1986) and in Tanzania (Mkwaja ranch) against *G. m. morsitans* (Williamson *et al.*, 1983V). However, the most recent and more successful SIT application in Tanzania has been against *G. austeni* in Unguja Island of Zanzibar (Bailey, 1998). Before release in both areas population suppression was achieved by insecticide use; endosulfan at Mkwaja and animal bait dipped in deltametrin and insecticides impregnated screens in Unguja respectively (Williamson *et al.*, 1983 IV & Horeth-Bontgen, 1992).

The shortcoming of SIT is that it cannot be used on its own, need to be integrated with other control methods and is most efficient when population densities are low. Secondly, the method targets one tsetse specie at a time, hence for areas where several species co-exist it is not possible to control all species concurrently. In Mkwaja, *G. pallidipes*, which was not also controlled by SIT, rapidly returned to pre-spraying population densities, and lack of a reliable barrier led to re-invasion of tsetse from untreated areas. The case is different with *G. austeni*. Unguja is an Island, 35 km off coast of mainland Tanzania, re-invasion is unlikely to occur.

The commonly practised method against tsetse is by chemical means. Usually it is directed towards the vulnerable adult stage of the insect (Davies, 1978). Insecticides are either formulated as residual or non residual. The insect acquires a lethal dose through tarsal contact in the case of residuals like endosulfan and DDT; or as droplets impinging on the body surface for the non-residuals like the pyrethroid compounds. Application is either by ground spraying and aerial spraying for example in Nigeria, Kenya, Zimbabwe, Uganda, Cote D'Ivoire as reviewed by Allsopp (1984), odour baited and insecticide treated targets for example in Zimbabwe (Vale *et al.*, 1988) and through live animal baits i.e. by dipping or spraying cattle (Elliot *et al.*, 1973; Fox,1991).

In Tanzania, this is the most widely used method, and it dates back to the DDT era. The country was graced by having the Tropical Pesticide Research Institute responsible for formulation, calibration and optimisation of droplets and flow rate of aerial spraying equipment (Burnet, 1970).

The effectiveness of endosulfan against tsetse was first demonstrated in the country against *G. morsitans, G. pallidipes* and *G. swynnertoni* by aerial spraying (Hocking *et al.,* 1966). Other applications recorded include DDT and endosulfan against *G. swynnertoni* (Robertson, 1971); ground spray of synergised pyrethrum against *G. swynnertoni, G. pallidipes, G. longipennis* and against *G. m. centralis* by endosulfan (Tarimo, 1971; Irving *et al.,* 1969). The use of live baits using synthetic pyrethroids has been reported by Fox (1991) & Horeth-Bontgen (1992). Although chemical application achieves good tsetse control, the problem of re-invasion and the creation of barriers remain a serious setback. Examples of areas where re-invasion have occurred after control are the Okavango Delta, Nigeria, Niger and Senegal (Allsopp, 1984). This also applies for Tanzania.

Apart from that, insecticides used also have undesirable side effects on the physical and biotic environment because most of them are not selective and they will kill non-target organisms. Also, tsetse has shown to have a level of genetic variation similar to that in other invertebrates, and like other pests, responds to selective pressures hence a possibility of developing insecticide resistance (Gooding, 1992).

Tsetse control by traps and targets (with or without odours and attractants), have achieved significant results in Zimbabwe against *G. m. morsitans* and *G. pallidipes* from an Island in Lake Kariba (Vale *et al.,* 1986). In the Zambesi valley similarly baited black targets at a density of 3-5 traps/km^2 in six months reduced the population of *G. m. morsitans* and *G. pallidipes* to less than 0.01% in an area of 600 km^2 (Vale *et al.,* 1988). Similar achievements have been recorded in Kenya against

G. pallidipes (98-99% reduction) over 100 km^2 using traps (Dransfield *et al.,* 1990). However in Tanzania, this method has not been applied to a significant level.

Factors limiting the control of tsetse and tsetse borne diseases

Despite the fact that several methods have been employed to control tsetse and trypanosomiasis since 1900, the distribution of the two in Tanzania and the tsetse belt as a whole, seem to have been altered not to a significant level. It is unfortunate that for a variety of social, political and economic reasons, the necessary control programs have not been maintained. Active surveillance, treatment of infected individuals with a limited available drugs and control of vectors are no longer carried due to civil conflicts in countries like DRC, Sudan and Angola. Civil disturbances in Uganda saw the resurgence of the disease as a result of uncultivated and unattended farms being occupied by shrubs such as *Lantana camara*, which is a suitable vector habitat for *G. fuscipes*. However control efforts including active surveillance, rural treatment facilities and subsequent vector control with impregnated pyramidal traps led to a rapid decline in transmission (Abaru, 1985 & Kuzoe, 1991).

In Sudan, because of political and civil unrest, only 5% of the population were under passive surveillance in 1989. As a result, this has an effect on her neighbours as well. There is a persistent outbreak of the Gambian sleeping sickness in the Northern area of Uganda arising out of infection imported by people returning from Sudan. Out of 425 patients diagnosed for the Gambian disease in the first quarter of 1990, 59.1% were southern Sudanese, 39% Ugandans returning from Sudan and 1.8% indigenous Ugandans (Kuzoe, 1991). In Tanzania, some of the Rhodesian cases reported in the country are from new immigrant refugees from Burundi. This indicates that refugee movements may also contribute to the transfer of the disease and strains of trypanosomes (Komba *et al.,* 1997). Thus political stability and co-ordinated efforts against the vector and disease are vital for controlling if not eliminating the disease (Maina, 1977 & Jordan, 1985b).

Economic constrain is another setback toward feasible tsetse control programs. The budgets set to meet the sleeping sickness control programme are usually so meagre to have any significant outcome. In most cases, disease which have a short incubation period are given a required priority compared to tsetse borne disease for human beings which have a relatively longer incubation period. As a result, there is a tradition of total or partial dependency on external aid by either bilateral or multilateral donors which in most cases are not reliable for a long term control programme as most of them will be given only in case of an emergency as it happened in the human African trypanosomiasis (HAT) flare up in Uganda (Kuzoe, 1991). Economic reasons are the major constrain towards a feasible and sustainable tsetse control programmes in Tanzania.

Other set back against control of tsetse and tsetse borne diseases include:-

- ***Changing nature of reservoirs***

It is now clear that domestic animals are involved in the transmission of HAT. *T. b. gambiense* and *T. b. rhodesiense* parasites were found in domestic pigs (Mehlitz, 1985) and in cattle (Robson *et al.*, 1972) respectively. Studies in South Eastern Uganda have again confirmed domestic animal involvement in HAT transmission (Hide *et al.*, 1996). The study showed that 25% of trypanosomes of the *brucei* subspecies isolated from cattle were isoenzymatically similar to trypanosomes infective to man. Change in environment, fauna and host availability following human activities has resulted into the modification of tsetse feeding patterns (Hide *et al.*, 1996 & Clausen *et al.,* 1998). The increasing number of reservoir hosts calls for an integrated HAT control strategy by involving regular medical surveillance of population at risk, tsetse control and treatment of infected animals with veterinary trypanocides mainly diminazene and, occasionally isometamidium. Removing infection from humans alone seems is not enough to remove the reservoir of infection of HAT (Kuzoe, 1991 & Welburn *et al.*, 2001).

- ***Presence of different genetic populations of trypanosomes***

Trypanosomes demonstrate a capacity to express different surface antigens (Vikerman, 1978; Gray & Luckins, 1976; Doyle, 1977). These antigens are glycoproteins that cover the surface of the trypanosome (Turner, 1982). During infection, different variable surface glycoproteins (VSG's) can be expressed, giving rise to trypanosome populations of different variable antigen types (VAT's). The antibody responses that can contribute to the protection of the infected host are thought to be directed against the exposed determinants of the VSG (Murray & Urquhart, 1977). The ability of antibodies to protect against trypanosome infection has only been suggested once (Olenick *et al.,* 1988). Antigenic variation, therefore, enables the parasite to evade the host's immune response and allows the infection to persist (Vos & Gardiner, 1990). Also there is a clear evidence of genetic exchange within the *T. b. brucei* population while in *T. b. rhodesiense* the exchange is limited. Furthermore there is a clear distinction between the *T. b. rhodesiense* populations from Tororo in Uganda and Zambian foci, which suggest that at least two different strains (and possibly subspecies) form the genetic makeup of *T. b. rhodesiense* (Hide *et al.,* 1996). Work done by Komba *et al.* (1997) has shown that Tanzanian stocks of *T. b. rhodesiense* collected in 1991 - 1994 were distinct from representative stocks from East Africa foci. This proves that *T. b. rhodesiense* stocks form a mosaic of different genotypes varying from focus to focus in East Africa. The fact that genetic exchange can take place between *T. b. rhodesiense* and *T. b. brucei* (Gibson, 1989), and the fact that 2 subspecies co exist naturally in vectors and reservoir hosts, leads to the speculation that *T. b. rhodesiense* stocks evolve locally through frequent genetic exchange with their sympatric *T. b. brucei* stocks. In the Lambwe valley there are different genetic population of *T. brucei* responsible for the epidemiology of *T. brucei.* The population genetics and epidemiology of *T. brucei* which is complex and evolve through time (Mihok *et al.,* 1990), complicates the epidemiology and control of human and animal sleeping sickness in affected areas.

- ***Difficulties of Diagnosis***

Diagnosis of trypanosome is relatively difficult. Not only there are no specific clinical signs, but the intermittent and usually low parasitaemias make detection of trypanosomes difficult. First, infection is not synonymous with disease and many sub clinically affected animals live in delicate balance with potentially pathogenic trypanosomes (FAO, 1992).

However, diagnosis is an essential requirement in the management of disease both at the level of the individual, when a decision has to be made whether to treat or not, and at the epidemiological level for evaluating the performance of disease control strategies. The centrality of diagnosis in disease management has therefore, led to the introduction of a variety of tests for the detection of trypanosome infections as follows:

I. Parasite detection.

This is based on demonstrating, by direct microscopy, *in vitro* culture at 25°C or animal inoculation, the presence of trypanosomes in peripheral blood, cerebrospinal fluid, bone marrow, lymph gland fluid or in chancre aspirates (Baker, 1970; Woo, 1971 & Lumsden *et al.,* 1979). However, this is often difficult to achieve. First, because in the clonic stage of the disease, there is a scarcity and periodicity of trypanosomes in the peripheral blood which often precludes the detection of parasites by direct microscopy (Nantulya, 1989). Secondly, examination of the bone marrow or cerebrospinal, lymph gland and chancre fluids requires surgical manipulations that are difficult to carry out on a large number of clinical suspects or in epidemiological surveys. Lastly, facilities for animal inoculations and *in vitro* culture are expensive and unavailable at the dispensary or health centre level in the endemic areas (Nantulya, 1989).

II. Indirect Diagnosis methods

Antigenic variation of trypanosomes in the blood has lead to application of numerous indirect tests in diagnosing trypanosomiasis. This includes the capillary

tube agglutination test and immunoprecipitin tests (Aiyedum *et al.,* 1976; Taylor & Smith, 1983), indirect immunofluorescence antibody test (IFAT) (Wery *et al.,* 1970), indirect haemagglutination (Bone & Charlier, 1975), card agglutination (CATT) (Magnus *et al.,* 1978) and enzyme immunoassays (ELISA) (Voller, 1977). The short comings of these methods include first that they detect antibodies but cannot distinguish between an active infection and one that has been cured. Secondly, with the exception of the CATT test, other assays do not utilise antigens of defined specificity or purity. In the CATT test, the variable surface antigens of selected trypanosome antigenic variants are stabilised on whole trypanosomes by the mild fixation technique described by Nantulya & Doyle (1977) and used for detecting variable antigen specific antibodies in patient sera. This is important in order to standardise and avoid false- positive reactions in the presence of other parasite diseases (Voller, 1977; Van Meirvenne & Le Ray, 1985).

In an effort to come up with technologies which are likely to give accurate information on the current status of infection, techniques for trapping trypanosome antigens (Ag-ELISA) and amplifying trypanosome DNA by the Polymerase Chain Reaction (PCR) are in place. These technologies, with some improvement, give prospects of revolutionising the diagnosis of trypanosomiasis in animals, man and tsetse flies. A brief review on molecular techniques is given below.

DIAGNOSIS OF TRYPANOSOMIASIS USING MOLECULAR TECHNIQUES

The problems discussed above have led to the investigation of DNA-based methods for identification of parasites in most blood sucking insects like mosquitoes and tsetse flies. DNA-based technologies differ from most other identification techniques in that they detect the DNA itself, the genetic blueprint of each living organism in its most simple chemical form. DNA remains constant irrespective of

life stages. The durability and stability of DNA create few problems for storage or handling, enabling testing of frozen, dried or alcohol-preserved specimens. The aim in all cases has been to develop a methodology which is cheap, accurate and easy to use. DNA-based methods of identification ultimately rely upon the use of either DNA probe hybridisation or polymerase chain reaction (PCR).

DNA probes are essentially single-stranded DNA sequences, tagged with a detectable moiety such as radioactivity, that recognise specific complementary single-stranded target DNA sequences in the species of interest and demonstrate their presence by annealing or hybridisation. Hybridisation to the immobilised target DNA is observed by fixation and detection of the labelled moiety. A specific species is identified by the fact that hybridisation of the probe has occurred. The diagnostic use of DNA probes is of advantage because of its relative rapidity using hybridisation assays, the ease of processing large numbers of samples, and the ability to distinguish between morphologically similar species. Also, unlike serological assays, the use of DNA probes yields information on current infection status, and does not depend upon the host's immune competence. However the focus is on PCR method, so DNA probe methodology will not be reviewed in detail.

PCR represents a powerful new technology with a variety of field application such as studies on the epidemiology of parasitic infections, on measures to control the disease and clinical evaluation of the infecting species. PCR is an enzymatic DNA amplification procedure. Specific DNA fragments are synthesised by successive rounds of thermal denaturation, annealing and synthesis using a thermostable DNA polymerase and single-strand primers derived from sequences flanking the target fragment. The reaction product may be visualised on an ethidium bromide-stained agarose gel under ultraviolet illumination. Either the presence of a PCR product or a PCR product of a given size indicates the species of the specimen (Hill & Crampton, 1994). The method is sensitive and specific thus allowing identification of parasite species and strain in situations which were not previously possible.

Trypanosome detection and species identification in tsetse flies are laborious because they require dissection and microscopical examination of potentially infected insect organs. The identification is based on the localisation of trypanosomes in different vector organs (Lloyd & Johnson, 1924). In addition, the sensitivity of trypanosome detection by visual examination of vector organs is low, and the presence of parasites is usually only detected in insects with relatively high levels of trypanosomes. Furthermore, microscopical methods fail to identify mixed or immature infections when parasites are only found in the insect midgut. Thus, a more sensitive technique like PCR for the detection and identification of parasites even at lower numbers of trypanosomes down to a single trypanosome, and to a different trypanosomes species infecting tsetse flies is essential for a better understanding of the epidemiology of these diseases, both human and animal, and has to be accurately determined (Masiga *et al.,* 1992). Species-specific primers are needed for DNA amplification of all trypanosome subgroups for PCR to be effective in identification of trypanosomes (Morlais *et al.,* 1998b). The use of DNA based diagnostic methodology like PCR is valuable in analysing the epidemiology in the field. PCR can also be used to detect trypanosomes from blood fluids (Boid *et al.*, 1999). Can be used in genetic characterisation of metacyclic and blood stream forms and can thus improve taxonomic knowledge, characterise the hybrid products of genetic exchange, take part in the study of the parasitic populations or contribute to the identification of possible animal reservoirs of human trypanosomiasis. The PCR based method is sufficiently sensitive to identify trypanosomes directly in the vector, which constitutes prerequisite for more efficient vector control in the field. It may also allow or enable the characterisation of drug resistant populations and can constitute a rapid method to distinguish between relapse and re-infection, or mixed infections (Biteau *et al.*, 2000).

This work was aimed at studying (i) the immune response of blood sucking insects with special reference to lectin. The aim was to purify, clone and sequence the

midgut extract of "lectin-associated trypsin in *S. calcitrans*" in Chapter Two; (ii) "identification of trypanosome infections in *G.* swynnertoni Austen, *G. morsitans morsitans* Westwood, *G. brevipalpis* Newstead and *G. pallidipes* Austen using a PCR-based technique" so as to verify if patterns of infection vary widely across Africa and or if they are conserved and whether the patterns vary among tsetse species. The segregation of mixed infections into different groupings have important implications for genetic exchange in trypanosome species as well as being an important factor determining patterns of disease transmission. This will be covered in Chapter Three and (iii) "identify unknown trypanosomes using 18S rRNA primers". This has been covered in Chapter Four. In this chapter, the aim was to identify trypanosomes which could not be identified using the universal primers commonly used in the identification of trypanosomes. This was a result of being unable to identify about 75.6% of PCR tested samples using universal primers which are commonly used to identify tsetse transmitted trypanosomes. The primer adopted for this work were those which are used in amplification of the small subunit ribosomal RNA (ssu rRNA) gene. Ssu rRNA genes have been used in analysis of polymorphism by analysing the evolutionary relationships between the various groups of trypanosomatid and also analysis of variation between strains. The use of 18S ribosomal RNA primers was aimed at identifying other trypanosomes of economic importance which were not identified by the universal primers.

Chapter Two

LECTIN - ASSOCIATED TRYPSIN IN STOMOXYS CALCITRANS

SUMMARY

This study reports the cloning and sequencing of a trypsin from the reservoir zone of the midgut tissue of the blood sucking fly, *Stomoxys calcitrans*. The trypsin is blood meal induced, and is midgut tissue specific. The cDNA analysis of a mature protein revealed that this protease consists of 260 amino acid residues and has significant structural similarity with trypsin - like proteinase Try29F of the fruit fly *Drosophila melanogaster* (57% sequence identity) with trypsin 7 precursor of African malaria mosquito *Anopheles gambiae* and trypsin 3A1 precursor of yellow fever mosquito *Aedes aegypti* (both 55%). The protein has a relative molecular weight of 28 kDa. It has both the serine and the histidine active site signatures, typical of trypsin with its catalytic domain between 60 to 240 amino acids. It has an N-glycosylation site (NES) at position 163 to 166 with a significant probability of occurrence (P = 0.005). The cloned trypsin was extracted from the midgut of 8 - 9 days old flies which have been starved for 24 hours prior to dissection. The extract was capable of agglutinating rabbit red blood cells and was strongly inhibited by N-acetyl-D-glucosamine. The molecule was purified by affinity chromatography designed to purify lectins and the subsequent isolation of lectin using SDS-PAGE. This purification produced four strong bands with approximate molecular weights of 30,026, 25,838 and 24,330 Da in reduced condition with mercaptoethanol; and a 28,579 Da band from non reduced condition. EDMAN sequencing of bands showed that the molecule present in bands 30026 and 24330 Da were similar to insect trypsin with a relative good homology of 30026 Da band to *D. melanogaster* trypsin Try29F by 53% and *A. gambiae* trypsin AGRY2A by 54%. A 24330 Da band was also similar to *D. melanogaster* trypsin Try29F by 53% & TRYP_SIMVI (a trypsin from the black fly, *Simulium vittatum*) by 56%. A band with molecular weight of 28579 Da matched a *Homo sapiens* blood anticoagulant IX factor (Christmas

factor), where as a 25838 Da band had no match with any Swiss – prot data bank. The amino acids obtained from a 30026 Da band (IVGGYETDIKKVPFQVSLQA) were used to design a degenerate primer, **5`-** TAC GAG ACC GA(CT) AT(ACT) AA(AG) AA **- 3`**, which was used in PCR amplification of trypsin - specific sequences from a cDNA library of *S. calcitrans* gut. These experiments suggest the presence of a trypsin - lectin molecule moiety in S. *calcitrans.*

INTRODUCTION

Blood sucking insects have developed mechanisms to defend themselves from pathogens. These include lectins (Maudlin, 1991), lysins (Stiles *et al.*, 1990) and proteases (Imbuga *et al.*, 1992). It is thought that these are either responsible for detection of the pathogens through direct interaction with molecules on the surface of the invader, or through intermediary molecules (opsonins) which act as a bridge between the invader and the insect response mechanism (Hoffmann & Reichhart, 1997). Other means of self, non - self recognition speculated upon are differences in surface charge and hydrophobicity between the insect's tissues and those of the invader.

After the invader has been detected, physiological changes take place in the insect leading to the production of lysins, precipitins, opsonins, complement-like microbicides agglutinins and antimicrobial peptides (Hoffmann & Reichhart, 1997; Lehane *et al.,* 1997). In this process metazoan invaders are rapidly coated with droplets of material which make its surface sticky and the coat gradually hardens and melanizes before the invader is phagocytosed and or encapsulated (Boman & Hulmark, 1987). Microbes and protozoa are often killed by molecules making their covering membrane leaky. Research shows that midgut lectin plays an important role in the defence of the organism by agglutinating invading micro-organisms that carry surface components containing the corresponding sugar residues. It has been suggested that this is a vital first step in insect immune responses, the main hypothesis presented is that agglutinated foreign cells could then be more easily phagocytosed, and killed or encapsulated and then melanized (Boman & Hulmark, 1987). Lectins have two or more binding sites which interact with those of the invader, and thus the pathogen is agglutinated. However, the function of lectins in the midgut lumen is presumably different because haemocytes and the machinery

necessary for melanisation are absent. Because lectins are believed to play such a vital role in protection of *Glossina* from trypanosomes, this work was aimed at investigating if the sympatric species, S. *calcitrans*, which does not support the development of trypanosomes, contains a midgut lectin which might explain this significant difference.

Occurrence and classification of lectins

Lectins comprise a structurally diverse class of proteins characterised by their ability to bind carbohydrates with considerable specificity. They are found in organisms ranging from viruses and plants to humans and serve to mediate biological recognition events. Although many plant and microbial lectins have been known for sometime, only relatively recently has the prevalence of animal lectins also been recognised. Animal lectin families include C-type lectins, Galectins (S-type lectins), I-type, Pentraxins and P-type lectins (Gabius, 1997). The C-type animal lectins are a family of carbohydrate-binding proteins characterised by a 15-kDa calcium-dependent carbohydrate-recognition domain (CRD) that is usually linked to an accessory domain. Among others, the family includes the endocytic glycoprotein receptors, the selectins, the macrophage mannose receptor, and the soluble collectins (Rini, 1995). The S-type lectin family, are lectins of diverse metazoan organisms which share a cation-independent binding capacity to β-galactosides, and are characterised by a highly conserved amino acid residues. Both intra and extracellular functions have been proposed for these lectins including a role in modulating cell-cell and cell-matrix interactions. To date, galectin CRD has been found in at least four different structural arrangements, including monomers and dimers, as well as larger polypeptides containing one or two copies of the CRD in association with an accessory domain or linker characterised by proline and glycine-rich sequences (Rini, 1995). I-type family of lectins belong to a large group of proteins commonly known as immunoglobulins (Ig). Their activities include various immune functions and cytoplasmic and extracellular association which seem to protect tropoelastin from premature self-aggregation or proteolytic degradation

by serine proteases. P-type lectins are believed to be a targeting signal for soluble lysosomal enzymes and positioning of matrix proteoglycan degrading enzymes on the surface of the membrane (Gabius, 1997).

Lectins in general are widely distributed within the body fluids and other tissues of many invertebrates (Renwrantz, 1986). Lectins have been reported to occur in the guts of reduviid bugs (Pereira *et al.,* 1981; Gomes *et al.,* 1988), phlebotomine sandflies (Wallbanks *et al.,* 1986), and in adult tsetse flies (Maudlin & Welburn, 1988). They are also found in the guts and haemolymph of mosquitoes (*A. gambie*) (Mohamed & Ingram, 1994) and stable flies (Msangi, 1988). Lectins are inducible and are up regulated by a blood meal for example in tsetse (Msangi, 1988; Welburn *et al.,* 1989), and stable flies (Abdally, 1996).

Lectin functions are associated with the presence of multiple binding sites specific for different carbohydrates, the presence of additional (other than carbohydrate) binding sites, and the aggregation of different specific subunits in a single isomer and the occurrence of multiple lectins. The multivalency of lectins is responsible for their characteristic ability to agglutinate cells or glycoconjugates and very high affinity interactions may occur with complex glycopeptides that contain two or more lectin-binding domains (Gallagher, 1989). In many cases the monosaccharide-binding specificity of a lectin indicates in a broad sense its functional role, although, as we have seen, affinity and fine specificity can be modulated in several ways. For example, mannose recognition by C-type lectins in higher organisms, provide a mechanism for distinguishing self from nonself (Karp, 1996).

Also, analysis of the three dimensional structures of several carbohydrate-binding proteins, including plant lectins, enzymes, fabs, and the bacterial periplasmic transport proteins, has led to the identification of two major subgroups of lectins.
The group I carbohydrate-binding proteins, typified by the periplasmic binding proteins and some enzymes, completely envelop their carbohydrate ligands in deep

binding pockets. They also show primary specificity for a single sugar in a complex oligosaccharide and will always recognise that sugar when it is found at the non-reducing end of a carbohydrate chain, hence the name exolectin. Some exolectins have a mandatory requirement for end-chain sugars and are called the obligate exolectins (class Ia). Others recognise both peripheral and internal sugars and are described as facultative exolectins (class Ib).

Group II carbohydrate-binding proteins bind their ligands in shallow pockets or grooves on the protein surface. These are endolectins. They display a more complex mode of carbohydrate binding than exolectins. They bind to specific carbohydrate sequences but no individual sugar in these sequences plays a predominant role in the binding process. It follows that an interaction with one or more internal sugars is essential for carbohydrate recognition (end-recognition). This class is also divided into two subclasses; the homotypic endolectins (class IIa) which recognise sequences of identical sugar units and the heterotypic endolectins (class IIb) which bind most strongly to sequences composed of two or more different monosaccharides (Gallagher, 1989 & Rini, 1995).

Abdally (1996) working on the characteristic properties of both midgut and haemolymph agglutinins in *S. calcitrans* found that, they do not appear to be nucleic acids, lipids or glycolipids but are probably glycoprotein in nature following reactions with some organic reagents and various enzymes. He found that these lectins or lectin-like molecules, were specific mainly towards galactose and the amino/acetylated derivatives of glucose, and that they are specific towards mannosyl moieties on the rabbit red blood cell surface membrane and are probably only specific towards mannosyl moieties on human (ABO) Red Blood Cell (RBC) and other animal RBC surface membranes. Hence *Stomoxys* lectins are likely to be heterotypic endolectins. According to Abdally (1996), lectins in *S. calcitrans* belong to C-type lectins because they need Ca^{2+} ions for optimum functional activity.

The role of lectins in the immunity of insects

In various bloodsucking insects, midgut lectin activities play an important role in the life cycle of pathogens they transmit. Grubhoffer *et al.,* (1997) had summarised the role of lectin as follows:

(a) As regulatory of differentiation processes and morphogenesis like in growth factors and cell adhesion (Yoshizaki, 1990; Natori & Kubo, 1996). Due to the fact that in most cases the gut lectins are blood meal induced, it is also suggests that they participate in blood - meal processing and digestion (Lehane, 1991).

(b) Refractoriness / susceptibility. Lectins play a part in the regulation of vector infections by transmitted pathogen/parasite, and killing factors (Maudlin & Welburn, 1987; Welburn *et al.,* 1989; Welburn & Maudlin, 1992). For example when lectin are inhibited by the addition of carbohydrate inhibitors to the infective blood meal, establishment of parasites like *Trypanosoma* spp within the gut of tsetse flies is enhanced (Maudlin & Welburn, 1987), and also there is an increased number of *Brugia* microfilariae successfully migrating through the midgut wall of the mosquito *A. aegypti* (L) (Ham *et al.*, 1991). More work by Welburn *et al.,* (1996) showed that lectins are involved in triggering cell death in *Trypanosoma brucei rhodesiense* by a process of apoptosis. It was found that when *T. b. rhodesiense* were treated *in vitro* with *concanavalin A,* the lectin induced cleavage of DNA into oligonucleosomal fragments, suggesting activation of an endogenous nuclease in the parasite. The trypanosome showed condensation and blebs on its surface which are typical of an apoptotic cell (Welburn *et al.,* 1989).

Another major role of lectin is the ability to agglutinate trypanosomatid parasites and exhibit a strong specificity reaction. *Rhodnius prolixus* lectins were able to agglutinate the epimastigote forms of *T. cruzi*, a protozoan parasite of insects and human beings (Pereira *et al.,* 1981). Agglutinins of sandflies (*P. papatasi*) were active against promastigotes of *Leishmania ethiopica, L. major* and *L. donovani* (Wallbanks *et al.,* 1986). Work done on *S. calcitrans* midgut lectin showed agglutination on procyclic trypanosomatid flagellate parasites like *T. brucei, L.*

hertigi and *Crithidia fasciculata* at a dilution range of 2^{-13} to 2^{-14} at day 10 -14 post emergence (Abdally, 1996). Tsetse lectins were found to be active against trypanosomes commonly encountered in tsetse flies such as *T. brucei, T. vivax* and *T. congolense*; but not against trypanosomes unusual to tsetse flies such as *T. dionisii* from bats (Croft *et al.,* 1982; East *et al.,* 1983), *L. hertigi* promastigotes and *C. fasciculata* (Ibrahim *et al.,* 1984). They also strongly agglutinated calf red blood cells but not chicken and guinea pig cells, species which would rarely be fed on by tsetse flies (Ibrahim *et al.,* 1984). The killing of procyclic trypanosome by agglutinins found in tsetse flies is evidence that interaction exists between lectins and trypanosome membranes (Welburn *et al.,* 1989).

(c) Differentiation factors of a vector - specific developmental stage of the parasite/pathogen (e. g. signalling factors of parasite maturation) (Welburn & Maudlin, 1989, 1992). The duration of lectin signal required to induce maturation was determined by sequential addition or removal of a specific inhibitor, the D+ glucosamine to the diet of infected *Glossina* males. Midgut trypanosomes retained their ability to mature throughout their life in the fly when activity in the midgut was inhibited. When sugar inhibition was removed, maturation of trypanosome procyclic forms was unable to continue (Welburn & Maudlin, 1989).

(d) Lectins are also responsible for the recognition of self/non self in immune and defence reactions (opsonins, encapsulation and phagocytosis) (Lackie & Vasta, 1988; Vasta, 1991). The presence of rickettsia like organisms (RLO's) now known as *Sodalis glossinidius* (Dale & Maudlin, 1999) in insects like tsetse is believed to modulate the activity of lectin. These are maternally inherited and they produce the enzyme (chitinase) which hydrolyses insect chitin to glucosamine. Glucosamine production in pupae is thought to be responsible for the increased susceptibility to trypanosome infection of RLO infected tsetse flies because they inhibit the functions of lectins in the first feed after emergence (Welburn *et al.,* 1994).

Lectins are also responsible for killing trypanosomes that enter the guts of refractory flies. However, this applies only to trypanosomes that pass through a midgut stage in the fly (e.g. *T. congolense* and *T. brucei*). *T. vivax* which restricts its life cycle to the fly's mouth-parts is not affected by lectin activities (Maudlin & Welburn, 1988). Midgut lectins from different species of tsetse, showed some antitrypanosomal activities. Flies fed before an infective meal were able to clear trypanosomes from their midgut faster than flies infected at the first blood meal, though, starvation prior to infection increased the susceptibility to trypanosome infection at any blood meal (Welburn *et al.,* 1989).

Characteristics of lectins

Lectins, being proteinaceous in nature are thermo labile (>40°C), affected by freezing and thawing treatments, and require Ca^{++} and Mg^{++} ions for optimum functional activity (Abdally, 1996). However, the major characteristic property of midgut lectins is their ability to agglutinate erythrocytes (red blood cells - RBC) and other cells. Both male and female sandflies (*Phlebotomus papatasi*) midgut extracts agglutinated human RBC (Wallbanks *et al.,* 1986). Extracts from three *Glossina* species (*G. m. morsitans*, *G. p. gambiense* and *G. tachinoides*) exhibited wide specificities for carbohydrate residues on the surface of human erythrocytes, indicating heterogeneity which varied according to the tsetse species examined and the type of erythrocyte used (Ingram & Molyneux, 1988). RBC from sheep, horse and man were also agglutinated using both midgut and haemolymph extracts of *S. calcitrans* (Abdally, 1996). Rabbit RBC were the most sensitive cells with the titre value of 2^{-15} and 2^{-16} respectively, followed by human RBC groups B, O, horse, human group A and AB and lastly sheep RBC with titre values of 2^{-7} and 2^{-6}; for both midgut and haemolymph extracts.

Lectins also exhibit agglutination specificities towards polysaccharides. Midgut lectins of *S. calcitrans* show specificity towards α-D- and / or β-D-galactose

residues and to a lesser degree towards α-D- and β-D-glucose moieties on the surface of RBC. Their activities can also be inhibited by some polysaccharides. D+-glucosamine and melibiose showed a 100% inhibition of haemolymph and midgut agglutinins of *S. calcitrans* at a very low concentration; followed by galactose, mannose simple sugars and their derivatives or moieties with other combinations (Abdally, 1996). Strong inhibition by D-glucosamine followed by N-acetyl-D-glucosamine was reported on midgut lectins from *G. longipennis* (Osir *et al.,* 1995), despite their specificity towards sugars like galactose, mannose, galactosamine. Variation could be due to the differences in configuration and availability of binding sites (Gallagher, 1989).

Observation on trypanosome differentiation and lysis suggests the involvement of trypsin or trypsin like molecules (Imbuga *et al.,* 1992; Abubakar *et al.,* 1995). These molecules are closely related to lectin (Osir *et al.,* 1993). Osir *et al.*, (1995) purified the midgut lectin-trypsin complex of *G. longipennis* by anion-exchange chromatography. A dimeric molecule with both trypsin and lectin activities was isolated which had a native molecular weight (M_r) of 61000 $\pm$ 3000 Da, composed of two nonvalently-linked subunits designated α (M_r,~ 27,000 Da) and β (M_r, ~33000 Da). The trypsin activity and the glycosyl (lectin) residues were present on the α- and β- subunits respectively. The β-subunit exhibited properties which compares with those from tsetse species like *G. p. palpalis* which had the relative molecular weight of 24,000 and 26,000 Da and in *G. m. morsitans* where the β-subunit had a native molecular weight of 24,000 Da (Vanden Abbelle & Decleir, 1992). Similar β- subunit properties have been reported in other insect haemolymph lectins of *Calliphora vomitoria* and *Sarcophaga pergrina* (M_r, ~30,000 - 32,000 Da) (McKenzie & Preston, 1992; Komano *et al.,* 1980). Abdally (1996), purified and characterised the midgut lectins of *S. calcitrans* and the results of SDS-PAGE of midgut lectins gave three bands with relative molecular weights of 28,300, 16,214 and 14,600 Da.

Osir *et al.,* (1993) & Abubakar *et al.*, (1995) suggested that the midgut lectin in *G. m. morsitans* has both the lectin binding site (with affinity for D-glucosamine) and the enzyme activity of the trypsin. Also as shown by Imbuga *et al.*, (1992), it certainly suggests the trypsin or trypsin- like enzymes may be involved in the same functions. Goto *et al.* (1992) showed that high agglutination titres were a result of trypsinization of RBCs; and Sharon & Liz (1989) reported agglutination of cells by lectins can be increased by mild proteolysis. Thus, in the case of the *G. longipennis* lectin-trypsin complex, it may be that the enzyme is required to cleave off specific surface molecule(s) from the bloodstream-form trypanosomes exposing lectin-binding sites thus, facilitating agglutination.

As reviewed above, it is obvious that lectins play an important role in the immunology of insects and the response varies between species. Lectins used in parasitological investigations have been shown to influence growth and survival of parasites.

The focus of this work was on the purification of the midgut lectin (s) of *S. calcitrans* using biochemical and molecular approaches. Purification was carried out using affinity chromatography column utilising the known carbohydrate binding properties of lectins (Abdally, 1996).

MATERIALS AND METHODS

Maintenance of flies

Flies were obtained from a self supporting colony of *S. calcitrans* maintained in the insectary as described by Blakemore *et al.,* (1993). The insectary conditions were maintained at 25 - 28°C and a 12 hour light/dark cycle. Emerged flies were collected in a cage and when they were two days old (2 days p.e) were fed on cotton

wool swabs soaked either in heparinised pig blood or 5% glucose solution. Flies were then fed blood once a day until 7 or 8 days p.e when they were starved for 24 hours before being dissected.

Reservoir extraction

Carbon dioxide anaesthetised flies were held on ice prior to dissection. The reservoir zones of the midgut (Abdally, 1996) were carefully removed from groups of 40 flies and placed in 1ml of cold phosphate buffer saline (PBS), pH 7.4 which was kept in an ice bath until the dissection was complete. Midguts which still contained blood were discarded. Tissues were washed twice in two changes of cold PBS to remove any haemolymph and then homogenised in chilled PBS using a 1ml homogeniser (UNIFORM Jencons, England). Gut homogenates were stored in 1ml aliquots and left at 4°C overnight to permit tissue release of lectins. The gut extracts were then centrifuged at 6000 rpm for 5 minutes after which the supernatant was dispensed into another aliquot (1ml), labelled and stored at -20°C until required. The concentration of this homogenate was adjusted to 40 reservoirs/ml as indicated by Ingram & Molyneux (1988).

Agglutination assays

Agglutination assays were carried out as explained in Abdally, (1996) to familiarise with the properties of midgut lectin. As mentioned in the introductory part, the aim was to clone the midgut lectin which the chemical and physical properties was already investigated by Abdally (1996).

The required volumes of blood (2-5 ml) were withdrawn aseptically from the ear vein of a rabbit via a syringe containing anticoagulant. The 5% erythrocyte suspension was prepared according to Kabat (1961). The degree of agglutination was assessed using a seven score relative scale according to Cunningham & Vickerman (1962) and Ingram & Molyneux (1988). Scoring was:- 100% agglutination as (3+), 75% (3+/2), 50% (2+/1), 25% (2+/+), 10-20% (1+), trace (tr.)

and no agglutination as (0). The agglutination assays are expressed as the reciprocal of the highest dilution showing visual agglutination of the test erythrocytes. The dilution which just failed to give visible agglutination was regarded as the end point titres. They were expressed as $\log_2$ i.e. 2^{-n}

To make sure that the agglutinations were not due to the presence of naturally occurring phenomena (rouleaux formation or intra-erythrocytic haemoglobin crystals) and / or lysis against the RBC types, fresh normal rabbit serum (NRS) was used as a negative control. Other negative control experiments included RBC and PBS alone.

Doubling dilution with PBS pH 7.4 of 5 µl of reservoir lectin extract (RE) sample was made in 96 well microtitre plates (Dynatech). Equal volumes of rabbit RBCs were added to each well. A lectin, *Concanavalin* A (Con A), a glucose/mannose - specific lectin from jackbean (*Canvalia ensiformis)*, both obtained from Sigma were used as positive controls (Msangi, 1988). Then 5 µl (0.5%) of diluted RBC were added to all wells in rows A, B & C. Plates were covered with parafilm and incubated at 25-26°C for 2 hours after which the end point of haemagglutination activity was determined by eye.

A total of three agglutination assays were carried out as follows:

I. Agglutination assay of crude RE supernatant i.e. before being loaded into the affinity column for purification. In this case a total of 50 µl was used for five replicates, i.e. 10µl for each neat reaction per lane of plate wells.

II. Agglutination assay of pure RE extract after the sample has been dialysed, lyophilised and concentrated. The assay was carried out to test the agglutination activity of purified RE, before loading the sample into the NATIVE PAGE and SDS gels. 10 µl of RE for neat reaction was used.

III. Agglutination assay from the electro-eluted samples of corresponding bands cut from the NATIVE gel. From the NATIVE gel 5 bands were cut and electro-eluted.

From each band 10 µl was used for the assay. The remaining samples were used for the SDS PAGE gel.

Assay of inhibitor sugars

Type of inhibitor sugar used was N-Acetyl-D-glucosamine (GlcNaC) from Sigma. 0.05 g of GlcNaC was dissolved in 2.5 ml PBS pH 7.3 to make 20 mg/ml. After doubling the dilution of RE with PBS pH 7.3 glycoproteins was added to each well (i.e. GlcNaC) in replicates number 2 & 4. Plates were incubated at 25°C for 1 hour (Ingram & Molyneux, 1988) after which RBC suspension was added. Plates were further incubated at 25°C for 1 hour. The experiment was set as follows:

1. RE + RBC + PBS
2. RBC + PBS + GlcNAc
3. RBC + Con A
4. RE + RBC + GlcNAc

The concentration of RBC used was 0.5%. The degree of inhibition (DI) was scored following the scale of Ingram & Molyneux, (1988). A reduction in titre by 2 wells was scored as 1+, reduction by 3 wells was scored as 2+; 4 as 3+; 5 as 4+; 6 as 5+; and total inhibition as 6+ and lastly, a reduction by 1 well was $\pm$. If there was no inhibition, then the score was 0. The amount of both crude and pure lectin used was 10 µl for the neat reaction.

PURIFICATION OF MIDGUT LECTIN EXTRACT RESERVOIR

Preparation of the column

1ml of N-acetyl-glucosamine (GlcNac) agarose beads (Sigma) was gently packed in a 2.5 ml minicolumn. The column was then equilibrated with 0.05M sodium acetate (CH_3COONa) buffer pH 7.2 for 45 minutes at a speed of 4. Flow rate of the column was calibrated by collecting elutes on a dry boat from the column for 5 min, and

then reweighing the wet boat. The difference in weights (Wet boat - Dry boat) divided by elution time gave the flow rate of the column.

Running the RE sample in the column

The RE extract from approximately 1366 guts, was loaded into the column and eluted at a flow rate of 180 µl/min. The column was washed to remove the unbound RE using 0.05 M sodium acetate buffer pH 7.2, 3 x 3 the volume of the column. The column was washed again by using 0.2 M GlcNac dissolved in 0.05 M sodium acetate buffer pH 7.2, 3x 3 of the column volume, aimed at competing the occupied sites with excess GlcNac. The collected elute was the sample of interest and was kept for further processing. A total of four different purifications were carried out. In the second purification a cocktail of protease inhibitors (2µl aprotinin, 2µl leupeptin and 6µl 50 mM chloro-tosylamido amino heptanone hydrochloride, (TLCK)) (Sigma) were added to the crude RE before loading into the column.

Concentrating (Dialysing) the eluted sample

The solution was eluted from the column by using a 0.2 M GlcNac mixed with 0.05 M sodium acetate buffer pH 7.2. Elutes were packed in membranes which have been soaked in PBS pH 7.2 for 30 minutes. The membrane was tied at both ends and then placed in a beaker half filled with PBS pH 7.2 and stirred with a magnetic stirrer. Three changes of fresh buffer were carried out after every 1hour. The concentrated solution (the remnant in the tube) was apportioned into sample vials, quick frozen in liquid nitrogen, and lyophilised over night. The lyophilised sample was reconstituted in ultra pure water and desalted at 7500 RCF using a Centricon 3 membrane (Amicon) at 4°C.

NATIVE PAGE gel

The gel was made using 10% acrylamide (Table 2A). A maximum of 150 µl were loaded into a gel (in a ratio of 1:4 marker to RE sample) and run at 200V for about 1.40h in a cold room (4°C). The sample was loaded into a single well which can

accommodate up to 150ml of sample. A total of three NATIVE gels were made after three purification of crude lectin

The gel sides which contain the low molecular weight markers were cut and placed in a fixing solution (40% ETOH, 10% Glacial Acetic acid) for 30 minutes. Strips were washed in three changes of distilled water each change 5 minutes on a rocking platform, and developed in 0.2% Silver nitrate for 45 minutes. The remaining gel was transferred in an electrode buffer and kept at 4°C. The corresponding bands (on the remaining NATIVE gel) to those found in the developed strips were cut and placed in the membrane cap of the electroeluter model and eluted at 8mA overnight in electrode buffer at room temperature on a magnetic stirrer. The RE was then concentrated using a centricon membrane (Amicon). Portions were used in agglutination assays, and a portion of the sample was loaded into the SDS page gel and stained in 0.2% silver nitrate.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Two different kinds of SDS-PAGE gels were made. One was made using the RE material which was electro-eluted from the NATIVE gel and the other SDS gel was made using RE material obtained directly after purification i.e. without loading the RE in the NATIVE gel first. The method followed for SDS-PAGE was an adaptation of that described by Laemmli (1970). The gels were electrophoresed using BioRad Min- protean II equipment in accordance with the manufacture's instructions (BioRad Labs Ltd, Herts., UK). The resolving gel was made up to the required acrylamide concentration (Table 2A), and degassed, then 100 µl of ammonium persulphate (50 mg/ml), 100 ml of SDS and 10 µl TEMED (N,N,N',N'-Tetramethylethylenediamide) was added and mixed to start the polymerisation process. 3.4 ml of this mixture was cast in each chamber and a small amount of distilled water layered above the gel to prevent the admission of air to the setting of the gel. After setting the gel surface was dried with filter paper pieces. The stacking gel was then added and the comb inserted before the gel had set. After setting the combs were carefully removed and the wells rinsed with distilled water. The RE

sample (35 µl) was mixed with 5 µl of loading buffer + mercaptoethanol and denatured by boiling for 5 minutes and loaded into a gel. Low molecular markers (Table 2B) were also loaded as a reference to the migration distance of RE fragments. The gels were run at 200V, 58mA for 45 minutes, i.e. until the bromophenol blue in the sample had reached the bottom of the gel. The gels were then soaked in the eletroblotting buffer (Table 2A) for 5 min.

Polyvinylidene difluoride (PVDF) blotting

Bands on the SDS-PAGE gel were electrotransferred to PVDF membrane (Sigma) after being wet blotted with methanol for a few seconds. The gel was placed on the transblotting sandwich on the membrane placed between 2 filter papers on each side and rolled on top to remove air bubbles and sandwiched tightly. The transblotting sandwich was placed in the cassette and electroblotting buffer added and run at constant Voltage of 50, (170-100 mA) at room temperature for 30 min.

Coomassie blue staining of PVDF membrane

The membrane was stained by blue Coomassie for 45 minutes, rinsed in distilled water and left to dry between two blotting papers. The distance migrated by bands relative to the distance of low molecular weight markers was determined. A standard curve (Regression fitted line) was obtained by plotting the distance migrated by low molecular weight markers against the log 10 of their molecular weights (Table 2B). The relative weight of each band was calculated from respective regression equations (Figures 2:2a & 2:2b). The bands were cut and commercially sequenced by the Edman degradation method using an automatic protein sequencer (Applied Biosystems 473A) at Alta Bioscience (University of Birmingham).

Assay of trypsin activity

This assay was carried out to determine the substrate specificity of trypsin present in situ in the NATIVE gel. A piece of chromatography paper was soaked in 4 mM

BAPNA (α-N-Benz-oyl-DL-arginine-P-nitroanilide) (Sigma) in 10% DMF (N, N-DimethylFormamide) and 50 mM Tris buffer pH 7.9 and then blotted until just moist before pressing onto the surface of the gel (Gertler *et al.*, 1973).

Protein content determination in the RE extracts

Protein content of the sample was determined using the Bio-Rad protein assay method. Protein estimation was carried out at every stage before loading the samples into the column and gels, both the SDS and NATIVE PAGE gels.

The standard curve was prepared by plotting the average net absorbance at 595 nm for each known concentration of BSA (bovine serum albumin) which was used as a standard protein as follows: (I) Bio Rad dye reagent diluted to 1 in 4 with distilled water. (II) Six clean cuvette onto which was added a dye in the following amounts, 1ml & 980, 960, 940, 920 and 900 µl. (III) BSA added on II cuvettes in the following amounts, 0, 20, 40, 60, 80 and 100 µl. (IV) The seventh cuvette had 980 µl dye + 20 µl RE and the eighth had 980 µl dye + 20 µl elute. The absorbency was then determined and a standard curve was obtained. Regression analysis was used in order to obtain a line of best of fit for the data. The equation was used in the calculation of protein concentration of RE and the elute.

PCR AMPLIFICATION

A degenerate primer was designed based on the sequence information obtained from the upper, darkest band on Figure 2:1b. This band had the relative molecular weight of 30026 Da and the Edman sequencing derived from this band was IVGGYETDIKKVPFQVSLQA. The primer designed had a potential mismatch of 15%, an A and T rich 3' terminal region, a 12% degeneracy rate and was 21 base pairs long, as follows: **5`-** TAC GAG ACC GA(CT) AT(ACT) AA(AG) AA **- 3`** (Mal1). The designed primers were used in the amplification of the cDNA library.

Reactions were performed in a thermal cycler (Techne Progene), in a volume of 50 µl containing 6 µl of cDNA library (template), 5 µl deoxynucleotides (dNTPs, 50

mM), 5 µl 10 x buffer, 2.5 µl $MgCl_2$ (50 mM), 2.5 µl detergent (1-W%), 2 µl of 1mM degenerate primer (Mal1), 2 µl M13F primer (~200ng), 0.2 µl Taq polymerase (1unit/µl) and sterile distilled water added to a final volume of 50 µl. The reactions were 30 cycles of 1 min at 94°C, 55°C and 72°C respectively. The products were electrophoresed through a 1.5% agarose gel in TAE (Tris- acetate 40 mM, 1 mM EDTA) with 0.5 µg/ml ethidium bromide. DNA fragments were photographed in the stained gel illuminated under short wave UV.

Bands were excised individually from the gel and spun in agarose separation spin columns (Millipore) at maximum speed (12,000 x g) in a microcentrifuge (Jouan A 14). The elute were precipitated with 3M sodium acetate (pH 4.0) : ethanol (100%, 0.1:2, v/v) and held at -20°C overnight. The products were then centrifuged at 12000 x g speed for 15 minutes and the supernatant discarded. The pellets were washed with 70% ethanol, centrifuged at maximum speed for 2 minutes, and supernatant discarded. Pellets were vacuum dried and resuspended in 10 µl of 1x TE (10 mM Tris.Cl pH 7.5, 1 mM EDTA pH 8.0).

Cloning of the PCR product into TOPO-TA vector

E. coli cells were made competent to take up plasmid DNA by being incubated with cold calcium chloride. Using a sterile tooth pick, *E. coli* cells (XL1 Blue MRF) were streaked onto STA tetracycline (12.5µg/ml) plate and incubated overnight at 37°C. A single colony from each plate was selected and inoculated into 4 ml of LB, and incubated overnight at 37°C in a shaker. 1ml starter was used to inoculate 2 x 50 ml of LB in a sterile bijou flask and incubated at 37°C with vigorous shaking for 5 hours until the OD_{600} was 0.5. Cells were cooled down to 4°C and subdivided into sterile centrifuge tubes (JAZO tubes) and cells harvested by centrifugation at 3500 rpm, for 5 minutes at 4°C.

Cells of one tube were resuspended very gently in 1/4 (12.5 ml) volume of cold sterile 100 mM $MgCl_2$. This was used to resuspend subsequent tube followed by addition of another 12.5 ml of cold $MgCl_2$. The mixture was recentrifuged and cells resuspended in 1/4 volume (12.5 ml) of cold sterile 100 mM $CaCl_2$. The suspension was left to stand for 20 minutes on ice, and recentrifuged. Finally, the cells were gently resuspended in 1/20th final volume (2.5 ml) of 0.1M $CaCl_2$ + 14% glycerol.
Each PCR product obtained was cloned into the vector TOPO-TA. Ligation of DNA into a vector was carried out by gently mixing 1 µl DNA, 1 µl PCR-TOPO vector and 3µl sterile distilled water followed by incubation at room temperature for 5 minutes. Immediately, 1 µl of the 6 x TOPO cloning stop solution was added and mixed for 10 sec at room temperature and held on ice after wards. From this mixture, 2 µl of TOPO-TA (1 µl DNA, 1 µl PCR-TOPO vector, 3 µl sterile distilled water) was taken up by 100 µl of *E. coli* XL1 blue cells (ampicillin selected) incubated on ice for 30 min, heat shocked for 30 sec at 42°C and incubated on ice again for 2 min before being added to 250 µl of pre-warmed LB and incubated at 37°C for 30 minutes with gentle shaking. The cells were spread on STA ampicillin (50 mg/ml) plates which had been overlaid with 40 µl of 5-bromo-4-chloro-3-indolyl β- D- galactopyranoside (X-Gal) (40 mg/ml) and 40 µl of IPTG (isoproptylthio-β-D-galactoside, 100 mM), dried for 15 min and incubated overnight at 37°C.

Preparation of high copy plasmids (Qiagen midi prep)

For each band, a single colony (white colony with insert), was inoculated into a starter culture 4 ml LB + 4 µl Ampicillin (50 mg/ml), and incubated for 6 hours. From the mixture, 250 µl of cells was taken and inoculated in 25 ml LB + 25 µl ampicillin (50 mg/ml) and grown for 16 hours. Cells were always incubated at 37°C with vigorous shaking. DNA from bacterial cells was harvested according to the Qiagen Instructions. The absorbance at 260 nm and 280 nm was read spectrophotometrically using quartz cuvettes before calculation of the concentration

of DNA extracted. The ratio of A_{260} to A_{280} was used to estimate the purity of DNA extracted.

Determination of the product sizes of plasmid DNA for each band was carried out by restriction with *EcoR*I in a 10 µl reaction (0.2 µl (0.2 µg) of DNA, 1 µl *EcoR*I, 1 µl (10 x buffer) *EcoR*I buffer and 7.8 µl sterile distilled water). The reactions were incubated for 1 hr at 37°C and then eletrophoresed through 1.5% agarose gel in TBE (45 mM Tris-phosphate, 1 mM EDTA) with 0.5 µg/ml ethidium bromide. The DNA was visualised by photographing the stained gel under short wave UV illumination. The plasmid DNA samples representing the four bands with sizes of 925, 897, 545 and 545 bp, were sent to Alta Biosciences for sequencing.

SINGLE COPY DETERMINATION

Genomic DNA preparation from insects, *S. calcitrans*

Newly emerged *S. calcitrans* were quick frozen in liquid nitrogen for easier breakage of tissues, and ground into fine powder in a mortar. The powder was immediately transferred with a spatula to a Dounce homogenizer containing a 2-3 x volume of Holmes-Bonner buffer and homogenised with vigorous shaking. The mortar, pestle and spatula were pre-cooled in liquid nitrogen, Dounce homogeniser was placed on ice throughout the whole process of homogenising.

To the homogenate above, a 1x volume of saturated phenol : chloroform (1:1) was added and shaken for 10 minutes; before being centrifuged at 3000 rpm for 5 minutes. The upper phase (the aqueous phase) was transferred into a clean tube and more 1x volume of saturated phenol: chloroform added to the homogenate, shaken and centrifuged at 3000 rpm for 5 minutes. Addition of 1 x volume of phenol: chloroform was repeated until there was no more formation of interphase layer.

To the aqueous solution, 1 volume of chloroform was added, shaken and centrifuged at 3000 rpm for 5 minutes. This step was repeated again until there was no more formation of the interphase solution. 2 x volume of absolute ethanol was added to

the aqueous phase and the sample stored at -80°C for 1 hour, followed by spinning at 3000 rpm for 10 minutes. The pellet was dissolved in 1x TE buffer, and RNase was added to the final concentration of 20μg/ml and incubated at 37°C for 30 minutes. The mixture was shaken for 1 minute after the addition of 1x volume of phenol: chloroform and centrifuged again for 5 minutes. The aqueous phase was transferred into a clean tube, and mixed with 1x volume of chloroform shaken and spun down for 5 minutes.

The aqueous phase was transferred and 1/10 volume of 3M sodium acetate pH 5.2, 2x volume of absolute ethanol added and mixed well before being stored at -80°C for at least 1 hour. The mixture was spun for 15 minutes, and the pellet washed with 70% ethanol, vacuum dried and resuspended in TE buffer.

Southern Blotting

Genomic DNA of *S. calcitrans* was digested with six restriction endonucleases: *Bam HI, Pvu II, EcoRI, Xba I, Kpn I* and *Hind III* (Promega). 30 μg of digested DNA per lane were separated on 0.7 % agarose gel. Fragments were separated by gel electrophoresis and stained with ethidium bromide (200ng) solution. The gel was then photographed next to a metric ruler, so that accurate measurements of the migration of restriction fragments can be obtained. The marker used was bacteriophage λ DNA cleaved with *Hind* III.

The gel was first depurinated in a 0.2M HCl solution for 1 hour in two changes of the solution and washed in two changes of distilled water. Then denatured in a 0.5M NaOH + 1.5M NaCl solution for 30 minutes and lastly neutralised in a 0.5M Tris + 3M NaCl, pH 7.4. The single- stranded restricted fragments from the gel were then transferred by blotting onto the surface of a nylon membrane for approximately 24 hours in a blotting solution (Table 2A), and positions of gel slots on the membrane were marked with a pencil before being air dried, visualised under the UV light and baked at 80°C to bind the DNA to the membrane for 2 hours.

Preparation of DNA probe

Probes were prepared from plasmid DNA as follows: Plasmid DNA was amplified in a 50 µl PCR reaction as follows: 1 µg (1 µl) plasmid DNA, 5 µl of 10 x buffer, 5 µl dNTP, 2.5 µg $MgCl_2$, primers M13 Forward and Reverse each 2µl and 29.8 µl sterile distilled water. The reactions were run in the PCR machine for 30 cycles of 1 minute each at 94° C, 55°C and 72°C respectively. The plasmid DNA was then extracted using phenol/chloroform and ethanol precipitated at -20 °C for 1 hour. The pellet was washed in 70% ethanol and vacuum dried before being resuspended in sterile distilled water. The suspended DNA was then digested with *EcoRI* to remove the vector in a 40 ml reaction (34 µl DNA, 2 µl *EcoRI* and 4 µl enzyme buffer) and incubated for 1 hour at 37°C. The mixture was electrophoresed on a 1.5% agarose TBE gel and visualised by photographing the stained gel under UV illumination. The insert (DNA) with ~925 bp was obtained and purified using an agarose spin column (Qiagen), and quantified using an ethidium bromide plate.

The Oligonucleotide probe consisted of 1 µl (1 µg) of Lambda DNA, 6 µl of sterile distilled water. This was denatured by heating for 10 minutes at 95-100°C, and chilled on ice for 5 minutes. The probe was then labelled with 4 µl of NonaPrimer, 1 µl each of dATP, dTTP, dGTP (Appligene Nona Primer Kit), 5 µl of alpha ^{32}P dCTP (3000 Ci/mmol, approximate 50 mCi) and lastly 1 µl of Klenow enzyme. The mixture was incubated for 30 minutes at 37°C. After the incubation, the purification of the probe followed by addition of 60 µl of Adsorb solution, 2 µl of DNAprep solution. The mixtures was vortexing and incubated for 15 sec, (at room temperature), and spun down at 10,000 rpm. The supernatant was carefully discarded for decontamination. The pellet was washed with 100 ml of wash solution, vortexed to resuspend the pellet, and spun down at 10,000 rpm. The washing process was repeated three times. The pellet was resuspended in 100 ml of elution solution, vortexed thoroughly and incubated for 5 minutes at 65°C. During this last incubation, tubes were vortexed from time to time and spun down at 10,000 rpm to recover the supernatant that contains the purified DNA probe. The elution

was repeated for a full recovery of the probe. The probe was then denatured by boiling on a water bath for 3 minutes and kept on ice, before adding onto the membrane.

Hybridisation and Washing of Hybridised membrane

The hybridisation and initial washing steps were carried out as described (Perbal, 1988). The membrane was pre-hybridised with a protein blocking reagent to prevent the probe from binding non-specifically to the membrane because eukaryotic DNA have T-rich sequences. The pre-hybridisation solution was poured off followed by the addition to the membrane of hybridising solution which contained the labelled probe. The hybridisation solution was first warmed to 65°C before the addition of the probe. The membrane was incubated overnight with the hybridisation probe. Both pre-hybridisation and hybridisation were carried out at 65°C. The membrane was washed with 2 changes of 2 x SSC + 0.1% SDS. The first wash was carried out for 5 minutes with 150 ml and the second wash for 15 minutes, both at room temperature. The second wash was carried out using a 1 x SSC + 0.1% SDS, first washed for 5 minutes at room temperature and then for 15 minutes using a pre warmed washing solution (65°C) and the washing carried out at 65°C. The third wash was carried out using a 0.1 x SSC + 0.1% SDS at 30°C intervals at 65°C. The wet membrane was slip into a heat-sealable bag. Air bubbles smoothed out and sealed with a heat sealer. The membrane was exposed on Kodak X-Omat AR films with Ilford Fast Tungstate screens at -50°C for 10 days.

SCREENING OF THE cDNA LIBRARY

Plating and titring

XL1-Blue MRF cells were streaked on tetracycline STA plates and incubated over night at 37°C. A single colony was then inoculated into the medium 10 ml LB supplemented with 1ml of 100 mM magnesium sulphate ($MgSO_4$) and 0.5 ml of 4% filtered maltose. Cells were grown at 37°C, shaking for 4-6 hours, until when the

OD_{600} = 1.0. Bacterial cells were pelleted at 3500 rpm for 5 minutes and gently resuspend in half the original volume with sterile 10 mM $MgSO_4$. Dilution with sterile 10mM $MgSO_4$ continued until the OD_{600} = 0.5.

To plate the packaged ligation product (λ-cDNA), the following were mixed together; 1 µl of the final packaged reaction (neat), mixed with 200 µl of XL1-Blue MRF cells at OD_{600} = 0.5. Other 1:10 dilutions were 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. The phage and bacterial cells were incubated at 37°C for 15 minutes with gentle shaking to allow the phage to attach to the cells. The phage and bacterial cells were mixed with the 3 ml top agar (LB + 0.7% agarose) and immediately plated onto the agar plate (STA). After 0 minutes, plates were kept upside down and incubated at 37°C overnight. Plaques were counted and the number of plaque forming units (pfu/ml) obtained was used to estimate the number of packaged cells to be used in titring large plates. Hence in large plates (150 mm 20 x 22 cm) the amount of packaged ligation used was 1.6, 4, 5 and 10 µl for 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions respectively. These were added to 2 ml of host cells and incubated as above for 15 min at 37°C with gentle shaking. The aliquots of infected bacteria mixtures were added into 20 ml melted top agarose (48°C) and spread immediately and evenly in 150 mm 20 x 22 cm agar plates which had been pre-warmed at 42°C for ~6 hours. Plates were incubated at 37°C overnight until when plaques were approximately 1.5 mm larger. Plates with ~ 10,000 plaque forming units were chilled for 4 hours at 4°C to prevent the top agar sticking to the nylon membrane.

Colony and plaque lifts

Plates were removed from the cold room, and at room temperature, a nylon membrane (BIOTRANS 1.2 µm membrane) was placed neatly onto the surface of the top agarose so that it comes into direct contact with the plaques, avoiding trapping air bubble as much as possible. The membrane was handled using grooved hands, to prevent finger oils from wetting membrane which could in turn affect the

transfer of DNA. The membrane was marked in three or more asymmetric locations by stabbing through it and into the agar beneath with an 18 gauge red hot needle. After 5 minutes of DNA transfer, the membrane was peeled off using a blunt clean and sterile forceps and laid on top of Whatman 3MM blotting paper moistened in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes (DNA side upward). The membrane was transferred into another tray containing neutralising solution (1.5 NaCl, 0.5 M Tri.Cl pH 7.4) and laid on top of blotting papers. After 5 minutes, it was rinsed in 2 X SSC and placed, on paper towels to dry (DNA side facing upward). After the membrane has dried for 30-60 minutes at room temperature, it was sandwiched between sheets of Whatman 3MM paper, and the DNA fixed to the membrane by baking for 90 minutes at 80°C in a vacuum oven. The membrane was sealed in a plastic bag and kept in a dry and dark place. Later the DNA immobilised on a membrane was hybridised to alpha ^{32}p-labelled probe as explained below. Agar stock plates of the transfers were stored at 4°C for use after screening.

Prehybridising and Hybridisation of membrane

The membrane was loaded into the hybridising bottle and prehybridising solution added and agitated for in the oven at 65°C for 15 minutes before the addition of the 10 μg/ml of denatured salmon sperm DNA (boiled for 10 minutes and kept on ice). The membrane was prehybridized for 2 hours. The prehybridising solution was decanted and hybridisation solution plus the labelled probe added and hybridised overnight at 65°C. Labelling of the probe was conducted as indicated above. The membrane was rinsed two times in each of the following solutions, 2 x SSC solution followed by a wash buffer (2 x SSC, 0.1% SDS) at room temperature with some agitation.

The membrane was sealed in a plastic bag and exposed to a film at -50°C for 5 days, and the film developed afterwards.

Secondary screening

To orient the filters, the film was lined up and dots marked where the needle poked through, and strongest putative clones determined. Each strong clone was numbered. A square centimetre window was cut out using an inverted blue tip from the stock plate where the putative clone lined up with the film spot. The clone was transferred into a 1.5 ml eppendorf which contain 1 ml SM buffer and 20 µl of chloroform and vortexed. A total number of 99 plaques were collected and out of these, 30 plaques were used for PCR amplification from the library in a 50 µl reaction i.e. 10 µl plaque, 5 µl dNTPs, 5 µl 10 x buffer, 2.5 µl $MgCl_2$, 1.5 µl Mal1, 1.5 µl M13 FOR, 1 unit (0.2 µl) Taq and added up to 50 µl reaction with sterile distilled water.

The reactions were run in the PCR machine for 30 cycles of 1 minute each at 94° C, 55°C and 72°C respectively. The reactions were then eletrophoresed through 1.5% agarose gel in TBE (45 mM Tris-phosphate, 1 mM EDTA) with 0.5 µg/ml ethidium bromide. The DNA were visualised by photographing the stained gel under short wave UV illumination. The results showed products at 924 and 970 bp for two plaques numbered 1 and 20.

Plaques from number 1 and 20 were then diluted and titring with host cells carried out on small STA plates (100 mm) so that each plate will have around 50 plaques. These were incubated overnight at 37°C. Individual plaques were once again cut and allowed to diffuse into 1 ml SM and 20 ml chloroform mixture and vortexed. A total of 40 plaques for each original plaque i.e. NO: 1 & 20 were obtained. Out of these 20 plaques from each secondary plaque were used for PCR amplification. A 50 µl reaction containing 10 µl plaque, 5 µl dNTPs, 5 µl 10 x buffer, 2.5 µl $MgCl_2$, 1.5 µl primer (Mal1), 1.5 µl M13 FOR (forward primer), 1 unit (0.2 µl) Taq and added up to 50 µl reaction with sterile distilled water. The amplification was carried out for 30 cycles of 1 minute each at 94° C, 55°C and 72°C respectively. The reactions were then eletrophoresed through 1.5% agarose gel in TBE (45 mM Tris-phosphate, 1 mM EDTA) with 0.5 µg/ml ethidium bromide. The DNA were

visualised by photographing the stained gel under short wave UV illumination. The results showed ten products (from plaques NO: 16, 17, 18, 20, 22, 24, 26, 28, 29 and 63 which were screened from plaque number 1 except for 63 which originated from plaque 20. All had products of ~ 830-900 bp.

SINGLE - CLONE EXCISION

The cDNA library inserts cloned into the phage ZAP XR vector was automatically excised and recircularized into a smaller and more easily manipulated plasmid vector (Bluescript) by using an ExAssist helper phage. This was carried out in order to recircularize the lambda vector to form a phagemid containing the cloned insert by the assistance of Uni-ZAP XR vector.

XL1-Blue MRF cells from LB - Tetracycline plate and SOLR cells from LB-Kanamycin plates which had been incubated overnight at 37°C, were separately grown overnight in LB medium at 30°C. The following day, 0.5 ml of overnight grown cells were inoculated into 50 ml LB (a 1/100 dilution) and grown at 37°C for approximately 3 hours to mid phase (OD_{600} = 0.2-0.5). Cells were then gently spun down at 1500 x g and resuspended in 10 mM $MgSO_4$ at OD_{600} of 1.0. Into a Falcon polypropylene tube, a mixture of 200 µl of XL1 - Blue MRF cells at an OD_{600} of 1.0, 250 µl of phage stock (λ + sm > 1 x 10^5 phage particles), and 1 µl of ExAssit helper phage (> 1 x 10^6 pfu/ml) were added and incubated at 37°C for 15 minutes. Onto the mixture, 3 ml of LB media was added and tubes incubated overnight at 37°C with vigorous shaking. The cells were spun down for 15 minutes at 2000 x g, and the supernatant transferred to a fresh tube. The supernatant was then heated for 15 minutes at 70°C and spun again at 4000 x g for 15 minutes. The supernatant (which contains excised phagemid pBluescript packaged vector as filamentous phage particles) were decanted into sterile tubes. To plate excised phagemids, 200 µl of freshly grown SOLR cells (OD_{600} = 1.0) were added into two

clean and sterile tubes, followed by the addition of 100 µl or 10 µl of the excised phagemid bluescript stock. The tubes were incubated for 15 minutes at 37°C. The cells were then plated on LB - ampicillin plates (50 µg /ml) and incubated overnight at 37°C. The phage stock used was from plaques 16, 17, 18, 20, 22, 24, 26, 28, 29 and 63. Hence a total of twenty ampicillin plates were used, two for each phage stock. White colonies appearing on the plate which contain the pBluescript double-stranded phagemid with the cloned DNA inserts were used for further analysis.

Amplification of bacterial cells

Bacterial cells from ten clones above (16, 17, 18, 20, 22, 24, 26, 28, 29 & 63) were amplified in a 40 ml reaction as follows: - 5 µl dNTPs, 4 µl PCR buffer, 5 µl MgCl2, 1 µl of M13 Forward and 1 µl of M13 Reverse, and sterile distilled water added to 40 µl. Few bacterial cells were added and the mixture heated in a Thermocycler PCR machine for 15 minutes at 95°C. After 15 minutes, 10 µl of Taq (1 µl PCR buffer, 0.2 µl Taq and sterile water added to make 10 µl reaction) was added, and the reaction run in a Thermocycler PCR machine for 30 cycles of 1 minute each at 94° C, 55°C and 72°C respectively. The reactions were then eletrophoresed through 1.5% agarose gel in TBE (45 mM Tris-phosphate, 1 mM EDTA) with 0.5 µg/ml ethidium bromide. The DNA was visualised by photographing the stained gel under short wave UV illumination. The results of single clone excision using SOLR cells yielded the amplification products at about 800-937 bp. Clone 18 had the longest insert, ~ 937 bp. Further analysis of STET preparation of 10 plasmids yielded identical restriction maps, by using *EcoRI* + *Kpn1* and *Pvu II*. However clone 18 still had a slight longer insert than others (at ~ 900 bp). This was selected for further analysis.

From a plasmid material obtained from clone 18, 1 µl of DNA was dissolved in 250 µl of sterile distilled water for use as a template. The reaction mixture was as follows: 2 µl template, 5 µl dNTP, 5 µl PCR buffer, 2.5 mM $MgCl_2$, 1.5 µl M13Forward, 1.5 µl primer Mal2 (**5'**- GAG CGG CCG CCA GTG TGA - **3'**)

primer, 1 unit (0.2 µl) Taq and sterile distilled water added to make a 50 µl reaction. The reactions were run in a Thermocycler PCR machine for 30 cycles of 1 minute each at 94° C, 55°C and 72°C respectively. After electrophoresis, the product obtained was around 900 bp. The similar reaction was carried again using primer Mal1(**5`-** TAC GAG ACC GA(CT) AT(ACT) AA(AG) AA **- 3'**) primer instead of primer Mal2. The product obtained for this clone (18) was around 810 bp.

Purified DNA was further digested with *EcoRI, EcoR1+ Kpn1, Kpn1* and *Pvu II.* In each digestion, 0.2 µg of DNA was used. The mixture was incubated at 37°C for one hour, followed by electrophoresis through 1.5% agarose as explained above. Results showed a product (insert) of ~ 900 bp with *EcoRI* + *Kpn1* mixture and an insert of 1268 bp when the DNA was digested with *Pvu II.* Following these results, 10 µl of plasmid DNA was sent to Alta Biosciences Lab for sequencing, and latter to MWG Biotech lab for detailed sequencing.

RESULTS

Agglutination assay

The lectin activity of RE was monitored throughout the purification by agglutination assays. The summarized activity of agglutination is presented in Table 2.1. The result shows that the activity of purified RE was as strong as in the crude lectin. In both cases the lectin activity end point was 2^{-8}

A purified lectin was then loaded into a NATIVE gel page and the RE bands obtained by electroelution. The lectin activity of these bands was then monitored. As indicated below, the activity in three bands was similar (2^{-3}), but the activity of the fourth band was a bit lower (2^{-2}).

Table 2:1 Agglutination activity of crude, purified and electro-eluted lectin

	Crude			Purified		Electro-eluted
Total loaded	Total lectin	activity	Total lectin	activity	Total lectin per band	Activity
10 ml	1ml = 2.2mg	2^{-8}	1µl = 1.25µg	2^{-8}	1. 1µl = 9ng 2. 1µl = 3.2ng 3. 1µl = 1.1ng 4. 1µl = 1.1ng	2^{-3} 2^{-3} 2^{-3} 2^{-2}

The lectin was also inhibited by N-acetyl-D-glucosamine (GlcNAc). A minimum of 290µM GlcNAc inhibited the RE by a score of 6+ i. e. total inhibition. The amount of RE for the neat reaction in the inhibition assay was 1 µl in 1.25 µg.

SDS-PAGE gel

Two sets of SDS- PAGE gels were used. The first set was used to load the RE obtained after purification i.e. without loading the RE in the NATIVE gel. Two gels were made one under reduced condition and the second under non-reducing condition (Fig. 2:1a & 2:1b).

The corresponding molecular weight (in Daltons) of RE bands obtained from SDS - PAGE gels are tabulated in Table 2:2. Four bands with relative molecular weights of 30026, 28579, 25838 and 24330 Da, were sequenced by Edman degradation. These were chosen for sequencing based on the work done by Komano *et al.,* 1980; Vanden Abbelle & Decleir, 1992; McKenzie & Preston, 1992; Osir *et al.,* 1995; and Abdally, 1996 which indicate that proteins responsible for immunity in dipterans have relative molecular weights between 24 - 30 kDa. These were the strongest bands as shown in Figures 2:1a & 2:1b.

Table 2:2 Relative molecular weights of RE bands on PVDF membranes after being transblotted from SDS-PAGE gel. Result shows bands from midgut lectin (RE) extract as migrated relative to the molecular weights of markers. Bold numbers shows bands which were sequenced by Edman degradation; bold and underlined show bands with sequence homology to insect trypsins. (ME = Mercaptoethanol)

Molecular weight Markers (MW) (Da)	Molecular weights of RE bands on PVDF membrane as transblotted from SDS-PAGE gels (Da)	
	I^{st} GEL + 2ME	2^{nd}GEL
94000	63643	73663
67000	50044	38419
43000	**30026**	**28579**
30000	29137	26152
20100	**25838**	22609
	24330	14471
14400	15502	
	10489	

Proteins revealed in the N-Terminal sequencing of a band with relative molecular weights of 30026 Da, showed a homology to a trypsin from *D. melanogaster* Try29F trypsin by 53% and *A. gambiae* trypsin AGRY2A by 54% (Muller *et al.*, 1993 and Wang *et al.*, 1994). A band with relative molecular weight of 24330 Da was also similar to *D. melanogaster* Try29F trypsin by 53% (Wang *et al.*, 1994) and by TRYP_SIMVI, a trypsin of black fly (*Simulium vittatum*) by 56% sequence identity (Ramos *et al.*, 1993). The band with 28579 Da matched a *Homo sapiens* blood anticoagulant IX factor, Christmas factor (Yoshitake *et al.*, 1985). The sequenced band with $M_r \sim 25837$ Da, which was found to have no match with any Swiss- prot data bank in 1998, have been found recently to be similar to HEMA_INBGL by 100% (a heamaglutinin responsible for attaching the virus to the cell receptors and for initiating infection) (Yamashita *et al.*, 1988). Both results of earlier and new searches are tabulated in Table 2:3 below and Table 2F in the appendix.

Table 2:3 Protein sequence obtained from Edman degradation of RE bands on PVDF membranes; and their similarity.

Band size (Da)	**Protein sequence**	**Similarity**	**Reference**
30026	IVGGYETDIKKVPFQVSLQA	Try29F AGRY2A	Muller *et al.*, 1993 & Wang *et al.*, 1994
28579	VNGE D/L AKPGQF	Christmas factor	Yoshitake *et al.*, 1985
25837	I V/T G/N G/G E/E	No match	
24330	IV G/N GQPT T/K INQFPYQV	Try29F TRYP_SIMVI	Wang *et al.*1994 Ramos *et al.*, 1993

Nucleotide sequencing of the plasmid DNA

The amino acids obtained from a 30,026 Da band (IVGGYETDIKKVPFQVSLQA) was used to design a degenerate primer, **5`-** TAC GAG ACC GA(CT) AT(ACT) AA(AG) AA **- 3`**, which was used in PCR amplification of trypsin - specific sequences from a cDNA library of *S. calcitrans* gut. From this, five bands were obtained as follows:- 814, 627, 573, 496 and 405 base pairs. After ligation into a vector and screening for white bacteria, for each band above the following plasmids were obtained with sizes of 925, 897, and two bands with 545 bp which all four were sequenced by Edman degradation. Blast searching using the nucleotide sequence of plasmid DNA from the first band (925 bp), produced a significant sequence similarity to a serine protease of *A. gambiae* (the malaria mosquito) with P = 7e-10 (100% identical) (Dimopoulos *et al.*, 1997). The nucleotide sequence from the second band had no match with any BLAST nucleotide data bank entry. The third and fourth bands had nucleotide sequence similar to beta 2- tubulin of various organisms like *D. melanogaster*, with P = 5e-11 (88% identical) (Michiels *et al.*, 1987), and *Achlya klebsiana* (Cameroon *et al.*, 1990).

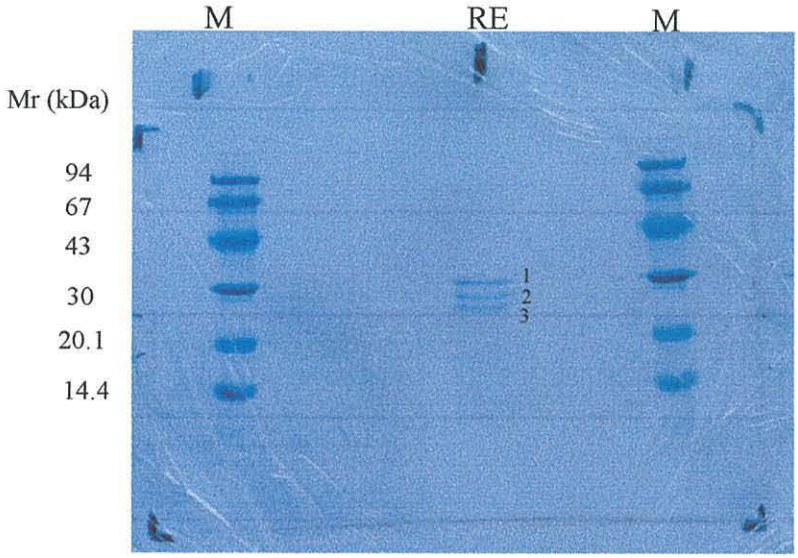

Figure 2:1a: PVDF blot of purified midgut lectin from *S. calcitrans* under non reducing conditions.

Mr - relative molecular weight
M - low molecular weight marker
RE - midgut reservoir extract bands
1,2,3 - RE bands
1 - 28579 Da
2 - 26152 Da
3 - 22609 Da

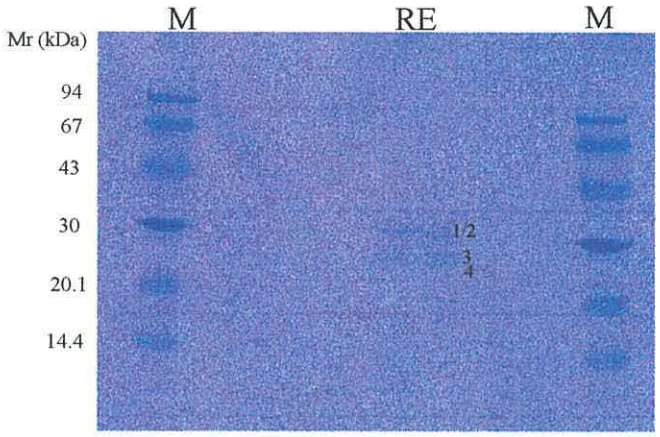

Figure 2:1b. PVDF blot of purified midgut lectins from *S. calcitrans* Under reduced condition (+2ME), for amino acid sequencing.

M_r - relative molecular weight
M - low molecular weight markers
RE - midgut Reservoir Extract
1,2,3,4 - RE bands
1 ~ 30026 Da
2 ~ 29137 Da
3 ~ 25837 Da
4 ~ 24330 Da

Nucleotide sequencing of the plasmid DNA after lambda excision

By using the probe obtained above (from band 925 bp), a cDNA library of the gut was screened and a plasmid insert of about 900 bp was obtained which by using internal primers (Mal1 and Mal2 respectively), and by using restriction enzymes *EcoRI, EcoRI* + *KpnI, KpnI* and *PvuII,* an insert of ~ 900 bp was obtained and sequenced by Edman degradation. The final result from BLASTP indicated that the plasmid (pMAL1) is a serine protease of a trypsin family (100%) with serine active site at DSCQGDSGGPMA (207 – 218) and the histidine site at LTAAHC (65 – 70), which are embedded in conserved sequence blocks GDSGGP and LTAAHC (Fig 2.1c).

```
   1          11          21          31          41          51
 1 MRFFFAIAVL  FVASCSAANL RSKPRLDGRI VGGYETDIKK  VPFQVSLQAG WHFCGGSLIA 60
61 KRFVLTAAHC TDGDPEFNPN FKVRVGSSYS EKGGLLLKVN RIHQHHQYSA SVVDYDFSIL 120
121 ELEDYDTSAL GFELQYAKLP TADDVADGTL VTVSGWGNTK NPNESSDVLR AVQVPKVNQK 180
181 VCQEAYTNFG DVTDRMICAG YTQGGKDSCQ GDSGGPMALD GTLVGVVSWG FGCAEPNYPG 240
241 VYARVASVLP WIAEKTGLSL
```

Figure 2:1c: Amino acids sequences of a purified and cloned RE extract from the midgut of *S. calcitrans*. Bold, underlined amino acids show sites with relative degree of significant biochemical activity.

The trypsin has a homology similarity to trypsin - like proteinase Try29F of the fruit fly *Drosophila melanogaster* (57%), with trypsin 5 (TRY5) precursor of yellow fever mosquito *Aedes aegypti* and with trypsin 7 precursor of African malaria mosquito *Anopheles gambiae* both by 55% (Muller *et al.,* 1993; Dimopoulos *et al.*, 1996 & Paululat, 1996). The trypsin has a preproenzyme of 289 amino acids (Mr ~ 31369.7 Da and pI 6.897), a putative signal peptide of 12 amino acids and a putative activation peptide of 29 amino acids. The mature protein has a relative molecular weight of 28094.7 Da, with 260 amino acids and a theoretical pI of 5.73. The protein has an Asparagine (ASN) glycosylated site, NES. By using the ScanProsite

programme, results obtained indicate that the trypsin catalytic domain lies between 60 to 240 amino acids of the mature protein with a significant probability of occurrence for both the histidine and serine (P = 2.601e-07 and P = 7.319e-08 respectively). The N-glycosylation site (NES) occurs at 163 - 166 amino acids with a significant probability of occurrence (P = 0.005). The occurrences of other glycosylation site are of minor significance. These include the protein kinase C phosphorylation site at SEK (90 - 92) and TDR (193 - 195). The Casein kinase II phosphorylation site at TDGD (71 - 74), SYSE (88 – 91), SVVD (111 – 114), SILE (118 – 121) and TADD (141 – 144). The trypsin has 7 N-myristoylation site at GGYETD (32 – 37), GSLIAK (56 – 61), GSSYSE (86 – 91), GTLVTV (148 – 153), GGKDSC (204 – 209), GGPMAL (214 – 219) and GTLVGV (221 – 226).

DISCUSSION

Agglutination activity was observed at 2^{-8} for both crude and purified lectin. This indicates that the extracted RE was very strong even in purified state. The lectin collected from electreluted bands obtained from the NATIVE gel showed activity at an overall level of 2^{-3} and the lectin was still able to agglutinate rabbit red blood cells. Agglutination of red blood cells is one of a typical characteristic of lectin (Abdally, 1996).

The aim was to purify midgut reservoir lectin, however from Edman sequence information's; purification appears to have produced a trypsin. Osir *et al*, (1995), reported that midgut lectin from tsetse fly are associated with trypsin activities. In this purification, a cocktail of protease inhibitors (aprotinin, leupeptin and chloro-tosylamido amino heptanone hydrochloride, (TLCK)) (Sigma) was only used once. However, the attempt to use this cocktail of protease inhibitors, lead to the disappearance of bands i.e. no band was obtained in the SDS-PAGE gel, an

indication that the trypsin like property of the RE was inhibited. When inhibitors were not used, RE bands were recovered on the SDS PAGE gel as previously obtained from the NATIVE gel. A total of five strong bands were obtained from the NATIVE by cutting at the approximate distance migrated by the RE bands, relative to the low molecular weight markers of 94000, 67000, 30000-43000 (triplets), 20100 and lastly around 14400 Da. Between 30000 and 43000 Da, there were three strong bands together whose resolution was poor such that they could not be obtained individually. These were cut and grouped as one band. Samples of the eluted RE for the respective five bands were loaded in SDS-PAGE. Results from SDS PAGE gel showed various bands and one band of interest was obtained at ~ 30,000 Da. The size of this band was consisted as the one obtained in the first purification which on Edman sequencing was similar to insect trypsins.

More verification of the trypsin like property of RE was carried out by repeating the purification without the addition of inhibitors, but this time using the NATIVE gel first to isolate the molecule before loading it on the SDS-PAGE gel. This was carried out in order to preserve to the maximum the biological activities of the molecule (lectin). It was suspected that the biological activity of lectin was lost when the sample was loaded in the SDS-PAGE gel because SDS denatures protein. The disappearance of bands when inhibitors were used, and the fact that the lectin extract still retained the agglutination activity on red blood cells, is in accordance with the findings of Osir *et al.*, (1995) that the midgut lectin is a dimer with both trypsin and lectin molecules. Also, the triplet bands showed a yellow colouration when a chromatography paper soaked in 4mM BAPNA and 10% DMF was pressed onto the surface of the gel, an indication of the presence of trypsin *in situ.*

Gooding (1974) partially purified trypsin from *G. m. morsitans* females. The trypsins were inhibited by TLCK and PMSF (Phenyl methane sulphonyl fluoride). The inhibition is an indication that histidine and serine are present at the centre of the trypsin. In this study, a faint yellow coloration obtained on the second band

background of the native gel, suggest the presence of a serine protease *in situ*. Active bands appear as yellow patch which is due to the formation of a coloured compound, P-Nitroaniline (Gertler *et al.*, 1973).

It is estimated that a minimum of 24 independent serine proteases occur in the midgut of *S. calcitrans*. Lehane *et al.* (1998) sequenced two highly abundant serine protease cDNAs (Ssp1 and Ssp2). Ssp2 was found to be a typical trypsin where as Ssp1 had similarities to both trypsin and chymotrypsin. The purified and sequenced trypsin could be among the minimum 24 proteases. The final sequence of the plasmid (pMAL1) is different from ssp1 and ssp2, while pMAL1 has a major glycosylation site (NES), ssp1 & ssp2 do not possess such a site. However, ssp1 has two rare glycosylation sites at NAC and NIC, and they lack in ssp2 and in this sequenced trypsin.

Protease as is the case for most trypsin family members, are enzymes that catalyse the hydrolysis of peptide bonds. They catalyse a broad spectrum of important biological reactions, including prohormone processing, blood coagulation and fibrinolysis, protein metabolism, immune reactions and tissue remodelling for example during insect metamorphosis (Tsuji *et al.*, 1998). As a result they have been found to play a number of critical roles in pathogenesis of parasitic diseases. Parasite proteases facilitate invasion of host tissues, allow parasites to digest host proteins, help parasites evade the immune response and prevent blood coagulation (McKerrow, 1989). In blood sucking insects, serine proteases are among enzymes that play a crucial role during the digestion of blood meal in the gut (Muller *et al.*, 1993).

Interdependency of lectin, protease and trypsin in defence mechanism of insects

Maudlin *et al.* (1984), Ingram & Molyneux, (1990) found that midgut lectins of tsetse flies were induced in response to serum components of the blood meal. In

mosquito (*A. aegypti),* lectin activity in the midgut was strongly induced by protein meal (Grubhoffer & Noriega, 1995). Briegel & Lea (1975) and Felix *et al.* (1991) reported that various proteins are also responsible for inducing the proteolytic activity, and the extent of trypsin synthesis correlates positively with the concentration of soluble protein in the meal. It has been also reported that stretching of the midgut or abdominal wall might activate neural mechanisms and lead to increased production of some gut enzymes in some insects, such as mosquito esterase's (Volf & Palanova, 1996), although this stimulus appears to be insufficient to trigger lectin secretion in sandflies. Molyneux & Stiles, (1991) reported that in some *Glossina* species, a blood meal stimulates the release of different molecules apart from lectins which include proteolytic enzymes and lysins.

Lectins and proteases released into the midgut of some bloodsucking Diptera are thought to form functional complexes which participate in blood meal digestion as well as in interaction with ingested pathogens. For example, the proportion of tsetse acquiring midgut infections can be significantly increased *in vivo* by adding inhibitory oligosaccharides to the blood meal for the first few days after infection. This is an indication that lectin is the first line of defence in tsetse against infection and serves to kill incoming trypanosomes (Maudlin & Welburn, 1987). The clearance is thought to involve the trypsin-like enzyme and midgut lectin. This is because a trypsin-like enzyme and the main midgut lectin share several specificities and are closely related (Osir *et al.*, 1993). In *G. morsitans* the trypsin activity was inhibited by D-glucosamine, the same way like lectins; and the blood meal-induced agglutination activity in *G. morsitans* followed a quite similar pattern to that of the trypsin (Van den Abbelle & Decleir, 1992), with both activities being co-eluted when separated by anion-exchange chromatography (Abubakar *et al.*, 1995). The enzyme cleaves off the specific surface molecule(s) from the bloodstream-form trypanosomes, a process which is thought to expose the lectin-binding sites and thus facilitating agglutination by lectins (Osir *et al.*, 1995). It is also thought that the

trypsin-like molecule cause lysis of trypanosomes (Imbuga *et al.*, 1992 & Abubakar *et al.*, 1995).

Trypsin is the most important and prevalent endopeptidase in the adult female bloodsucking insects. In insects like *A. aegypti*, trypsin account for at least 75% of the proteolytic activity in midgut homogenates (Briegel & Lea, 1975), while in *Stomoxys* midgut, 95% of trypsin activity are found in posterior midgut (Lehane, 1991). Serine proteases are a class of proteolytic enzymes characterised by the presence of uniquely reactive serine side chain. They are of extremely widespread occurrence and diverse function. One of the most studied serine protease family member is trypsin. Members of the trypsin family occur not only in the vertebrates and other phyla or higher organisms, but also among prokaryotes (Kraut, 1977).

Biochemical analysis suggested that in *Anopheles* mosquitoes the digestion of the blood meal is carried out in the gut by trypsins. Shortly after blood feeding, increasing trypsin activity could be detected in the gut lumen and remained elevated for several hours until digestion was completed (Billingsley & Hecker, 1991; Horler & Breigel, 1995). Purified mosquito enzymes with trypsin-like activity showed a range of properties in common with other known serine proteases. The properties include molecular weight range, substrate and inhibitor specificity, and the level of amino acid homology with known trypsin sequence (Graf *et al.*, 1991). This is exemplified by the cloning of a gene family in *A. gambiae* consisting of seven trypsin-related coding sequences (Muller *et al.*, 1993).

It is postulated that before the adaptive immune mechanisms evolved, innate mechanisms served to prevent infections. Antimicrobial proteins are common agents of innate immunity. They play a central role in immune defence in the lower phyla and they are also increasingly recognised as part of the host defence mechanisms in the animal kingdom. In mammals, they can serve both as a first defence before adaptive immune strategies. Proteolytic enzymes and other molecules structurally

related to proteases also play a role in innate immunity and are often found in close association with antimicrobial proteins. Protease cascades contribute to the immobilization and killing of invading micro-organisms, as in the complement cascade (Esser, 1991). Proteases participate in the processing and activation of various cytotoxic molecules (Zanetti *et al.*, 1990). They are important components of the antimicrobial weapon/arsenal of phagocytic cells, and they are found in storage organelles that are traditionally involved in microbial killing or that may be brought into contact with microbes upon lysosome fusion. They may have a number of roles; they may exert a cytotoxic effect through their proteolytic activity or independently from it, and they may contribute to the digestion of previously killed target cells. They may act alone or in combination with other antimicrobial agents. In addition, they may convert other cytotoxins from inactive to active forms. Thus proteases and analogues in many different ways can arm the cell to achieve its full potential in host defence. Although multiple and sometimes overlapping biological functions is a feature of this family, yet they all seem to pertain to host immunity (Gabay, 1994).

It is beyond doubt that the trypsin – lectin dimer cloned in this study is responsible for defence mechanism in *S. calcitrans* and is released along with proteolytic enzymes, lectins and lysins in the midgut following the ingestion of blood / proteinous meal and pathogens. These have multiple and sometimes overlapping biological functions which all seem to pertain to host immunity. Hultmark (1993) and Hoffmann, (1995), concluded that serine protease participate in immune signalling pathways as well as in the activation of immune reactive components such as a prophenol oxidase to phenol oxidase during melanotic encapsulation. Work by Abdally (1996), showed that the absolute concentration of both the haemolymph and midgut reservoir lectins were high in *S. calcitrans* than in tsetse flies. This possibly explains why *Stomoxys* in not a cyclical vector of trypanosomiasis despite being sympatric with *Glossina.*

Chapter Three

IDENTIFICATION OF TRYPANOSOME INFECTIONS IN GLOSSINA SWYNNERTONI AUSTEN, G. MORSITANS MORSITANS WESTWOOD, G. PALLIDIPES AUSTEN & G. BREVIPALPIS NEWSTEAD USING A PCR - BASED TECHNIQUE.

SUMMARY

Trypanosomes in the dissection-positive proboscis of four tsetse species (*Glossina swynnertoni, G. morsitans morsitans, G. brevipalpis* and G. *pallidipes*) were identified by PCR using species-specific primers. Of the 14702 flies dissected 1287 were proboscis positive. PCR was performed on 1041 dissection-positive proboscis giving an overall positive identification in 254 (24.40%) only. Of the 254 PCR identified infections 193 were single, 57 were double and 4 were triple infections. The triple infection recorded was from *G. pallidipes*. Chi-squared analysis revealed no significant differences in terms of grouping. Segregation of trypanosomes into three groups was only true with *G. pallidipes* and not obvious with the other three species *G. swynnertoni, G. m. morsitans & G. brevipalpis*. The result suggest that the patterns of infection varies among species and are also conserved within species; and that the grouping of trypanosomes into different infection categories in different fly species is a result of the differing feeding patterns among different fly species and members of each fly population, and or the ability of the trypanosomes in each of the infection categories to significantly influence the maturation of trypanosomes in the other categories.

INTRODUCTION

Trypanosomes are widespread in sub-Saharan Africa causing disease in man and his domesticated animals. They are cyclically transmitted by tsetse flies although some mechanical transmission by other vectors also occurs (Hoare, 1972). Despite many years of study our understanding of the epidemiological processes which determine the prevalence of trypanosome infection in tsetse flies is still far from complete. For years trypanosome identification in tsetse flies have based upon the location of the trypanosome in the dissected fly (Lloyd & Johnson, 1924) and this technique has been the foundation for much of our understanding of the epidemiology of trypanosomes in tsetse flies. The introduction of DNA-based methods and particularly PCR for trypanosome identification (Moser *et al.* 1989; Masiga *et al.* 1992, 1996; Majiwa *et al.* 1994; McNamara, Laveissiere, Masiga, 1995; Solano *et al.* 1996; Woolhouse *et al.* 1996; Morlais *et al.* 1998a, 1998b & Lehane *et al.*, 2000) has made an assessment of the accuracy of the Lloyd & Johnson (1924) technique a practical possibility. The epidemiology of vector transmitted disease in general and trypanosomiasis in particular has benefited a lot from the development of molecular biology tools during the last decade. The tools have enabled the characterisation of parasites and their vectors and also the study of their genetic variation to be more precise. They play key roles in developing more focused and efficient control programs. Analysis of the variability of the trypanosome species, especially *Trypanosoma congolense*, and the accurate characterisation of the parasites in the host and in the vector has helped in understanding the epidemiological cycles, the study of the vectorial capacity of various tsetse species in the lab and in the field aimed at checking if in each endemic focus a particular strain of trypanosomes is present. Sometimes epidemics may be due to re-emergence in a local strain; or as a result of an introduction of new strains of trypanosomes from another endemic region.

One of the shortcomings of Lloyd & Johnson (1924) technique is its failure to identify many of the mixed infections which exist. However, DNA methods of trypanosome identification (e.g. Majiwa and Otieno, 1990; Majiwa *et al.* 1994; Woolhouse *et al.* 1996; Masiga *et al.* 1996) has been instrumental in identification of mixed infections. Recent findings by Lehane *et al.*, (2000) showed that mixed infections segregate into three largely separate divisions among tsetse populations (i) *Trypanosome congolense* savannah & *T. congolense* Kenya coast (ii) *T. simiae, T. congolense* Tsavo & *T. godfreyi* and (iii) *T. vivax*. This finding was reliant on the use of DNA based methods. The cause of the segregation of these mixed infections in tsetse is unknown but could have several possible explanations. One is that differing feeding patterns among members of the fly population, so that the pattern would arise as a function of the feeding history of the fly and the infection status of the host animals (Clausen *et al.*, 1998).

It is accepted that *T. simiae* (Roberts, 1971) and *T. godfreyi* (McNamara *et al.*, 1994) are mainly associated with suids. Hence fly populations which frequently feed on these animals are more likely to contract *T. simiae* and *T. godfreyi*. It is also known that *T. congolense* Tsavo will cause mild (Zweygarth *et al.*, 1994) or fatal disease in domestic pigs (Majiwa *et al.*, 1993b) but that mice, goats and a steer could not be infected (Zweygarth *et al.*, 1994). Consequently this group of *T. simiae, T. godfreyi* and *T. congolense* Tsavo may separate from the other two because of an association with suids. It is also interesting to note that in a recent Phylogenetic analysis based on 18S rRNA these three trypanosomes all group on one branch within the *T. brucei* clade while the other *congolense* trypanosomes group on a separate branch (Stevens *et al.*, 1999). The animal associations of the other two divisions, *T. congolense* savannah/ *T. congolense* Kenya coast and *T. vivax*, are virtually unknown. Knowles *et al.*, (1988) reported that *T. congolense* Kenya coast at their site were mainly restricted to cattle, sheep and goats. If *T. congolense* savannah/ *T. congolense* Kenya coast and *T. vivax* are differently distributed in the ruminants at the field site studied by Lehane *et al.*, (2000) then

particular host feeding choices in different cohorts of flies may explain the separate infection categories.

The second explanation for segregation of mixed infections other than feeding preferences may be the ability of trypanosomes in each of the infection categories to significantly influence the maturation of the trypanosomes in the other categories (Reifenberg *et al.*, 1997a).

The novelty of patterns of infections prompted a need to carry out another survey at a new field site using other species of tsetse fly, *G. swynnertoni* Austen, *G. m. morsitans* Westwood, *G. brevipalpis* Newstead and *G. pallidipes* Austen. This was to verify if patterns vary widely across Africa or if they are conserved and whether the patterns vary among tsetse species. The segregation of mixed infections into different groupings have important implications for genetic exchange in trypanosome species as well as being an important factor determining patterns of disease transmission. Also, most studies on the patterns of mixed infection which exist in the field have been small sample sizes. Hence, it was the intention in this study to accumulate sufficient PCR identified data so as to investigate the different patterns of mixed infections which may exist in the field in *G. m. morsitans, G. swynnertoni, G. brevipalpis* and *G. pallidipes*.

MATERIALS AND METHODS

Description of study sites

Sangaiwe, at the Periphery of Tarangire National Park

Fieldwork was organised from the Tsetse and Trypanosomiasis Research Institute (TTRI), Tanga, Tanzania. The first field site chosen was Sangaiwe (4°00' S 36°00'E) near Sangaiwe Post at the periphery of the Tarangire National Park, in

Northern Tanzania. Sangaiwe is a human settlement in a location of Magugu, which popularly is a known foci of human sleeping sickness. In the past, the areas bordering the Sangaiwe Post were infested by *G. morsitans s.l.*, *G. pallidipes* and *G. swynnertoni* (Tsetse Monthly report Arusha region 1965 – 1991 Unpublished). However, current tsetse population includes *G. morsitans s.l.* and *G. swynnertoni*. Animal and human sleeping sickness was rampart in the area before 1990, and deliberate efforts had to be made to control the disease by surveillance and screening sick people who were being treated at Magugu sleeping sickness centre. Aerial spraying of insecticides and bush clearance were very effective in bringing the tsetse population to a lower density. Currently the use of animal bait (animal dipped in insecticide like pyrethroid compounds) have continued to bring the tsetse population down and the cases of human disease being reported are in negligible number. Nevertheless nagana is still present. Parasitological screening carried out between August 1999 to January 2000 in Babati district (which include Sangaiwe), showed a high trypanosomiasis infection rate in animals (cattle), especially in areas bordering Tarangire National Park; and other villages of Minjingu ward. A total of 209 cattle were bled for trypanosome screening and 23.92% were positive to trypanosomiasis and 8.61% to tick borne disease. Report from cattle owners also shows big losses of cattle every year due to trypanosomiasis. No person screened was found to be positive to trypanosomiasis infection during the study (TTRI 2000 – Unpublished).

The field site borders the wooded, bushed, open and thicketed grasslands with dense to medium forests. *Acacia tortilis* is the most abundant species of vegetation. Game animals frequently seen on the other side in the park include Zebra, Ostrich, Widebeast, Impala, Giraffe, Lesser Kudu, Buffalo, Warthog, Elephants, Cheetah and Jackal. The village supports a dense population of cattle, goats, sheep, donkeys, dogs, chicken and cats. Cattle are ranked as the most important livestock followed by goats. These are the most abundant animal in the area. Sheep and donkeys had equal rank in terms of importance, though are not as abundant as cattle and goats.

Rainfall and temperature records for the period of study were obtained from the meteorological station at Sangaiwe Post, owned by Tanzania National Parks (TANAPA).

Description of *G. swynnertoni* Austen

G. swynnertoni has a wide range of vertebrate hosts including bovids, suids, elephant, hippopotamus, primates, carnivores, aardvark and avians, of which buffalo, warthog and giraffe were the hosts most generally favoured. Comparison of the feeding patterns of this species by Moloo *et al.*, (1971) in Serengeti, showed that this species is readily adaptable. The local tsetse species were primarily zoophilic and, since game was abundant they attacked man only through chance meeting. Blood meal analyses by Ndegwa (1997) showed the host preferences as suidae with 64% of blood meal from pigs. Others included ruminants (3.9%), hippos (3.5%), Felidae (3.5%) and man (3.5%).

The activity pattern carried out by Ndegwa (1997), showed that the activity peaked between 1100hr and 1200hr and remained relatively active throughout but decreased at sunset (1800hr) especially for female flies. The activity of male flies peaked in mid afternoon (1500 - 1600hr) and decreased rapidly at sunset. Like most tsetse flies, *G. swynnertoni* is most active when the ambient temperature and/or light intensity are at a certain level. When these are above or below this optimum, the activity is less.

***G. m. morsitans* Westwood**

G. m. morsitans were collected from the same traps as *G. swynnertoni*. However, more *G. m. morsitans* were trapped in areas of relatively open woodland characterized by a predominance of medium to large isolated trees and a few small thickets. According to Clausen *et al.*, (1998) blood meal analyses showed that this species mostly fed on suids, ruminants and mammals like hippopotamus.

Pangani field site

Flies were also collected at Pangani (4 64' S 38 44' E) in Pangani district which is along the coast of Tanzania. The climate is influenced by it's near to equatorial latitude and proximity to the Indian Ocean. The area is characterised by high relative humidity and temperatures. The vegetation types include the communities of wooded grassland, bushed grassland, evergreen tree grassland, palm-high grass Savannah, riverine gallary forest; high, mixed forest and bush land thicket. As in Sangaiwe, game animals are very common. These include hippopotamus, buffaloes, warthogs, elephants, baboons, monkeys, giraffe, reedbuck, bushbuck, duicker, sable antelope, hartebeest and domesticated animals. Wild animals do migrate between Pangani and Mivumoni in search of water and food especially during the dry season. Tsetse species found in the area include *G. pallidipes*, *G. m. morsitans*, *G. brevipalpis* and *G. austeni*. However only *G. pallidipes* and *G. brevipalpis* were retained for dissections, because the trapped number was high compared to other species found in the area. No meteorological data could be obtained for Pangani as the meteorological station was not functional by the time this study was conducted.

***G. brevipalpis* Newstead & *G. pallidipes* Austen**

In Pangani, the forest forms the habitat for *G. brevipalpis* and *G. pallidipes* inhabits the thickets. While *G. brevipalpis* was trapped inside thickets and bushes, *G. pallidipes* was trapped on the periphery of the forest on open areas. However, all two species disperse to some extent into the open grassland. *G. pallidipes* disperses into the inland edge of the forest. The procedure of collecting flies was the same as in Sangaiwe. The relative abundance of *G. brevipalpis* to *G. pallidipes* was 1:5.

Fly collection

Flies for trypanosome infection were collected from a single 1 km stretch of earth road running along the road and adjacent to a stretch of forest (this road is a boundary between the park and the village). They were caught in open areas especially those that are used as game trails (Quentin & Brady, 1992). Flies were

collected using 5 biconical traps (Challier and Laveissiere, 1973) and 28 modified Pyramidal traps (Gouteux & Lancien, 1986) each baited with acetone (release rate of 0.5 - 1 g/h) and a 4 ml. sachet containing 3-n-propylphenol, 4-methylphenol and octenol at a ratio of 1:8:4 (Hargrove and Langley, 1990). To avoid undue fly mortality, traps were set in the shade of trees and flies were normally collected twice a day: at 11 am and 6 pm local time. The population density showed that *G. m. morsitans* and *G. swynnertoni* occurs at Sangaiwe in a ratio of 2:1. Flies were stored alive at 4° C in the dark and returned to the laboratory for dissection on the same or on the following day. Only living flies were dissected, irrespective of sex.

Fly dissection

Flies were dissected within 24 hours of their return to the laboratory. Dissection of mouth-parts were carried out in normal saline and screened under the light microscope for the presence of trypanosomes. The mouth-parts of positive flies were transferred into a small eppendorf for further analysis by PCR. To avoid cross contamination during dissection between flies, dissecting instruments were immersed in 5% sodium hypochlorite for two minutes, followed by extensive rinsing in distilled water followed by immersion in 0.9% saline. Re-used slides were washed thoroughly in detergent, sterilised by immersion in sodium hypochlorite and rinsed extensively in distilled water. The focus of this study was on trypanosome positive mouth-parts only. A total of 2893 dissections were carried out for *G. swynnertoni* and 5160 for *G.* m. *morsitans* in Sangaiwe while in Pangani, 979 *G. brevipalpis* and 5670 *G. pallidipes* were dissected.

PCR analysis

PCR template buffer was prepared from a mixture of 10 mM Tris-HCl pH 8.3, 50mM KCl, 1.5 mM $MgCl_2$, 0.9% Nonindet P40 and 2 μg Proteinase K. The buffer was portioned in 40 μL aliquots and kept at 4°C. After infected mouthparts have been added to a tube the sample was incubated at 55° C for 1 hour followed by 10 minutes at 95° C to inactivate the proteinase K.

PCR primers used for analysis are listed in Table 3A (Appendix). PCR was performed as 50µl reactions containing 1 unit of Taq polymerase, 4µl of template, 1 x PCR buffer (Promega), 200µM dNTPs, 1.5mM $MgCl_2$, 1µM of each primer except for the *T. vivax* primers where 5µM of each primer was used. Cycling conditions were 1 cycle of 94°C for 3 minutes followed by 30 cycles of 94°C 1 minute, 60°C two minutes and 74°C 30 seconds on a Touchgene Techne Thermal cycler. A much lower annealling temperature was used for *T. vivax* (i.e. 55°C instead of 60°C). Reactions were performed in sets of 40 each set using only one pair of primers. All templates were tested with each of the seven pairs of primers. This is because for *T. vivax* two sets of primers were used as indicated in Table 3A. Positive control and no template negative control were also included in each run as a reference point for the experimental template. At the outset, all primer pairs were tested against all positive control DNA available to confirm the expected positive reactions. PCR products were analysed on 2% agarose gels. The results from the trypanosome identification using the two sets of primers, *T. simiae* (TSM) and *T. congolense* Tsavo (TST) have been grouped together as *T. simiae* Tsavo following the regrouping of the two subspecies into *T. simiae* Tsavo (Gibson *et al.*, 2001)

Data analysis

Chi-square analysis to test the significance of the discrepancy between the observed and the expected results was used (Hampton, 1994). The test was meant to find the significance if any of infection patterns in tsetse found in one site, infection in tsetse between the two sites and infection in tsetse between *G. pallidipes* species from Pangani and Mivumoni. The infection pattern of *G. pallidipes* from Mivumoni was obtained from Lehane *et al.*, (2000). The two sites, Pangani and Mivumoni were 60 km apart and the data from Pangani was collected in February 2001, while that from Mivumoni was collected in 1995 – 1996, an interval of 5 to 6 years.

RESULTS

PCR identification

A summarised account of dissected flies and the total positive for both parasitological and PCR results are tabulated in Table 3.1. The northern flies (*G. swynnertoni* and *G. m. morsitans*) had a high number of unidentified trypanosomes by PCR analysis compared to the unidentified in *G. brevipalpis* and *G. pallidipes* from the coast, Pangani.

Table 3:1 Summary of positive parasitological and PCR results

Tsetse species	Dissected	Parasitological Positive	% infection rate	PCR analysed	PCR positive	% PCR positive	% PCR unidentified
Gs	2893	437	15.1	368	43	15.04	84.96
Gmm	5160	269	5.21	266	40	11.68	88.32
Gb	979	106	10.83	106	54	50.94	49.06
Gp	5670	475	8.38	301	117	38.87	61.13

Gs – *G. swynnertoni*, Gmm – *G. m. morsitans*, Gb – *G. brevipalpis*, Gp – *G. pallidipes*

No attempt was made to use PCR to identify trypanosome infected flies which were dissection negative although in other studies this has been shown to raise the infection rate significantly (Morlais *et al.* 1998a). In order to minimise the number of immature infections identified and to avoid problems with residual trypanosome DNA in digested blood (Morlais *et al.* 1998a), PCR was only performed PCR on infected proboscis. The details of the trypanosomes positively identified are given in Table 3:2.

Table 3:2 Single and mixed infection categories found in tsetse species and the % of pooled trypanosome groups

Trypanosome groups	**Gs**	**Gmm**	**Gb**	**Gp**	**Total Infection / / trypanosome Group**	**Total % infection / trypanosome group**
Tcs	2	6	5	15	28	11.02
Tck	9	1	0	4	14	5.51
Tst	13	11	47	33	104	40.95
Dgg	8	4	0	17	29	11.4
Tv	2	8	1	7	18	7.09
Tcs/Tck	3	6	1	21	31	12.2
Tst/Dgg	6	3	0	3	12	4.72
Tck/Tst	0	1	0	1	2	0.8
Tck/Tv	0	0	0	4	4	1.57
Dgg/Tv	0	0	0	2	2	0.8
Tcs/Tv	0	0	0	6	6	2.4
Tcs/Tck/Tv	0	0	0	4	4	1.57
Total infected	43	40	54	117	254	100
Total PCR analysed	368	266	106	301		
Total infected (%)	11.68	15.04	50.94	38.87		

Tcs, *T. congolense* savannah; Tck, *T. congolense* Kenya coast; Tst, *T. simiae* Tsavo; Dgg, *T. godfreyi*; Tv, *T. vivax*; Gs, *G. swynnertoni*; Gmm, *G. m. morsitans*; Gb, *G. brevipalpis*; Gp, *G. pallidipes*.

Table 3.2 shows result of identified trypanosome subgenus per tsetse species and also the pooled result per trypanosome species. The result shows that the single infection scored high observations for each species compared with the double infection and triple infection. The highest single infection observed was from the *T. simiae* Tsavo with 104 (40.95%) observations, and most of them were from the coast flies (*G. pallidipes* and *G. brevipalpis*). For the double infection, the category of *T. congolense* Savannah & Kenya coast was the highest by having 31 (12.2%)

observations. The triple infection category had 4 (1.57%) observations which all were obtained from *G. pallidipes*.

Segregation

Due to the high % of unknowns, it was difficult to undertake an analysis based on the individual infection category. So infections were pooled together (Table 3.3) based on the trypanosome groups as reported by Lehane *et al.,* (2000) with regard to trypanosomes associated with suids (*T. simiae* Tsavo & *T. godreyi*), trypanosomes associated with ruminants (*T. congolense* Savannah & *T. congolense* Kenya coast), trypanosomes associated with ruminants and *T. vivax*, trypanosomes associated with suids and *T. vivax*, trypanosomes for suids and ruminants and lastly *T. vivax* infection alone. The groups are summarised as follows:

1. All single and double infections of *T. congolense* Kenya coast & Savannah
2. All single and double infection of *T. simiae* Tsavo, & *T. godfreyi*
3. All *T. vivax*
4. All *T. congolense* Kenya coast and Savannah mixed with *T. vivax*.
5. All infection in 2 above which occur together with *T. vivax*.
6. All infection in 1 & 2 which occur together in association.

When analysing the relationship of infection categories from the two sites (Pangani & Tarangire), groups 4, 5 & 6 were ignored because they composed infection from *G. pallidipes* only. Between sites, there was no significant difference between the infection in Tarangire and Pangani. The Chi-squared analysis within a site showed that there was no significant difference in infection in *G. swynnertoni* and *G. m. morsitans*, however, significant difference was obtained between *G. brevipalpis* and *G. pallidipes* by $\chi^2 = 17.9$, (df = 2) at $P \leq 0.05$. This could be attributed by the fact that there was a distinct difference in infection in categories 1 and 2, i. e. between the *T. congolense* Kenya coast & savannah infection, and the *T. simiae* Tsavo & *T. godfreyi*. *G. pallidipes* had high score of ruminant associated trypanosomes and suid

trypanosomes (53) compared to the observed infection in *G. brevipalpis* (6 and 47 respectively).

Table 3:3 Pooled results on grouping categories of trypanosomes as based on their association with suids, ruminants, suids + ruminants, suids + *T. vivax*, ruminants + *T. vivax* and *T. vivax* only

Species	Category 1	category 2	category 3	category 4	category 5	category 6
	TCK, TCS, TCS/TCK	TSM, TST, DGG, TST/DGG	TV	TV/TCS/TCK	TST/DGG/TV	TST/DGG / TCS/TCK
Gs	14	27	2	0	0	0
Gmm	13	18	8	0	0	1
Gb	6	47	1	0	0	0
Gp	40	53	7	14	2	1
Total	73	145	18	14	2	2

Tcs, *T. congolense* savannah; Tck, *T. congolense* Kenya coast; Tst, *T. simiae* Tsavo; Dgg, *T. godfreyi*; Tv, *T. vivax*; Gs, *G. swynnertoni*; Gmm, *G. m. morsitans*; Gb, *G. brevipalpis*; Gp, *G. pallidipes*.

Mixed infection

For *G. swynnertoni* the results showed that 79.07% were single infections and 20.93% were double infection. In *G. m. morsitans*, 75% were single and 25% double infection. 98.15% were single infection in *G. brevipalpis* and 1.85% double infection. For *G. pallidipes*, 64.96% were single, 31.62% were double and 3.42% triple infection. The single and double infections were recorded in all species (*G. swynnertoni, G. m. morsitans, G. brevipalpis* & *G. pallidipes*), but in addition, *G. pallidipes* had a triple infections as well (Table 3.1 & 3.4).

Table 3:4 Trypanosome infection groupings in % per individual tsetse species

Species	Single	Double	Triple
G. m. morsitans	75	25	0
G. swynnertoni	79.07	20.93	0
G. brevipalpis	98.15	1.85	0
G. pallidipes	69.96	31.62	3.42

The number of identified *T. vivax* infection was very low from all samples collected from the two areas, Tarangire and Pangani. Two sets of *T. vivax* primers (Masiga *et al.*, 1992 & Masake *et al.*, 1997) were used in order to identify more of *T. vivax* infections. Primers by Masake *et al.*, (1997) identified few *vivax* infections from *G. m. morsitans* and *G. pallidipes*. With the two primers, the total numbers of positive T. vivax infection identified are tabulated in Table 3.5.

Table 3:5 *T. vivax* primers used to identify *T. vivax* infection and the number of infection identified per tsetse specie.

Species	TVM	TVW
G. m. morsitans	5	3
G. swynnertoni	0	2
G. brevipalpis	1	0
G. pallidipes	5	18
Total	11	23

TVM – primers by Masake *et al.*, (1997), TVW – primers by Masiga *et al.*, (1992)

G. pallidipes from Pangani and Mivumoni

A Chi-squared (χ^2) test of the infection pattern from *G. pallipes* Mivumoni (Lehane *et al.*, 2000, data summarised from Table 3B in the Appendix) and Pangani was carried out following the grouping of infection categories as in Table 3:3, based on the association of trypanosomes with different host as explained by Lehane *et al.*, (2000). The groups of the infection pattern are indicated in Table 3.6. Between the two species from the two sites, there was a significant difference between infections ($\chi^2 = 41.97$, df = 5, $P \leq 0.05$). This could have been attributed by *T. vivax* and *T. congolense* + *T. vivax* categories which were in high numbers in Mivumoni than in Pangani

Table 3:6 Comparison of infection segregation of trypanosomes in *G. pallidipes* from Pangani and Mivumoni.

Tsetse species	Category 1	Category 2	Category 3	Category 4	Category 5	Category 6
G. pallidipes Pangani	40	53	7	14	2	1
G. pallidipes Mivumoni	133	99	89	8	2	4

DISCUSSIONS

G. brevipalpis had the highest infection of *T. simiae* Tsavo than that which was found in *G. pallidipes*, a sympatric specie. From the other site, the infection of *T. simiae* Tsavo in the two species (*G. swynnertoni* and *G. m. morsitans*) was almost the same. The result shows that *T. simiae* Tsavo is well distributed in all tsetse species and at the two sites.

It is now clear that *T. simiae* Tsavo and *T. godfreyi* are more or less distributed throughout the tsetse belt, and have established themselves in different tsetse species. *T. simiae* Tsavo has been reported from Kenya (Majiwa *et al.* 1993b), Central African Republic (D'Amico, 1996) and from the Coast of Tanzania (Lehane *et al.* 2000). *T. godfreyi* has previously been reported from Southern (Woolhouse *et al.* 1996) and West Africa (McNamara *et al.* 1994; Masiga *et al.* 1996) and the coast area of Tanzania (Lehane *et al.* 2000); and this study, reports the presence of *T. simiae* Tsavo and *T. godfreyi* in the coast and the northern part of Tanzania collected from *G. swynnertoni* and *G. m. morsitans, G. pallidipes and G. brevipalpis*.

The *congolense* – type (Savannah & Kilifi) were also common at the two study sites, Pangani and Tarangire. The Savannah subgroups are also widespread in Africa and common in domestic livestock (Nyeko *et al.* 1990; Reifenberg *et al.* 1997a). The Kenya coast/ Kilifi subgroups have also been reported from various areas of East Africa (Kukla *et al.* 1987; Gibson *et al.* 1988; Nyeko *et al.*, 1990) as well as the Central African Republic (D'Amico, 1996), Côte d'Ivoire (McNamara *et al.* 1995, Masiga *et al.* 1996), Zimbabwe and Zambia (Woolhouse *et al.* 1994, 1996).

T. vivax, though widely spread in Africa, was recorded at a lower rate in the field sites studied here than recorded by Lehane *et al.,* (2000). In this study 18 observations were single infection and 16 observations as mixed infection. It is also interesting that all mixed infection with *T. vivax* came from *G. pallidipes* and not from other species. It was unfortunate that there were high numbers of unidentified trypanosomes which prevented a more clear-cut analysis of the data. The possible reason could probably be due to the presence of new strains of trypanosome at the field sites studied here which could not be picked by the primer sets currently in use (see Chapter Four).

The presence of mixed trypanosome infections as well as segregation into different groupings has been reported by several authors (e.g. Majiwa & Otieno, 1990, Majiwa *et al.* 1994, Woolhouse *et al.* 1996 & Masiga *et al.* 1996) and most recently by Lehane *et al.*, (2000). The grouping and segregation is however not consistent in this study. In Lehane *et al.* (2000), 36% of the infections were mixed with 31.5% double infections, 3.5% triple infections and 1% quadruple infections, whereas in this study, the overall single infection was 76%, 22.44% double and 1.57% triple infection. Only *G. pallidipes* showed consistent results with *G. pallidipes* at Lehane *et al.*, (2000) site. Even for individual species, the single infections were high than mixed infections (Table 3.4). This shows that even from relatively close sites in Tanzania trypanosome groupings in tsetse flies vary considerably. This strongly

suggests that segregations are not a fixed entity but are chance events driven by external considerations such as host availability.

In this study, a total of 1041 samples were PCR analysed and only 24.40% were positive. Comparing this with the data set of Lehane *et al.*, (2000), is difficult because of the relatively high % of unknowns in this study. Woolhouse *et al.* (1996) studied *Nannomonas* infections of *G. pallidipes* at two field sites in Zimbabwe and one in Zambia. In their 179 molecularly identified infections only 9 involved mixed infections of *T. simiae* Tsavo and *T. godfreyi* with each possible pair occurring 3 times. A clear grouping of *T. simiae* Tsavo and *T. godfreyi* is not evident in this study.

Mixed infections of the *T. congolense* types were also common as previously reported by various authors. In this study, similar patterns of mixed infections as in Lehane *et al.* (2000) have been noted in a few cases. The highest group for mixed infection was *T. congolense* Savannah and *T. congolense* Kenya Coast with 31 (12.2%), observations followed by *T. simiae* Tsavo and *T. godfreyi* which had 12 (4.72%) observations (Table 3.2).

Mixed infections might be contracted simultaneously from single infected animals or may be a result of sequential infections over a number of feeds. There is published evidence that both mechanisms are possible. Thus mixed trypanosome infections have been reported in mammals (Noireau *et al.* 1986; Nyeko *et al.* 1990) and it has been shown in the laboratory that flies can acquire mixed infections from a single feed albeit inefficiently (Reifenberg *et al.* 1997a). Laboratory studies have shown that sequential infection of tsetse with trypanosomes is possible (Gibson & Ferris, 1992). Although the data set was large enough for comprehensive analysis, a high % of unknowns derailed the anticipations. Tentative interpretation of the data by Lehane *et al.*, (2000) showed that the fly with a triple infection with *T. simiae* Tsavo and *T. vivax* was 31 days old and the mean age of 3 flies with a triple

infection with *T. congolense* Savannah, *T. congolense* Kenya coast and *T. vivax* was 48 days. Given the small proportion of infected flies in the population it seems reasonable to conclude, in line with many other studies that the probability of infection at any one feed is low. Consequently, given a maturation period of 20 or so days, the complex infections at a young age suggest simultaneous infection from a single infected animal. In contrast those flies with quadruple infections were all old flies (>95 days) compared to flies in the other, less complex infection categories. From that it could be tempting to conclude that the older the fly the more likely to end up with multiple infection whether as a result of sequentially acquired infections or simultaneous infections. Based on this, it may suggest that flies collected for this study were young flies only, probably the traps used were biased towards young flies, and this could explain why more of triple and quadruple infections were missed. But also, this is the first time molecular analysis have been carried out on infected mouthparts of *G. brevipalpis, G. swynnertoni* and *G. m. morsitans*, probably this is the real pattern of infection in these species.

Msangi *et al.* (1998) noted that changes in nutritional status with month, was a result of seasonal migration of animals thus affecting the availability of hosts for flies to feed from. Nutritional status influences infection status of the *T. congolense* savannah /*T. congolense* Kenya coast and the *T. simiae* Tsavo & *T. godfreyi* categories but not the *T. vivax* category.

There are also clear differences between species and habitat, both possibly related to diet. Levels of *T. vivax* and *T. congolense* infection are well known to be related to the proportion of bovidae and suidae blood meals respectively. Moloo *et al.*, (1971) noted that there is a positive correlation between the infection rate of *G. swynnertoni* with *T. vivax* and the percentage of the blood meals of the flies that were derived from bovidae. It has been suggested that bovids act as the main source of *T. vivax* infections, because warthogs, the other main hosts of *G. swynnertoni*, are refractory to such infections. This could be a reason for a very low number of *T. vivax*

infections in *G. swynnertoni* in Northern Tanzania, which feeds mostly on suids (Ndegwa, 1997).

G. pallidipes from Mivumoni (Lehane *et al.*, 2000) had high observations of *T. vivax* (89) compared to 7 observation in this study. However *G. pallidipes* at our site had high numbers of *T. congolense* Kenya Coast & *T. congolense* Savannah + *T. vivax* (14 observations) compared to 8 in Lehane *et al.*, (2000). The two samples were collected from sites 60 km apart.

G. swynnertoni and *G. m. morsitans* were collected during the dry season (May 2000), which was a result of drought in the Northern part of Tanzania, Arusha. As a result there were thousands and thousands of game congregation along the river and Lake Burunge, both inside the park, just few kilometres from our study site. These animals had migrated from the Masai Mara in search of water and pastures. Also pastoralists will take their cattle illegally into the park in search of pastures. Consequently there was plenty of food for the two species (*G. m. morsitans* and *G. swynnertoni*) from all these animals. However, the case was different for *G. brevipalpis* and *G. pallidipes*. These were collected during the end of the dry season and the area was very dry during collection and most wild animals had migrated away from the Pangani forested areas in search of food. Changes associated with season could be a factor towards the kind of infection or trypanosome harboured by flies trapped.

G. brevipalpis feed mainly on hippopotamus, suidae & to a lesser extent on ruminants (Clausen *et al.*, 1998). *G. brevipalpis* gave the highest % of *T. simiae Tsavo* infection compared to *G. Pallidipes* from the same locality (Table 3:2). It shows clearly that its preference for suidae was probably the source of this infection pattern. This was true for *G. swynnertoni* also which had the highest of *T. simiae Tsavo* compared to other trypanosome identified. In general the result indicates that feeding patterns are the determining factor for infection patterns in flies.

Identifications based on the dissection methods of Lloyd & Johnson (1924) are inconsistent with PCR identification results. Given that so much of our understanding of the epidemiology of trypanosomes in tsetse flies is based on the Lloyd & Johnson (1924) dissection technique, it is clear we should move to molecular tools for the precise identification of trypanosomes for better understanding of epidemiological studies.

Susceptibility to trypanosome infections

It was shown by Janssen & Wijers (1974) that the virulence of *T. simiae* Tsavo is determined by the species of tsetse transmitting the trypanosome. In *G. brevipalpis* the trypanosomes become virulent and in *G. pallidipes* they lose part of this virulence. *G. pallidipes* is thus more resistant to infection with *T. simiae* Tsavo than G. *brevipalpis*. This could be the reason why the natural infection with *T. simiae* Tsavo in *G. brevipalpis* was higher than in *G. pallidipes*. The possible explanation for this could be due to difference in susceptibilities of *G. brevipalpis* and *G. pallidipes* for trypanosomes. The second possibility is that the pattern seen is a result of different feeding patterns in the two fly species and the fact that the different hosts fed upon have different infections. The two species were collected from the same site.

CONCLUSION

Segregation of trypanosomes into three groups was only true with *G. pallidipes* but not obvious with other three species *G. swynnertoni, G. m. morsitans* & *G. brevipalpis*. Pattern of infection varies among species and are also conserved within species.

Chapter Four

PCR ANALYSIS OF THE UNKNOWN TRYPANOSOMES USING 18 S RIBOSOMAL RNA

SUMMARY

The use of 18S ribosomal RNA primers has enabled the amplification of trypanosomes species/subgroup sequences which could not be identified by species-specific primers. Groups of three different Mivumoni type *T. (Nannomonas) godfreyi* obtained through DNA sequence analysis are presented. Group I: represented by clone T223. These were all collected from the coastal area of Tanzania from *G. pallidipes*. Group 2 represented by clone T104 and were from *G. brevipalpis* in the coast and *G. m. morsitans* in Tarangire. Group 3 represented by clones T93 from *G. brevipalpis* and *G. swynnertoni* from Tarangire. The new isolate of *T. (Nannomonas) simiae* Tsavo from the coast (Mivumoni type) is represented by clones T1 & T70 which were isolated from *G. pallidipes* and the Tarangire type *T. simiae* Tsavo (Clone T10) was isolated from *G. swynnertoni*. This study also presents a *T. vivax* variant (clone T78) from Tarangire, isolated from *G. m. morsitans*. The results indicate that the Mivumoni type of *T. godfreyi* are not restricted to the coast area only, but seem to be widespread in the northern tsetse belt as well. The results also show strongly that more work is needed to describe the diversity of trypanosomes in Tanzania.

INTRODUCTION

In the tsetse belt, trypanosomiasis is a disease which continues to be a constraint to livestock production as well as to the welfare of human beings. A wide range of domestic and wild animals serve as hosts of trypanosomes and there are large numbers of different tsetse and trypanosome species, subspecies and strains involved. As mentioned in earlier chapters, tsetse transmitted trypanosomes include *Trypanosoma vivax, T. congolense, T. evansi, T. simiae* Tsavo, *T. godfreyi* and *T. brucei.* In addition different subspecies and strains exist and they can cause different disease syndromes in different hosts.

Trypanosome parasites exhibit diversity in their genetic make - up. The considerable intra-specific genetic diversity at the level of individual genes has been useful for characterisation of the parasite species and strains, and has been used extensively for epidemiological analyses. The classical method of trypanosome identification to subgenus level in tsetse flies is based on the location of trypanosomes in the dissected fly (Lloyd & Johnson, 1924). This technique has been the foundation for much of our understanding of the epidemiology of trypanosomes in tsetse flies. Various trypanosome species have been characterised by morphology and morphometrics, using stained blood smears, movement characteristics of the organisms in fresh blood films, localisation of the various developmental forms of the parasite in the tsetse fly, and the infectivity and virulence of the trypanosomes in various animal hosts (Hoare, 1970a, b). However, it is difficult and sometimes impossible to identify a parasite solely on the basis of its morphology (Majiwa *et al.*, 1993a). For example it is not possible to distinguish *T. simiae* Tsavo from *T. congolense*, or *T. brucei* from *T. evansi* on the basis of morphological characteristics alone. Sometimes, to a certain extent trypanosomes can be classified on the basis of their developmental cycle in the vector as *vivax* type, *congolense* type or *brucei* type according to the presence of parasites in the proboscis alone, or in the proboscis and

gut, or in the proboscis, gut and salivary glands, respectively (Stephen, 1986). Similarly, this by itself is also insufficient, because some species such as *T. simiae* Tsavo and *T. congolense* have identical developmental cycles in the vector. Furthermore, the identity of the different species, in cases of a mixed infection, cannot be confirmed using morphological criteria as immature, procyclic vector stages are practically indistinguishable (Hoare, 1970a; Stephen, 1986).

Other approaches used have been to inoculate samples of the parasite type into experimental animals and then observe the infectivity and virulence of the parasites in the various hosts. For example *T. brucei, T. rhodesiense* and some strains of *T. congolense* multiply readily in laboratory mice and rats while *T. simiae* Tsavo and East African *T. vivax* does not infect rodents (Baker, 1970; Nantulya, 1990). Similarly, some field isolates of *T. congolense* do not infect rodents (Godfrey, 1961; Young & Godfrey, 1983; Masake *et al.*, 1987). These traditional methods of identification and classification of trypanosomes, though useful, still are inadequate when used on their own. The inadequacy of this technique with regard to mixed and immature infections has been known for some time and has been confirmed by several field studies (Majiwa & Otieno, 1990; Majiwa *et al.,* 1994; Solano *et al.*, 1996; Woolhouse *et al.*, 1996; Masiga *et al.*, 1996; Morlais *et al.,* 1998a & b; Lehane *et al.*, 2000). It is clear that much work remains to be done to understand the molecular and genetic basis of these differences. It is particularly important to correlate molecular variation at the gene level with changes in patho-physiological properties of the parasites.

The introduction of DNA - based methods and particularly PCR, for trypanosome identification (Majiwa & Webster, 1987; Kukla *et al.,* 1987; Gibson *et al.,* 1988; Moser *et al.*, 1989; Masiga *et al.,* 1992) and their application to field studies has been a major step forward. It has improved the general understanding of the epidemiology of tsetse transmitted disease, and has revealed major gaps in our understanding of the epidemiology of trypanosomiasis. It is now clear that a

significant proportion of trypanosome infections isolated from tsetse flies cannot be identified using current probes (specific probes for *T. brucei ssp, T. congolense* Savannah, Forest, Tsavo and Kilifi subgroups, *T. simiae* Tsavo, *T. godfreyi* and *T. vivax,* East and West African forms). Lehane *et al.*, (2000) performed 406 PCR analyses on dissection-positive probosces, giving positive identifications in 352 (86.7%). The identity of the remaining 13.3% of infections is unknown. Similar studies, but with smaller sample sizes achieved successful identification rates of 94% and 54% in Cote d'Ivoire (McNamara *et al*, 1995; Solano *et al.* 1999 a & b respectively). Some of the unidentified infections were suspected to be due to reptilian trypanosomes, such as *T. grayi*, which develop in tsetse hindgut (Gouteux & Gibson, 1996). However, when unidentifiable infections from tsetse in The Gambia were cultured *in vitro* and brought back to the lab, a new species of subgenus *Nannomonas*, subsequently named *T. godfreyi*, was discovered (McNamara *et al*, 1989; McNamara, Mohamed & Gibson, 1994; Masiga, McNamara & Gibson, 1996). Similarly, in Kenya a new subgroup of *T. congolense* the Tsavo type was described (Majiwa *et al.*, 1993b).

Recombinant DNA technology has provided a more accurate tool for identification and characterisation of African trypanosomes. Trypanosome species or subspecies can now be identified with a high degree of specificity and sensitivity (Hide & Tait, 1991; Majiwa *et al.*, 1993a). For example within *T. congolense*, at least four different genotypes or subspecies were identified, which included Savannah, Kilifi, West African Forest / Riverine and Tsavo genotypes (Young & Godfrey, 1983; Majiwa *et al*, 1985, 1993b). The value of characterisation of trypanosomes to the subspecies level is seen in the fact that some of the genotypes show differences in clinical picture of the disease caused in the animals. The Savannah type *T. congolense* isolates, are known to cause skin reactions or chancres in cattle (Akol & Murray, 1983), while Kilifi type *T. congolense* isolates do not cause chancre (Masake *et al.*, 1987). However, within the Kilifi genotype, a large number of strains (serodemes) have been identified which do not confer cross protective

immunity (Masake *et al.,* 1988). Different trypanosome strains have been also found to have variable degrees of sensitivity to drugs (Silayo *et al.*, 1992: Gray & Pergrine, 1993). Recently, the use of a species specific PCR test and DNA sequence analysis has been instrumental in regrouping *T. congolense* Tsavo type as *T. simiae* Tsavo (Gibson *et al.*, 2001).

This work was aimed at identifying trypanosomes which could not be identified using the universal primers commonly used in the identification of trypanosomes (Chapter 3). As reported in Chapter 3, the PCR based analysis of infected trypanosomes resulted in 75.6% unidentified trypanosomes. The method commonly used to identify multiple bacterial species in the environment by PCR amplification of the small subunit ribosomal RNA (ssu rRNA) gene was adapted for trypanosomes. Conserved primers to the ssu rRNA gene were used to amplify an approximately 500 bp region spanning the most variable region of the trypanosome gene (Stevens *et al.*, 1999). Ssu rRNA genes have been used in analysis of polymorphism by analysing the evolutionary relationships between the various groups of trypanosomatids and also analysis of variation between strains. The use of 18S ribosomal RNA primers will be vital in the identification of other trypanosomes of economic importance which were not be identified by the universal primers. The isolated PCR-derived DNA from the unknown trypanosomes will be ligated into a vector (plasmid) and sequenced to determine its composition.

MATERIALS & METHODS

Materials used in this study were collected from Pangani and Tarangire as explained in Chapter Three. These were PCR tested using seven sets of primers listed in Table 3A. Those which were negative were then tested for mouthpart trypanosomes by using the 18S rRNA primer.

PCR tests

The PCR reaction to determine which mouth parts were positive by 18s rRNA was performed as 50µl reactions containing 1 unit of Taq polymerase, 4µl of template (10- 20ng), 1 x PCR buffer (Promega), 200µM dNTPs, 1.5mM $MgCl_2$, 1µM of each primer. Cycling conditions were 1 cycle of 94°C for 3 minutes followed by 30 cycles of 94°C 1 minute, 50°C for 30 seconds and 72°C 60 seconds, followed at the end of 30 cycles with extension at 72°C for 10 minutes and hold at 4°C on a Touchgene Techne. PCR products were analysed on 2% agarose gels.

Primers used were as described by Maslov *et al.,* (1996) and are tabulated below (Table 4: 1). The primer combinations gave the following PCR product, CJ 300 bp, CI 600 bp.

Table 4:1: Ssu rRNA primer sequences used to identify unknown trypanosome species.

Code	Sequence	Short code
S-662	GACTACAATGGTCTCTAATC	I
S-826	CCAACAAAAGCCGAAACGGT	J
S-713	CCGCGGTAATTCCAGCTCC	C

To ligate the PCR products, a TOPO Cloning reaction (Invitrogen) for positive samples was made as follows: 2 µl of fresh PCR product, 1 µl salt solution, sterile water 2 µl and TOPO vector 1µl. The mixture was gently mixed and incubated at room temperature (22-23°C) for 5 minutes. Then from the above mixture 2 µl of the TOPO Cloning reaction was added into a vial of One Shot Chemically Competent *E. coli* and mixed gently followed by incubation on ice for 30 min. The cells were heat shocked for 30 seconds at 42°C in water bath without shaking, and followed by an immediate transfer of tubes to ice. 250µl of room temperature SOC medium was added and the tubes capped tightly before being placed horizontally on a shaker (200 rpm) at 37°C for 1 hour. From each transformation, 10 - 50 ml cells were

spread on prewarmed selective plate and incubated overnight at 37°C. White colonies from each transformation were screened by PCR amplification using internal primers and those with inserts were inoculated in the 4 ml LB medium containing 50 μg/ml ampicillin and incubated at 37°C with shaking overnight. Plasmid extraction was carried out from 3ml of LB following the QIAprep Miniprep Handbook (2002). The remaining 1 ml of each positive colony was glycerol preserved by addition of 500 μl of sterile LB: Glycerol, quick frozen in liquid Nitrogen and stored at -80°C for future analysis.

Plasmid sequencing

Accurate DNA quantification was carried out and the appropriate concentration diluted to about 100 ng and denatured at 96°C for 1 minute, cooled before the addition of sequencing primer 2 μl, DTCS Quick Start master Mix 8 μl and loaded into a Thermal cycling program at 96°C for 20 sec, 50°C for 20 sec, 60°C for 4 min for 30 cycles followed by holding at 4°C. The products were ethanol precipitated as follows: Into labelled tube 4 μl of stop solution (1.5M NaOAc + 50 mM EDTA prepared fresh daily by mixing equal volumes of the 3M NaOAc and 100mM EDTA) and 1 μl of 20mg/ml glycogen. In these tubes, the sequencing reaction was added into appropriately labelled 0.5ml microtubes and mixed thoroughly. Followed by the addition of 60 μl cold 95% (v/v) ethanol / dH_2O from -20°C freezer and mixed thoroughly. The mixtures were immediately centrifuged at 14,000 rpm at 4°C for 15 minutes, and the supernatant carefully removed. The pellet was rinsed twice with 200 μl 70% (v/v) ethanol / dH_2O from -20°C freezer. For each rinse, the precipitate was centrifuged at 14,000 rpm at 4°C for a minimum of 2 minutes. Pellets were vacuum dried for 40 minutes and resuspended in 40 μl of the Sample Loading solution provided in kit by Beckman Coulter. The samples were loaded into CEQ sample plate wells and overlaid with mineral oil and loaded into the sequence machine (Beckman Coulter).

Sequence alignment

Sequences were edited to remove the vector and the trypanosome universal primer sequence and compared with database sequences using BLASTn software (Altschul *et al.*, 1990). Sequences were aligned with the following known tsetse transmitted trypanosomes: *T. vivax* (U22316), *T. godfreyi* (AJ009155), *T. simiae* Tsavo (AJ009162), *T. congolense* Kilifi (AJ009144), Savannah (AJ009146) and Riverine Forest (AJ009145) types and *T. brucei* (AF306772) using Clustal W (Thompson *et al.*, 1994) in the DNAstar suite of programmes. Phylogenetic analysis was also performed automatically during sequence alignment by Clustal W. The Alignment programme enabled the alignment of sequences by Multiple alignment of nucleotide sequences and automatically computed the sequence Distances, Residue substitutions, Phylogenetic Tree and made available the Alignment reports for every alignment performed.

Also another approach used to analyse the phylogeny of the obtained sequences was PAUP* 4 (beta version 4.0b4a; Swofford, 2001). The default options of PAUP were used: TBR branch swapping, zero length branches collapsed. The Phylogram constructed by bootstrapped (100) replicates maximum parsimony analysis of 38 (*Nannomonas* and *Duttonella*, and 32 isolated in this study) unrooted 18S ssu rRNA sequences. The sequence addition was done by 3 random replicates. The final tree is derived from the 195 retained trees by choosing the most consensus tree option. Bootstrap values for all major nodes are given and all branches receiving bootstrap support values > 50% are shown; relationships failing to achieve this level of support are shown as polytomies.

RESULTS

PCR identification

A total number of 314 infected mouth parts analysed by 18s rRNA primers from four different tsetse species are tabulated below in Table 4:2.

Table 4:2 Total number of unknown trypanosomes cloned and sequenced per individual tsetse species.

Source	Analysis by 18s rRNA primers	Positives	Total cloned	Inserted	Sequenced	Similarity to Trypanosomes
G. m. morsitans	74	18	13	11	11	10
G. brevipalpis	50	9	9	8	8	8
G. pallidipes	183	72	17	17	17	15
G. swynnertoni	7	7	5	5	5	5

Summarised results of sequenced clones and their similarity as obtained from BLAST search are presented in Table 4A (in the appendix). A total of 38 sequences were obtained which had resemblance with tsetse transmitted trypanosomes.

DNA sequence comparison and similarity

T. godfreyi

Clustal W alignment of all sequences obtained, showed that 16 clones were similar *to T. godfreyi* (Table 4B & Fig.4:1 in the appendix), and these segregated into four groups as follows:-

1. This included clones like T228, T229, T223, T225, T51 and T253. These were all collected from the Coastal area and all except T51 were from *G. pallidipes*. T51 originated from *G. brevipalpis*. The overall identity of this group to *T. godfreyi* was about 96%.

2. This consisted of clones T12, T159, T2, T76, T8 and T104. Clones T2 and T104 were collected from *G. brevipalpis* and the rest originated from *G. m. morsitans* in Tarangire. Their similarity to *T. godfreyi* was around 95.5%.

3. This group was made up of T3, T93 and T7. Their similarity to *T. godfreyi* was around 95.5%. T7 & T93 were collected from *G. brevipalpis* and T3 from *G. swynnertoni*.

4. T30, was a strain of *T. godfreyi*, with sequence identity of 99.4%

***T. simiae* Tsavo**

Four groups were obtained which related to *T. simiae* Tsavo.

1. Clones T5, T231, T121, T84 and T21. The relatedness to *T. simiae* Tsavo was around 99%. This originated from Tarangire except for T21 which was from *G. pallidipes.*

2. T1, T22, T49, T70 and T79. The similarity of this group to *T. simiae* Tsavo was around 94%. This group was isolated from *G. pallidipes*.

3. T10 was similar to *T. simiae* Tsavo by 95.5%, which was collected from *G. swynnertoni*

4. T60 from *G. pallidipes* and was similar to *T. simiae* Tsavo by 98.4%.

Congolense - type

1. Kilifi type: included T75 (99.7%) and T40 (100%).

2. Savannah type: Included T20 and was similar by 97.8%.

T. vivax

One clone was similar to this species. This was T78 by 86.2%, and was isolated from *G. m. morsitans*.

Because many isolated clones were similar to each other by ~ 100% few clones representing each clade were selected as presented in Table 4:3 and Figure 4:2.

Table 4:3 Summary of % identity of sequenced clones as compared to tsetse transmitted – trypanosomes.

Clone Number	*T. vivax*	*T. godfreyi*	*T. simiae* Tsavo	*T.c.* Savannah	*T. c.* Kilifi	*T.c.* riverine Forest	*T. brucei*
T1	82.0	93.9	**94.5**	88.1	88.4	87.8	86.3
T10	79.9	94.2	**95.5**	89.0	87.4	88.3	85.9
T104	81.0	**95.2**	93.8	87.5	87.1	86.5	84.2
T20	82.0	88.4	89.3	**99.7**	97.8	98.7	91.3
T93	80.3	**94.8**	93.5	86.3	86.0	85.4	83.6
T223	79.7	**96.1**	93.5	87.8	87.8	87.1	84.5
T30	81.0	**99.4**	92.5	89.0	88.4	89.0	86.7
T75	78.2	89.0	88.0	97.8	**99.7**	97.8	88.5
T70	82.6	92.9	**93.8**	88.7	89.0	88.4	87.9
T78	**86.2**	72.6	73.4	72.6	72.2	72.3	74.1
T84	84.1	92.5	**99.0**	89.3	87.7	88.6	86.3

Bold numbers indicate % similarity of isolated DNA sequences to respective subgenus after Clustal W alignment against *Nannomonas, Trypanozoon and Duttonella* trypanosome groups from the Genebank.

Phylogenetic analysis

Usually, clones with similarities to known trypanosome species of about 99-100% represent strains of that species and if the similarities is about 96% it shows you are dealing with a new species (Dr. Wendy Gibson, pers communication). In this case clones T104, T93 and T223 represent new groups of *T. godfreyi*. Also from Table 4B (appendix), result shows that 16 of the cloned trypanosomes were *T. godfreyi*. This could certainly explain why a bulk of infected mouthparts could not be identified by the available species specific PCR test in Chapter 3. These species are well distributed in the two field sites which mean that they are found both along the coastal as well as the northern part of Tanzania. T30 represent a strain of *T. godfreyi*.

T. simiae Tsavo is represented by four groups as shown above. Group 1 which include T5, T231, T121, T84, T21 and the second group which is represented by clone T60. These represent strains of *T. simiae* Tsavo and the strains are also well distributed in the two field sites. However, Group 3 which include T1, T22, T70, T79 and T49 were similar to *T. simiae* Tsavo by 94%. This group certainly represent new isolates and these were only isolated from *G. pallidipes* collected from the Coast area. Isolate T10 is also certainly another unique clone (similarity by 95.5%) and the mouth part was collected from *G. swynnertoni* in Tarangire.

Congolense - types

T75 represents a strain of Kilifi type and this originated from Tarangire. If the strain is well distributed in the area could also explain why the positive samples identified by species specific primers for *T. congolense* Kilifi/Kenya Coast were very few (Chapter 3). T40 is similar by 100%, it is surprising that although the clone is 100% identical to *T. congolense* Kenya Coast/Kilifi, and it was not identified in the first place when species - specific primers were used. T20 by a similarity of 97.8% represent a strain of *T. congolense* savannah.

The *T. vivax* species (T78) was isolated from template material collected from infected mouthparts of *G. m. morsitans* in Tarangire. If this is the species circulating in the area it could explain why the infection of *T. vivax* picked by PCR was very low (Chapter 3). Out of 637 (269 Gmm and 368 Gs) analysed from Tarangire, the total *T. vivax* infection was only 10 (1.6%) which certainly indicates the lack of primer specificity. It is also surprising that none of the *T. vivax* were identified by the 18S rRNA primers from Pangani samples. Because the number of *T. vivax* identified from that area was also very low 24 (5.9%). This was a total from single, double and triple infections with *T. vivax* out of 407 (106 Gb and 301 Gp) PCR analysis (Table 3:2, Chapter 3).

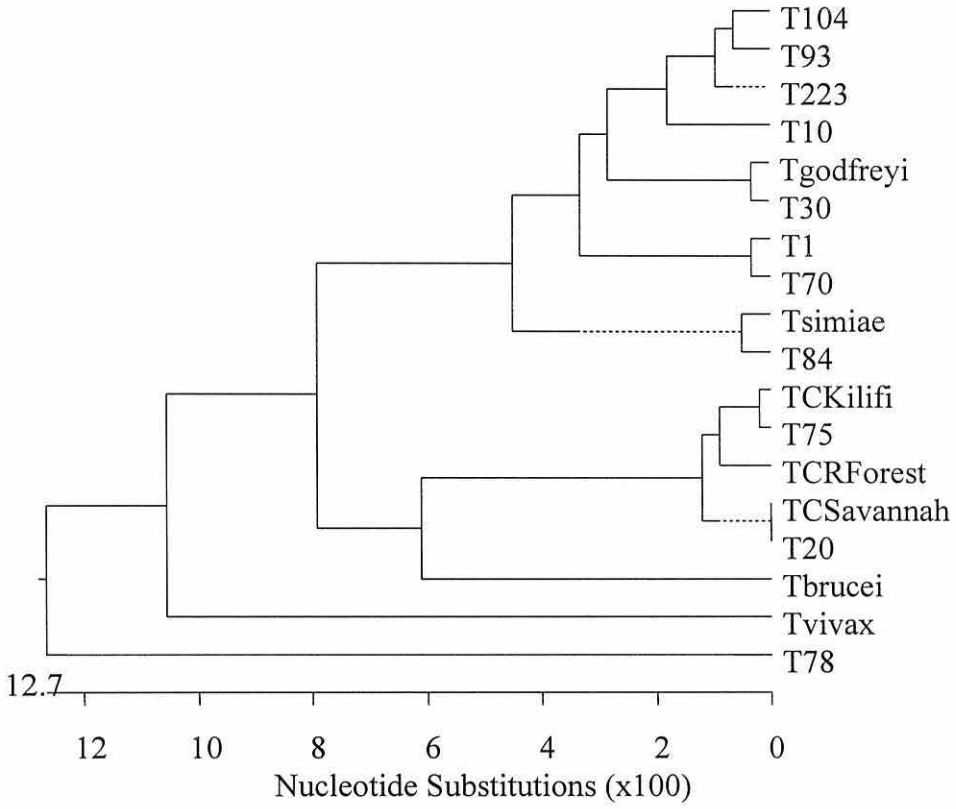

Figure 4:2. Phylogenetic tree (clustal W) of representative trypanosome isolated in this study and their relationship to the subgenera *Nannomonas*, *Trypanozoon* and *Duttonella*. The sequences of the subgenus used in this study were retrieved from the Genebank.

PAUP analysis

Parsimony analysis of the 38 taxa produced 3 equally parsimonious trees. The resulting bootstraped tree is shown in Figure 4:3. Robust bootstrap support is provided for subgenus *Duttonella* (100%) and for the subgenus *Nannomonas,* different subgroups are also well supported as all are above 50%. These include the subgroup *T. godfreyi* (95%); *T. simiae* Tsavo (58%) and other isolate which share the same subgenus, starting from T10 to T79. Isolate T60 is supported by 74%, T10

by 65%. By using PAUP, it was not easy to tell whether some of the isolates especially isolate T10 through to T79 belonged to *T. godfreyi* or *T. simiae* Tsavo as according to the phylogram (Fig 4.3) they are not rooted to any of the two named subgenera; rather they appear as separate groups within the *Nannomonas*. However, based on the distance analysis (Table 4.3) these have been grouped accordingly under different subgenera for example T10 shows close similarity to *T. simiae* Tsavo by 95.5% etc.

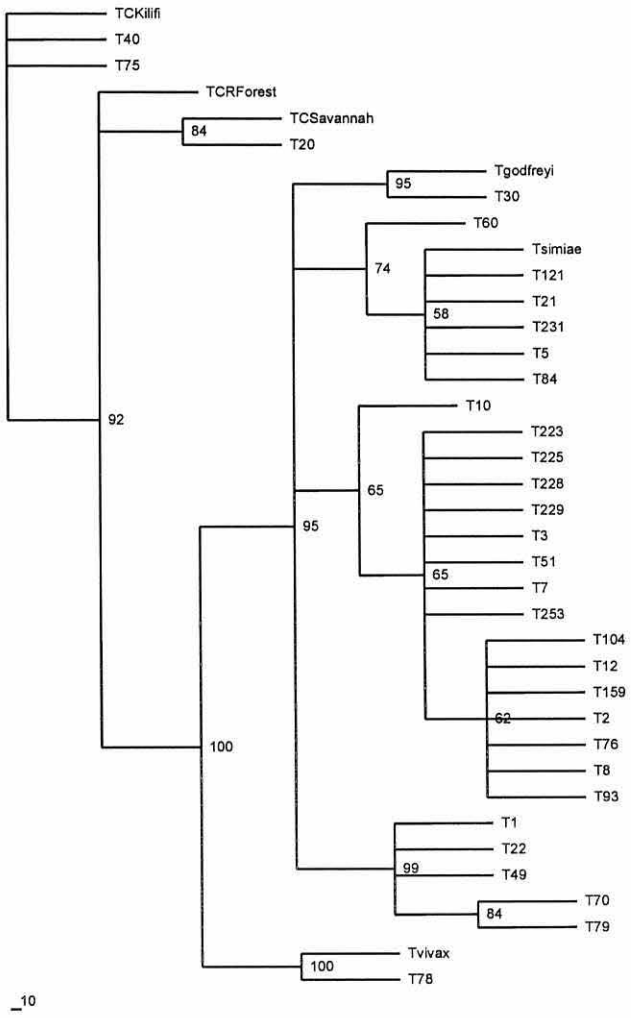

Figure 4:3 Phylogenetic tree (PAUP) of isolated and sequenced trypanosomes in this study. The tree is derived from 3 most parsimonious trees out of 195 trees. Bootstrap values for all major nodes are given and all branches receiving bootstrap support value > 50% is shown. Relationships failing to achieve this level of support are shown as polytomies.

DISCUSSION

Morphological appearance, vertebrate host range and developmental cycle in the insect vectors, were the traditional criteria for taxonomic classification of tsetse transmitted trypanosomes (Hoare, 1970). Sub grouping of *T. congolense* has been based on clinical differences in the diseases different subgroup causes (Godfrey, 1961), isoenzyme polymorphism among different isolates (Young & Godfrey, 1983) and genetic differences i.e. different DNA sequences which could be the cause for some pathological differences observed in the disease caused by each of the different groups of *T. congolense*. The reason which led to the conclusion that *T. congolense* comprised of species and subspecies (Hoare, 1970 & 1972; Godfrey, 1982) can be summarised as follows (1) Trypanosomes evolving one from the other. Hoare (1972) proposed that *T. evansi* had evolved from *T. brucei* by adopting life in different vectors and hosts in a different geographical environment. *T. brucei* and *T. evansi* share substantial sequence similarities (Majiwa *et al,* 1985). (2) Similar morphology: When trypanosomes evolve independently or simultaneously from ancestral trypanosomes, it is likely that the ancestral morphological form will be retained in a majority of the new isolates, but the genome of the two trypanosomes (new) could continue to evolve independently in different directions. Mutations and genetic drift will collectively result in genomically distinct trypanosome populations (Strachan *et al*, 1985). Theory predicts that in order for a complete genetic divergence to occur between any two populations, the two populations should be geographically isolated (Watterson 1985). However, sometimes it is not necessary. For example *T. congolense* isolates Savannah, Kilifi and Forest are found in the same localities and sometimes share the same host as in mixed infections (Lehane *et al.*, 2000). Other explanations for genetic diversity apart from geographical isolation of each groups, include the tsetse fly host preference of any of the groups and host preference of members of each of the groups.

Garside & Gibson (1995) used restriction length fragment polymorphism (RFLP) analysis of nuclear and kinetoplast DNA to investigate the taxonomic relationships between 5 groups within subgenus *Nannomonas*. Sequence conservation was generally high within a group, but low between groups, with the notable exception of the Savannah and Forest groups, which appear to be the most closely related groups within the subgenus, than they are to *T. simiae Tsavo* or *T. godfreyi.* Evidence to support this relatedness was based on numerical analysis of the major banding patterns generated with the tubulin and rDNA probes which showed that only the *T. congolense* Savannah and Forest groups had a high percentage similarity (63%), while all other pair wise combinations of groups had very low similarities (<10%).

Despite the relevance of such an approach, the criteria for determining species status by genetic distances or similarity indices are not distinct and it is difficult to define what level of similarity or extent of distance constitutes a new species. Some authors have suggested that only when genetic distances correspond to biological characteristics then the criteria should be used for classification (Tibayrenc, 1993).

Based on these criteria (morphology, behavioural, isoenzyme polymorphism and DNA probe analysis and the abundance genetic heterogeneity), *T. congolense* was subdivide into at least 4 subgroups of uncertain taxonomic status (Savannah, Forest, Kilifi and Tsavo). However, no subdivision within *T. simiae* Tsavo and *T. godfreyi* has been reported. The two share the restricted host range, differing only biochemically and the former in its virulence in pigs (McNamara *et al*, 1994). In this study, the isolated clones which are similar to *T. godfreyi* have segregated into three main groups as indicated in Figure 4:2. The first group which will be referred to as the Mivumoni type was isolated from flies collected from the coast largely *G. pallidipes* and only one *G. brevipaplis*. The second group consisted of isolates from Tarangire (five clones from *G. m. morsitans* and two from *G. brevipalpis* in the coast). The third group is a mixture of isolates from the coast (*G. brevipalpis*) and

from Tarangire (*G. swynnertoni*). Hence these new *T. godfreyi* subgroups are found from both sites the coast of Tanzania and the northern part of the country. These were distinct from *T. godfreyi* through DNA sequencing with regard to % similarity as indicated in Table 4B and Figure 4:1. The findings in this study could be interpreted based on the criteria that *T. godfreyi* and the isolated new *T. godfreyi* (from Mivumoni belt) are groups of trypanosomes evolved one from the other. *T. godfreyi* (Mivumoni type) isolated here is similar to *T. godfreyi* by 96% sequence identity. The new groups have been isolated from flies which share the same habitat and also probably the same hosts.

Similarly, Hoare (1972) pointed different hosts as being the reason behind the evolution of *T. evansi* from *T. brucei.* In this study, *G. m. morsitans* and *G. swynnertoni* were collected in the Northern part of Tanzania which is very much influenced by the migration of wild animals not only in Tarangire National Park but also from a dense population of livestock as explained in Chapter 3. However the Coastal fly lacks the influence of dense livestock following the closure of Mivumoni Livestock Multiplication Ranch. Hence the diet of flies is likely to depend mostly from wild animals and very few livestock owned by individual farmers which are insignificant. But the fact that still, a similar *T. godfreyi* Mivumoni type was isolated in the northern part (T3; T12, T8, T159 & T76) is an indication that the type of host and geographical isolation in our study, was not a reason for the evolution of this *T. godfreyi* Mivumoni type, rather the species is well distributed in the two study sites. Certainly, the species have been in the area for a long time such that it is well established in the local ecology. Flies from this area were collected during the dry season of February, 2001 in Pangani and flies from Tarangire were collected in May, 2000. When *G. pallidipes* midguts collected between 1995 - 1996 in Mivumoni were analysed, a sequence exactly similar to sequences from T223, T225, T228, T229, T253 and T51 was isolated (Dr. W. Gibson, pers communication, the sequence appears as number 23 in Figure 4:5 in appendix). Dr. Gibson's samples were from the same fly belt to Pangani site but collected six years

before the collection for this study was made. In Figure 4:5, clone 23 is grouped as *T. godfreyi* together with clone T223 (which appears as RCT223). The sequence similar to sequences of five clones mentioned above, certainly suggests that the trypanosome type has been in the area for a long time but it remained undetected due to lack of species - specific primers. It appears that the *T. godfreyi* Mivumoni type has been circulating in the two areas for a long time, and it has established itself in different tsetse groups: The *Fusca* group - as represented by *G. brevipalpis* and the *Morsitans* group as represented by *G. pallidipes*, *G. m. morsitans* and *G. swynnertoni*. These two groups have different host preferences (Table I & II appendix). Similarly, more work is needed to verify the distribution and establish the geographical limit of the new trypanosomes.

***T. simiae* Tsavo**

The result shows the presence of probably two novel trypanosomes. One subgroup was isolated from *G. pallidipes* (T1, T22, T49, T70 and T79) (Mivumoni type *T. simiae* Tsavo); and the second group is represented by isolate T10 (Tarangire type *T. simiae* Tsavo). It is possible that these groups (new isolates) have evolved independently or may be simultaneously from an ancestral *T. simiae* Tsavo.

T. vivax

Clustal W alignment shows that T78 is similar to *T. vivax* by 86.2% (Table 4:3). Recent 18S ribosomal RNA-based phylogenetic studies of trypanosomes indicate considerable differences in genetic diversity within clades and, in particular, the apparently high rate of sequence evolution within the *Trypanosoma brucei* and related tsetse-transmitted species. Analysis of relative clade rates shows the salivarian trypanosomes to be the most rapidly evolving group of taxa, and that *T. vivax* 18S rRNA not only evolves at approximately 7 - 10 times the rate of non-Salivarian trypanosomes, but also evolves significantly faster than all other Salivarian trypanosome sequences (Stevens & Rambaut, 2001). Reasons for such a rapid evolution in this single taxon are unclear. However it is postulated that *T.*

vivax appears to be unique in having an 18S rRNA G+C content of 55.4%, a figure some 3% higher than any other trypanosomatid included in the study by Haag *et al.*, (1998) as first indicated by Hasegawa and Hashimoto (1993). It is also known that within the Salivarian group, *T. vivax* diverged before *T. congolense* and *T. brucei*; identical conclusions were drawn using the ribosomal RNA sequences. In this study both as reported in Chapter 3 and 4, the % of *T. vivax* identified was not as high as reported by Lehane *et al.,* (2000). The possible explanation could be due to faster evolution of *T. vivax* than any other Salivarian trypanosome. This could explain why PCR identification of *T. vivax* infection was very low in the two sites. A similar experience of few *T. vivax* has also been reported by Morlais *et al.,* (1998a) and Lefrancois *et al*, (1999). However, the closure of Mivumoni ranch could also explain the reason for fewer *T. vivax* identified especially for Pangani collection. Moloo *et al.,* (1971) reported that high percentages of *T. vivax* infection are associated with high % of the diet being obtained from bovid. Levels of *T. vivax* and *T. congolense* infection are well known to be related to the proportion of bovid and suid blood meals respectively. As an example, *G. longipalpis* feeds predominantly from bushbuck whereas *G. palpalis* is cosmopolitan with a preference for reptilian blood. The two flies have different levels of infection with *T. vivax. G. longipalpis* is usually found with high % of *T. vivax*, while *G. palpalis* which feeds on reptilian, the *T. vivax* infection are low. So the difference in *T. vivax* infection rates in tsetse at their study site was probably due to a difference in feeding pattern. Moloo *et al.*, (1971) explained that *G. swynnertoni* in the Serengeti area was an opportunistic feeder; and there was a positive correlation between the infection rate of *G. swynnertoni* with *T. vivax* and the percentage of the blood meals that were derived from bovids. High % of blood meal from bovidae resulted into high % of infection with T. *vivax*. It was also explained that bovids act as the main source of *T. vivax* infections, because warthogs, the other main hosts of *G. swynnertoni*, are refractory to such infections. Mivumoni ranch (which was on the western part of the coastal study site) had about 1000 animals at its peak. These animals provided a good biomass in terms of tsetse diet. So few *T. vivax* infection identified could also apart

from fast evolution, be due to the absence of bovids in high numbers. The case for the Northern part is different as the isolation of a Tarangire type *T. vivax* explains why the identification of *T. vivax* by using both *T. vivax* primers from West and East Africa (Masiga *et al.,* 1992 & Masake *et al.,* 1997 respectively), was very low. This indicates that the primers used to identify *T. vivax* were not specific for the Tarangire *T. vivax.*

New primers for *T. vivax* have been reported by Morlais *et al.,* (2001), however due to limited time and template, they could not be tested. It is also interesting to note that even with 18S rRNA primers, no *T. vivax* was identified from the coast. Hence, it will be significant to carry out more PCR tests from the coast in an attempt to isolate *T. vivax* by 18s rRNA primers; so as to find exactly why the identification of *T. vivax* in large numbers was not achieved, and try to ascertain whether the reason is due to fast evolution of *T. vivax,* and to establish the impact of Mivumoni ranch closure on the infection rate of *T. vivax* in tsetse. This could also be carried out by surveying the infection of the same trypanosome species in the very few livestock found in the nearby area from individual farmers.

G. brevipalpis

As reported in chapter 3, the PCR analysis of *G. brevipalpis* collected samples showed that 47 of the samples were infected by *T. simiae Tsavo*, 5 by *T. congolense* savannah, 1 *T. vivax* and a mixed infection of *T. congolense* Savannah and *T. congolense* Kenya coast by 1 (Table 3:2; Chapter 3). The use of ssu primer had indicated that *G. brevipalpis* was also infected by *T. godfreyi* trypanosomes as indicated by clustal W alignment which places clones T7, T93, T51 and T104 as identical to *T. godfreyi.* All clones were collected from *G. brevipalpis*. Usually, the *Fusca* group of tsetse flies are considered to be poor vectors of pathogenic trypanosomes compared to the *Morsitans* tsetse group (Jordan, 1974; Maudlin, 1991). Maloo, (1986) reported that the infection rate of *G. brevipalpis* collected

from Kenya coast was similar or higher than those in *G. pallidipes* up to 19.2%. The infections were due to *T. congolense* and *T. vivax*. The result from this study certainly shows that *G. brevipalpis* is another tsetse species for concern, and its potential in the transmission of trypanosomiasis shouldn't be underestimated.

Chapter Five

GENERAL DISCUSSION

Various methods are used by blood sucking insects to defend themselves against parasites as has already been explained in this study. The lectin extracted from the midgut of *S. calcitrans* is believed to play an important role in defence. The final sequence has proved to be a trypsin, suggesting that the lectin is a dimer similar to that found in *Glossina* (Osir *et al.*, 1995 & Abubakar *et al.*, 1995). In dipterans, they play a crucial role during the digestion of blood meal in the gut (Muller *et al.*, 1993). Work by Osir *et al*, (1995), reported that midgut lectin from tsetse fly is also associated with trypsin activities. The findings by Osir *et al.*, (1995) suggested that the midgut lectin is a dimer containing both trypsin and lectin activities. Maudlin & Welburn, (1987) reported that the proportion of tsetse acquiring midgut infections can be significantly increased *in vivo* by adding inhibitory oligosaccharides to the blood meal for the first few days after infection. This is an indication that lectin is the first line of defence in tsetse against infection and serves to kill incoming trypanosomes. The clearance is thought to involve the trypsin-like enzyme and midgut lectin, as the trypsin-like enzyme and the main midgut lectin are closely associated. It is suggested that the enzyme cleaves off the specific surface molecule(s) from the bloodstream-form trypanosomes, a process which is thought to expose the lectin-binding sites, thus facilitating agglutination by lectins (Osir *et al.*, 1995), whereas the trypsin-like molecule cause lysis of trypanosomes (Imbuga *et al.*, 1992 & Abubakar *et al.*, 1995).

It is possible that the trypsin - lectin dimer identified here is responsible for defence mechanism in *S. calcitrans* and is released along with proteolytic enzymes, lectins and lysins in the midgut following the ingestion of the blood / proteinous meal and any associated pathogens. These have multiple and sometimes overlapping

biological functions which all seem to pertain to host immunity. Work by Abdally (1996), showed that the absolute concentration of both the haemolymph and midgut reservoir lectins were higher in *S. calcitrans* than in tsetse flies. This possibly helps to explain why *Stomoxys* is not a cyclical vector of trypanosomiasis despite being sympatric with *Glossina* and sharing a very similar life style and digestive physiology.

Another way in which bloodsucking insects defend themselves against parasite invasion is by being able to support or not support the establishment, development and maturation of trypanosomes. The analysis of trypanosome infections in *G. m. morsitans, G. brevipalpis, G. pallidipes* and *G. swynnertoni* has shown that different tsetse species have different patterns of infection which vary between species but are conserved within species. In this study the variation in the rate of infection is probably brought about by different feeding preferences exhibited by different tsetse species. The rate of *T. congolense* Savannah infection in Pangani was high in *G. pallidipes* (15 observations) and low in *G. brevipalpis* (5 identified) although the tsetse species were sympatric (Table 3:2; Chapter 3). The same applies to the northern part where the number of *T. congolense* Kenya coast (Kilifi) identified in *G. swynnertoni* was 2 and 6 in *G. m. morsitans*. The number of *T. godfreyi* was higher in *G. swynnertoni* (8 observations) than in *G. m. morsitans* (4), while at the coast, the infection was zero in *G. brevipalpis* and 17 in *G. pallidipes*. High infection rates with *T. simiae* Tsavo are normally thought to be associated in tsetse with a preference for feeding on suids and hippos; and the same applies to *T. godfreyi*. Infection with *congolense* and *vivax* types is associated with feeding on bovids (Clausen *et al.*, 1998).

Moloo *et al.*, (1971) noted that there is a positive correlation between the infection rate of *G. swynnertoni* with *T. vivax* and the percentage of the blood meals derived from bovids. It has been suggested that bovids act as the main source of *T. vivax* infections, because warthogs are refractory to such infections. *G. m. morsitans*

preferred host is recorded by Clausen *et al.*, (1998) as ruminants and it had the highest % of *T. vivax* infections (20%) compared to *G. swynnertoni* (4.65%) which prefers suids. *G. pallidipes* which also had 52.2% of its blood meal from ruminants in Clausen *et al.*, (1998), had 5 (5.98%) of *T. vivax* infection in this study, relatively higher compared to the infection in *G. brevipalpis* which was 1 (1.85%) (Table 3.2). *G. brevipalpis* prefers to feed on hippos (Weitz, 1963). This certainly indicates that the levels of *T. vivax* and *T. congolense* infection are related to the proportion of bovid and suid blood meals respectively.

Blood meal is another factor for trypanosome establishment. The blood meal of the host plays an important part in establishment of gut adapted trypanosomes in tsetse flies (Vickerman, 1985). It is clear that blood meal at the first infective feed is critical, with subsequent meals of less importance. Similarly, the source and nature of the blood meal have different effect on procyclic population establishment (Olubayo *et al.*, 1994; Nguu *et al.*, 1996; Masaninga & Mihok, 1999). Infective feeds from goats' facilitated infection, whereas feeds from wildlife species, such as eland and buffalo, inhibit infection. The presence of trypanotoxins in the blood of wildlife (Muranjam *et al.*, 1997) is suggested to be related to the inhibition of midgut lectin - mediated killing of trypanosomes by serum and / or erythrocyte factors, depending on the blood and the tsetse species (Mihok *et al.*, 1995). More regular feeding enabled by the dense population of wildlife in the northern area could be one of the reasons for a low infection rate in Tarangire (overall 13.09%) compared to 42.01% in Pangani. The population of wildlife in Pangani is very low compared to that in Tarangire.

Success of trypanosomes

The attributes of trypanosomes as parasites are their ability to avoid being too host or vector specific; thus they are able to maintain viable populations even in times where the preferred vector or host populations have declined. Other attributes include avoidance of the immune response by variable antigen types (VAT). This is

where each parasite is able to alter a surface glycoprotein, known as the variable surface glycoprotein (VSG), thereby hindering its detection by the host's immune system (Vickerman, 1994). In addition to this are yet an undefined number of strains and clones with an ability to further, an incalculable range of VAT. Antigenic variation is one of the unique evolutionary mechanisms of trypanosomes. Despite the VSGs in trypanosomes, tsetse flies contain glucosyl and galactosyl lectins which are lytic to trypanosomes, presumably through interaction with the procyclic surface coat (Welburn *et al.*, 1993 & 1994).

The isolated trypanosome species isolated in this study are related to the known trypanosomes but it appears that mutations and genetic drift have collectively resulted in the evolution of genomically distinct trypanosome populations (Strachan *et al*, 1985).

Geographical isolation is predicted to be one of the prerequisite in order for a complete genetic divergence to occur between any two populations (Watterson 1985). However, sometimes this is not necessary as exemplified by *T. congolense* isolates Savannah, Kilifi and Forest which are found to occur in the same localities and sometimes share the same host like in the mixed infections reported by Lehane *et al.*, (2000) and in this study as shown in Table 3:2, Chapter 3. In this study, we have isolated three new groups of *T. godfreyi.* These have been isolated from tsetse species in the same group as those which were PCR positive when species specific primers for *T. godfreyi* were used. These flies were trapped from the same localities in both sites and emptied from the same traps. In this case, geographical isolation does not apply in the evolution of the isolated Mivumoni type *T. godfreyi.* It is without doubt that these groups of trypanosomes (*T. godfreyi* and the Mivumoni type *T. godfreyi*), have evolved one from the other, and certainly, the species have been in the area for a long time such that they are well established in the local ecology. Flies from Pangani area were collected during the dry season of February, 2001. When the midguts of *G. pallidipes* collected in Mivumoni between 1995 and

1996 were analysed, a sequence exactly similar to sequences from the group represented by T223, was isolated (Dr. W. Gibson, personal communication). Dr. Gibson's samples were from the same fly belt to our site in Pangani but collected six years before the collection for this study was made.

The sequence similar to sequence T223, certainly suggests that the trypanosome type has been in the area for a long time but it remained undetected due to lack of a species - specific primers, and it has been circulating in the two areas for a long time, such that it has established itself in different tsetse group: The *Fusca* group - as represented by *G. brevipalpis* and the *Morsitans* group as represented by *G. pallidipes*, *G. m. morsitans* and *G. swynnertoni*. The Mivumoni type *T. godfreyi* (T223) is similar to *T. godfreyi* by ~ 96%. Other Mivumoni types *T. godfreyi* (T93 & T104) are similar to *T. godfreyi* by ~ 95.6%.

The case is different with *T. simiae* Tsavo isolates. The Mivumoni type represented by T1 & T70 from *G. pallidipes* and the Tarangire type represented by T10 (from *G. swynnertoni*), certainly it suggest that these groups of *Nannomonas* have evolved independently or may be simultaneously from an ancestral trypanosome *T. simiae* Tsavo. In this instance, certainly geographical isolation of the two tsetse species harbouring the two *Nannomonas* could be the possible explanation for the genetic diversity of each group, plus the influence of host preference of each tsetse group (Hoare, 1972).

In this study both as reported in Chapter 3 and 4, the % of *T. vivax* identified was not as high as reported in Lehane *et al.,* (2000). The possible explanation could be as explained by Stevens & Rambaut (2001), that *T. vivax* evolves faster than any other Salivarian trypanosome. Certainly, lack of species - specific primer for this variant (T78) was a reason behind the few identification of positive *T. vivax*. A similar experience of few *T. vivax* has also been reported by Morlais *et al.,* (1998a)

and Lefrancois *et al*, (1999) which certainly is an indication that *T. vivax* is evolving faster.

The closure of Mivumoni ranch could also explain the reason for fewer *T. vivax* identified especially for Pangani samples. As pointed out by Moloo *et al.,* (1971), high percentages of *T. vivax* infection are associated with high % of the diet being obtained from bovids. Levels of *T. vivax* and *T. congolense* infection are well known to be related to the proportion of bovid and suid blood meals respectively. So the difference in *T. vivax* infection rates in tsetse in Pangani is probably due to the absence of high level of bovid contribution to the tsetse diet. Mivumoni ranch (which was on the western part of our study site) had about 1000 animals at its peak. These animals provided a good biomass in terms of tsetse diet. Although there is no proof to support that the absence of cattle is a reason for a big drop in the *T. vivax* infection but it cannot be ruled out.

The case is different for the Northern part where the isolation of the Tarangire type *T. vivax* explains why the primers used were not specific for *T. vivax* because the Tarangire type is only similar to *T. vivax* by 86.2%. So despite the fact that two different sets of primers for *vivax* type were used (*T. vivax* primers from West and East Africa by Masiga *et al.,* 1992 & Masake *et al.,* 1997 respectively), still they were not specific for the Tarangire *vivax*. New primers for *T. vivax* have been reported by Morlais *et al.,* (2001) but, they were not used in this study.

It is also interesting to note that even with 18S rRNA primers, no *T. vivax* was identified from the coast. As a result, it will be important to perform more PCR tests from the coast in an attempt to isolate *T. vivax* by 18s rRNA primers; this will help determine whether the reason is due to the fast evolution of *T. vivax*. It would also be interesting to establish the impact of Mivumoni ranch closure on the infection rate of *T. vivax* in tsetse. This could also be carried out by surveying the infection of

the same trypanosome species in the very few livestock found in the nearby area from individual farmers.

Apart from the novel trypanosomes, one *T. godfreyi,* six *T. simiae* Tsavo, and *T. congolense* (one of each Kilifi and Savannah) strains were also isolated by the 18S rRNA primers. The strains also reduced the number of trypanosome which could have been identified in Chapter Three because of the non-specificity of the primers used. More *T. simiae* Tsavo strains were obtained compared to other *Nannomonas*. These are represented by isolate T84. These appear as a subclade of isolate T60, and were isolated from different tsetse species. Other strains of *T. congolense* subgenus are represented by isolate T75 of *T. congolense* Kilifi/Kenya Coast and isolate T20 as *T. congolense* Savannah (Table 4B). The *congolense* type strains were all from *G. pallidipes*. T30 was a strain of *T. godfreyi* and was obtained from *G. pallidipes*. This is an indication that divergence between trypanosome sequences can exist according to geographical location as was observed for *T. vivax* by isoenzyme characterization (Fasogbon *et al.*, 1990) and the PCR method (Dirie *et al.*, 1993)

The 18S rRNA primers increased the kind of trypanosomes found in *G. brevipalpis*. The PCR analysis of *G. brevipalpis* collected samples showed that 47 of the samples were infected with *T. simiae Tsavo*, 5 with *T. congolense* savannah, 1.85% *T. vivax* and a mixed infection of *T. congolense* savannah and *T. congolense* Kenya coast by 1 (Table 3:2). Clustal W alignment placed clones T7, T93, T51 and T104 as identical to *T. godfreyi;* these were collected from *G. brevipalpis*. This certainly indicates that *G. brevipalpis* is not a poor vector (Jordan, 1974; Maudlin, 1991), and certainly is another tsetse species for which we need to be concerned.

Identifications based on the dissection methods of Lloyd & Johnson (1924) are inconsistent with PCR identification results. Given that so much of our understanding of the epidemiology of trypanosomes in tsetse flies is based on the Lloyd & Johnson (1924) dissection technique, it is clear that we should move to

molecular tools for the precise identification of trypanosomes for better understanding of epidemiological studies and infection found in different tsetse species.

The use of DNA based diagnostic methodology like PCR is valuable in analysing the epidemiological situation in the field. It can be used in genetic characterisation of metacyclic and blood stream forms and can thus improve taxonomic knowledge, characterise the hybrid products of genetic exchange, take part in the study of the parasitic populations or contribute to the identification of possible animal reservoirs of human trypanosomiasis. The PCR based method is sufficiently sensitive to identify trypanosomes directly in the vector, which constitutes prerequisite for more efficient vector control in the field. It may also allow or enable the characterisation of drug resistant populations and can constitute a rapid method to distinguish between relapse and re-infection, or mixed infections (Biteau *et al.,* 2000).

70% of Tanzania is infested by 10 species and subspecies of tsetse flies. Among them the important vectors of the human disease are *G. morsitans* (*s. l*), *G. swynnertoni*, *G. pallidipes* and *G. fuscipes* (Kilama *et al.*, 1981). Geerts & Holmes (1998) reported that all drugs currently in use throughout the tsetse belt have shown various resistances against all trypanosomes species i.e. *T. vivax, T. brucei* and *T. congolense* (Tanzania not spared). It is also interesting to note that while in this study novel groups responsible for animal trypanosomiasis are being reported, work done by Komba *et al.* (1997) reported the isolation of distinct stocks of *T. b. rhodesiense* in Tanzania which were distinct from representative stocks from East Africa foci. Since there is no indication of new products becoming available in the near future (for both human and animal diseases), it is of utmost importance that measures are taken to avoid or delay the development of resistance and to maintain the efficacy of the currently available drugs (Geerts & Holmes, 1998). This can only be done through proper diagnosis of both animals and human beings followed by proper treatment.

SUGGESTIONS FOR FURTHER WORK

More molecular diversity studies of tsetse transmitted trypanosomes need to be carried in Tanzania in order to have a clearer picture of the trypanosome population present in particular more work needs to be carried at the two sites, Pangani and Tarangire, so as to isolate the two novel trypanosomes reported in this work, first in order for a complete sequence of the 18S region in both directions of about 1200 bp to be obtained in order to confidently place the trypanosomes on the phylogenetic tree (Stevens *et al.,* 1999); secondly in order that all methods used to classify other trypanosomes (i.e. classification by morphology, tsetse transmission and DNA analysis of a full sequence as explained by Gibson *et al.*, 2001) be carried as well for a clear picture of their clinical features when passaged in mice and rats as in Garside & Gibson (1995). Full sequences of ssu RNA genes from the novel trypanosomes could not be obtained due to lack of sufficient DNA templates.

More PCR tests from the coast should be carried out in an attempt to isolate *T. vivax* by 18s rRNA primers; this will help determine whether the reason is due to the fast evolution of *T. vivax;* and secondly, to establish the impact of Mivumoni ranch closure on the infection rate of *T. vivax* in tsetse. This could also be carried out by surveying the infection of the same trypanosome specie in the very few livestock found in the nearby area from individual farmers.

Appendix

Appendix for Chapter Two

Figure 2:2a: Distance migrated by bands from midgut lectin extract against LMW protein standards under non reducing conditions.

$$Y = 5.05972 - 2.57E\text{-}02X$$

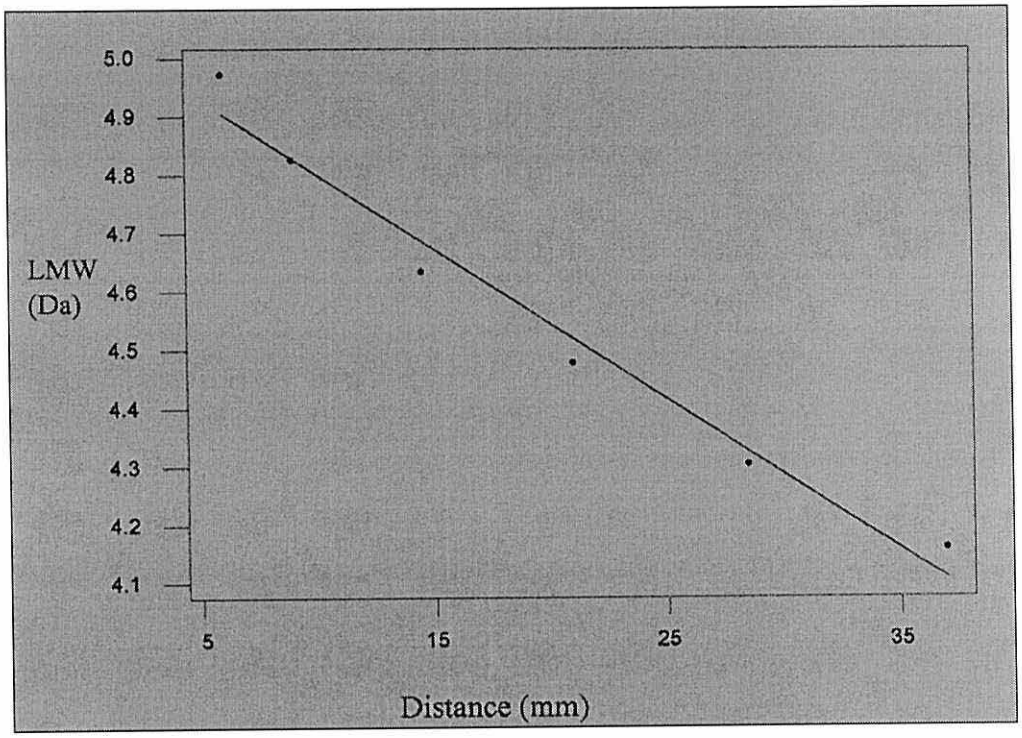

Figure 2:2b: Distance migrated by bands from midgut lectin extract against low molecular weight (LMW) protein standards under reducing conditions.

$$Y = 5.12689 - 2.61E\text{-}02X$$

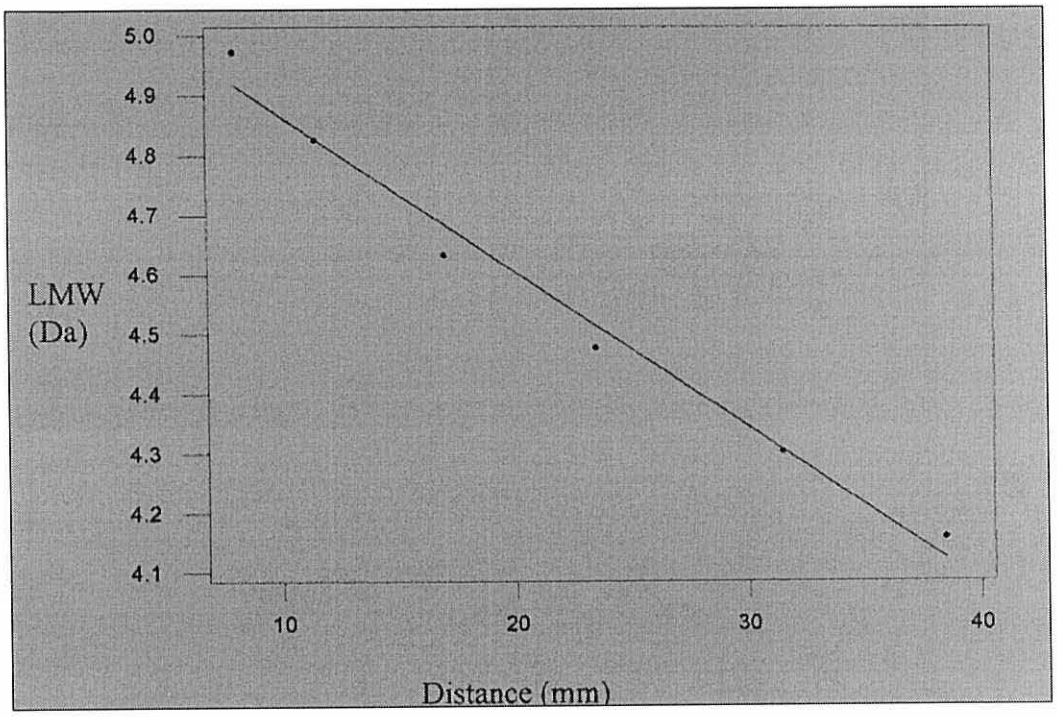

Table 2A: List of reagents used in Chapter Two

RESOLVING GEL 12%

Resolving buffer 2.5 ml, pH 8.8 (36.4gm Tris base 2.4M, added to 100ml of dH_20

Acrylamide (30% 37.5:1) 3.8 ml

Distilled water (dH_20), 3.7 ml

SDS 100 µl (10%SDS)

Ammonium persulphate (APS) 100 µl and TEMED 10 µl.

STACKING GEL (3%)

Stacking buffer 2.5ml pH 6.8 (3.0g Tris base 248mM, added to 100ml of dH_20).

Acrylamide (30% 37.5:1) 1ml

Distilled water 2.0 ml

SDS (10% SDS), 50 µl

APS (Ammonium per-Sulphate 50mg/ml), 75 µl

TEMED 10µl and Phenol red to colour

DESTAINER
300ml 100% Ethanol (ETOH)
70ml Glacial acetic acid
630 ml distil. water.

ELECTROBLOTTING BUFFER
CAPS solution (3-[cyclohexylamino]-1-propanesulfonic acid buffer.
10x CAPS (100mM, pH 11), dissolved 22.13g of CAPS in 900ml of Dioinized water. Titrated with 2N NaOH (approx. 20mL) to pH 11 and Deionized water added to final volume of 1L. Stored at 4°C.

Electroblotting buffer (1x Stock buffer in 10% Methanol, MEOTH). 2l of buffer by mixed with 200 ml of 10x CAPS buffer with 200 ml of MEOTH, and 1600 ml of deionized water.

NATIVE GEL.
Resolving gel (10%T, 2.67%C)
3.9 ml acrylamide (37.5:1)
1.5 ml resolving gel buffer
3.81 ml sucrose solution
750 µl APS and 2.05 ml dH_2O

STACKING GEL (4%T, 2.67%C)
2 ml stacking gel buffer
2.112 ml acrylamide (37.5:1)
2 ml APS
8 ml sucrose solution
1.89 ml distil water
Phenol red to colour

SAMPLE BUFFER
1ml stacking gel buffer, 1 ml glycerol
1 ml distil water containing 0.25mg bromophenol blue for colouring.

20 x SSC
175.3 g NaCl
88.2 g Sodium Citrate
800.0 ml distilled water
Adjust to pH 7.0 with few drops of 10.0 N NaOH, and adjust the volume to 1 litre with water.

50 x Denhardts solution.
5g Ficoll 400
5g Polyvinylprrolidine (Linda)
5g BSA
Dissolved in 500 ml of water. The solution filtered and kept at -20°C.

Sonicated solution (Salmon sperm DNA)
10mg dissolved in 1 ml of water. Dissolved in a magnetic stirrer for 90 minutes and passed several times through a 18G needle, and kept at -20°C.

Pre-hybridisation solution (100 ml)
25 ml of 5 x SSC
10 ml of 5x Denharts solution
5ml of 10% SDS
1 ml of 10mg/ml of Salmon sperm DNA (denatured by boiling for 10 minutes, and then kept on ice)
59 ml of sterile distilled water.

Hybridisation solution (10 ml)
5ml of 20 x SSC
2 ml of 5x Denharts solution
0.1ml of 10% SDS
0.1 ml of 10mg/ml of Salmon sperm DNA (denatured by boiling for 10 minutes, and then kept on ice)
6.3 ml of sterile distilled water.

SM (300 ml)
1.74g NaCl
0.6g MgSO4
15 ml of 1M Tris.Cl pH 7.5
2 ml of 2% gelatin

STET solution.
8% sucrose
0.5% Triton X-100
50 mM Tris-HCl pH 8.0
50 mM EDTA
The mixture is autoclaved.

Table 2B: Low molecular weight makers

Standard protein	Subunit molecular weight in Daltons	Source
Phosphorylase b	94000	Rabbit muscle
Albumin	67000	Bovine serum
Ovalbumin	43000	Egg white
Carbonic anhydrase	30000	Bovine RBC
Trypsin inhibitor	20100	Soybean
α-lactalbumin	14400	Bovine milk

Table 2C: Agglutination of crude RE from *S. calcitrans* against red blood cells.

Plate Wells	Dilution of titre	Scores					
		Replicate					
		1	2	3	4	5	Con A
1	Neat	3+	3+	3+	3+	3+	3+
2	1:2	3+/1	3+/1	3+/1	3+/1	3+/1	3+
3	1:4	3+/1	3+/1	2+/+	2+/+	2+/+	3+/1
4	1:8	3+/1	3+/1	3+/1	3+/1	3+	3+/1
5	1:16	3+/1	3+/2	3+/2	3+/1	3+/2	2+/+
6	1:32	3+/2	3+/1	3+/2	3+/1	3+/2	2+/+
7	1:64	3+/2	3+/1	3+/1	2+/+	3+/1	1+
8	1:128	3+/1	2+/+	2+/+	2+/+	2+/+	1+
9	1:256	1+	2+/+	2+/+	1+	1+	Tr
10	1:512	1+	1+	1+	1+	1+	Tr
11	1:1024	Tr	tr	tr	tr	tr	Tr
12	1:2048	Tr	tr	tr	tr	tr	Tr

Table 2D: Agglutination of purified RE of *S. calcitrans* against rabbit red blood cells before loading into a NATIVE PAGE gel.

Plate Wells	Dilution of titre	Scores		
		Replicates		
		1	2	Con A
1	Neat	2+/1	3+	3+
2	1:2	3+/1	3+/2	3+
3	1:4	3+/1	3+	3+/1
4	1:8	2+/+	3+	3+/1
5	1:16	2+/+	3+/2	2+/+
6	1:32	3+/1	2+/1	3+/2
7	1:64	3+/1	2+/1	3+/2
8	1:128	2+/1	2+/1	3+/2
9	1:256	2+/+	2+/+	3+/2
10	1:512	tr	tr	2+/+
11	1:1024	tr	tr	1+
12	1:2048	tr	0	tr

Table 2E: Agglutination of electro-eluted RE obtained from the NATIVE gel before loading into the SDS –PAGE gel.

Plate Wells	Dilution of titre	Scores					
		Bands					
		1	2	3	4	5	Con A
1	Neat	2+/+	2+/+	2+/+	2+/+	2+/+	2+/+
2	1:2	2+/+	2+/+	2+/+	2+/+	2+/+	2+/+
3	1:4	2+/+	2+/+	2+/+	2+/+	1+	2+/+
4	1:8	2+/+	2+/+	2+/+	2+/+	tr	2+/+
5	1:16	1+	1+	1+	Tr	tr	2+/+
6	1:32	Tr	1+	1+	Tr	tr	2+/+
7	1:64	Tr	1+	1+	Tr	tr	1+
8	1:128	1+	tr	tr	Tr	tr	1+
9	1:256	1+	tr	tr	Tr	tr	tr
10	1:512	1+	2+/+	2+/+	tr	tr	tr
11	1:1024	Tr	2+/+	1+	tr	tr	tr
12	1:2048	Tr	1+	tr	tr	tr	tr

Table 2F: Protein sequence by Edman degradation (Recent BLASTn search)

Band size (Da)	Protein sequence	Similarity	Reference
30026	IVGGYETDIKKVPFQVSL QA	Try29F AGRY2A	Muller *et al.*, 1993 & Wang *et al.*, 1994
28579	VNGE D/L AKPGQF	Christmas factor	Yoshitake *et al.*, 1985
25838	I V/T G/N G/G E/E	HEMA_INBGL	Yamashita *et al.*, 1988
24330	IV G/N GQPT T/K INQFPYQV	Try29F TRYP_SIMVI	Wang *et al.*, 1994 Ramos *et al.*, 1993

Appendix for Chapter Three

Table 3A: The primers (A.) and control DNA (B.) used in this study. TCS,*T. congolense* savannah; TCK, *T. congolense* Kenya coast; SM, *T. simiae*; TST, *T. congolense* Tsavo; DGG, *T. godfreyi*; TVW, *T. vivax.*

A

TCS1	CGAGAACGGGCACTTTGCGA	369	Masiga *et al.* (1992)
TCS2	GGACAAACAAATCCCGCACA		
TST1	GTCCTGCCACCGAGTATGC	450	Majiwa *et al.* (1993)
TST2	CGAGCATGCAGGATGGCCG		
TCK1	GTGCCCAAATTTGAAGTGAT	294	Masiga *et al.* (1992)
TCK2	ACTCAAAATCGTGCACCTCG		
TVWA	GTGCTCCATGTGCCACGTTG	175	Masiga *et al.* (1992)
TVWB	CATATGGTCTGGGAGCGGCT		
DGG1	CTGAGGCTGAACAGCGACTC	149	McNamara *et al.* (1994)
DGG2	GGCGTATTGGCATAGCGTAC		
TSMA	CGGGTCAAAAACGCATT	437	Masiga *et al.* (1992)
TSMB	AGTCGCCCGGAGTCGAT		
TVMF	TCGCTACCACAGTCGCAATCGTC GTCTCAAGG	399	Masake *et al.* (1997)
TVMR	CAGCTCGGCGAAGGCCACTTGG CTGGGGTG		

B

Trypanosoma (N.) godfreyi	GMOS/GM/88/Ken7
Trypanosoma simiae	GMOS/GM/88/Ken2
Trypanosoma vivax	MOVS/NG/50/Desowitz
Trypanosoma congolense savannah	MCAP/KE/81/WG81
Trypanosoma (N.) congolense Kenya Coast	MOVS/KE/81/WG84
Trypanosoma congolense Tsavo	

Table 3B: Mouthpart trypanosomes identified by PCR. Each row shows a separate category of single or mixed infections found in *G. pallidipes*. The count, column shows the number of flies with single or mixed infections of each category. The mean age of flies in each category is given. Tcs, *T. congolense* savannah; Tck, *T. congolense* Kenya Coast; Tsm, *T. simiae*; Tst, *T. congolense* Tsavo; Dgg, *D. godfreyi*; Tvw, *T. vivax.*

Tcs	Tck	Tsm	Tst	Dgg	Tvw	count	Mean age
*						51	80
	*					11	71
		*				14	77
			*			37	74
				*		23	77
					*	89	75
*	*					71	75
*			*			1	57
*				*		1	55
*					*	3	74
	*	*				1	77
	*		*			1	68
	*				*	2	66
		*	*			7	81
		*		*		6	76
		*			*	1	44
			*	*		12	67
			*		*	3	76
				*	*	2	61
*	*	*				3	47
*	*		*			2	91
*	*				*	3	53
*		*		*		1	91
		*	*	*		3	94
		*	*		*	1	31
*	*	*	*			1	117
*	*	*		*		1	110
*		*	*	*		1	95

Source: Lehane *et al.*, (2000).

Appendix for Chapter Four

Table 4A: Sequenced clones and their relatedness to tsetse transmitted trypanosomes obtained from BLAST searches. *Gb - Glossina brevipalpis; Gp - G. pallidipes; Gmm - G. m. morsitans; Gs - G. swynnertoni*

CLONE	SOURCE	PRIMER USED	SIMILAR TO	% SIMILARITY
1T	Gp	CI	T. simiae partial 18S rRNA gene, strain Tsavo	100
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	99
			T. simiae 18S rRNA gene, isolate KEN 2	94
2G	Gb	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7	95
3D	Gs	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7	94
			T. simiae 18S rRNA gene, isolate KEN 2	92
			T. simiae partial 18S rRNA gene, strain Tsavo	95
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	95
4N	Gs	CJ	T. simiae 18S rRNA gene, isolate KEN 2	98
			T. simiae 18S, 5.8S, 28S-LS1, srRNA1	94
			T. simiae partial 18S rRNA gene, strain Tsavo	92
5F	Gs	CJ	T. simiae 18S rRNA gene, isolate KEN 2	99
			T. simiae CP11, small subunit ribosomal RNA gene	97
			T. simiae 18S, 5.8S, 28S-LS1, srRNA1	95
7K	Gb	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7	95
			T. simiae 18S rRNA gene, isolate KEN 2	92
8P	Gmm	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7	94
			T. simiae 18S rRNA gene, isolate KEN 2	92
10G	Gs	CJ	T. simiae 18S rRNA gene, isolate KEN 2	95
			T. simiae partial 18S rRNA gene, strain Tsavo	93
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	93
11C	Gs	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7	95
			T. simiae 18S rRNA gene, isolate KEN 2	92
			T. simiae partial 18S rRNA gene, strain Tsavo	96
12C	Gmm	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7	95
			T. simiae 18S rRNA gene, isolate KEN 2	92
			T. simiae partial 18S rRNA gene, strain Tsavo	96
13B	Gb	CI	T. godfreyi 18S rRNA gene, isolate KEN 7	93
			T. simiae partial 18S rRNA gene, strain Tsavo	92

			T. simiae 18S rRNA gene, isolate KEN 2	90
20	Gp	CJ	T.congolense Savannah 18S rRNA isolate WG 81	100
			T. congolense IL1180	99
			T. congolense riverine isolate CAM	98
21C	Gp	CJ	T. simiae 18S rRNA gene, isolate KEN 2	99
			T. simiae 18S, 5.8S, 28S-LS1, srRNA1	94
			T. simiae partial 18S rRNA gene, strain Tsavo	93
22	Gp	CJ	T. simiae partial 18S rRNA gene, strain Tsavo	100
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	99
			T. simiae 18S rRNA CP11	94
30	Gp	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7	99
			T. simiae partial 18S rRNA gene, strain Tsavo	97
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	97
40	Gp	CJ	T. congolense Kilifi Isolate WG 5	100
			T. congolense forest isolate CAM	94
49F	Gp	CJ	T. simiae partial 18S rRNA gene, strain Tsavo	99
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	99
			T. simiae 18S rRNA gene, isolate KEN 2	93
			T. simiae partial 18S rRNA gene, strain Tsavo	92
51A	Gb	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7	95
			T. simiae 18S rRNA gene, isolate KEN 2	92
			T. simiae partial 18S rRNA gene, strain Tsavo	96
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	96
60D	Gp	CJ	T. simiae 18S rRNA gene, isolate KEN 2	98
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	94
70	Gp	CJ	T. simiae 18S rRNA gene strain Tsavo	99
			T. congolense Tsavo type 18S, 5.8S, 28S - LS1, srRNA1	98
			T. simiae 18S rRNA isolate KEN 2	95
75	Gmm	CJ	T. congolense Kilifi isolate WG 5	99
			T. congolense Riverine Forest 18S rRNA isolate CAM	94
			T. congolense Savannah 18S rRNA gene isolate W 81	94
76G	Gmm	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7	95
			T. simiae 18S rRNA gene, isolate KEN 2	92
			T. simiae partial 18S rRNA gene, strain Tsavo	96
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	96

78E	Gmm	CJ	T. vivax 18S, 5.8S, 28S-LS1, srRNA1 ribosomal RNA	88
79D	Gp	CJ	T. simiae partial 18S rRNA gene, strain Tsavo T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	99 98
84A	Gmm	CJ	T. simiae 18S rRNA gene, isolate KEN 2 T. simiae 18S, 5.8S, 28S-LS1, srRNA1 T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	99 95 93
87	Gb	CI	T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae partial 18S rRNA gene, strain Tsavo T. simiae 18S rRNA gene, isolate KEN 2	93 92 90
93I	Gb	CI	T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae partial 18S rRNA gene, strain Tsavo T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	93 93 93
104Q	Gb	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae 18S rRNA gene, isolate KEN 2 T. simiae partial 18S rRNA gene, strain Tsavo T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	94 91 95 90
121	Gmm	CJ	T. simiae 18S rRNA gene, isolate KEN 2 T. simiae 18S, 5.8S, 28S-LS1, srRNA1	99 95
149C	Gmm	CJ	T. simiae 18S rRNA gene, isolate KEN 2 T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae partial 18S rRNA gene, strain Tsavo	98 97 95
159J	Gmm	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae 18S rRNA gene, isolate KEN 2 T. simiae partial 18S rRNA gene, strain Tsavo T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	95 92 96 96
223B	Gp	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae 18S rRNA gene, isolate KEN 2 T. simiae partial 18S rRNA gene, strain Tsavo T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	95 92 96 96
225D	Gp	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae partial 18S rRNA gene, strain Tsavo	95 96

			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	96
228J	Gp	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae 18S rRNA gene, isolate KEN 2 T. simiae partial 18S rRNA gene, strain Tsavo	95 92 96
			T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	96
229E	Gp	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae 18S rRNA gene, isolate KEN 2 T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1 T. congolense riverine forest 18SrRNA gene, isolate CAM	94 92 95 94
231H	Gmm	CJ	T. simiae 18S rRNA gene, isolate KEN 2 T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1 T. godfreyi 18S rRNA gene, isolate KEN 7 T. congolense Kilifi type 18S, 5.8S, 28S-LS1,srRNA	99 93 98 94
246G	Gp	CJ	T. brucei RRP40 gene for Rrp40p homologue	100
253C	Gp	CJ	T. godfreyi 18S rRNA gene, isolate KEN 7 T. simiae partial 18S rRNA gene, strain Tsavo T. congolense Tsavo type 18S, 5.8S, 28S-LS1,srRNA1	95 96 96

Table 4B: Summary of % identity of sequences clones to tsetse transmitted – trypanosomes

Clone Number	*T. Vivax*	*T. godfreyi*	*T. simiae*	*T.c.* Savannah	*T. c.* Kilifi	*T.c.*riverine Forest	*T. brucei*
T1	82.0	93.9	**94.5**	88.1	88.4	87.8	86.3
T10	79.9	94.2	**95.5**	89.0	87.4	88.3	85.9
T104	81.0	**95.2**	93.8	87.5	87.1	86.5	84.2
T2	80.7	**95.5**	94.2	87.8	87.5	86.8	84.2
T121	83.8	93.2	**99.7**	89.3	87.7	88.6	86.6
T159	80.7	**95.5**	94.2	87.8	87.5	86.8	84.2
T12	80.7	**95.5**	94.2	87.8	87.5	86.8	84.2
T93	80.3	**94.8**	93.5	86.3	86.0	85.4	83.6
T20	82.0	88.4	89.3	**99.7**	97.8	98.7	91.3
T21	83.8	93.2	**99.7**	89.3	87.7	88.6	86.6
T22	81.3	93.5	**94.2**	88.1	88.4	88.1	86.6
T223	79.7	**96.1**	93.5	87.8	87.8	87.1	84.5
T225	79.7	**96.1**	93.5	87.8	87.8	87.1	84.5
T228	79.4	**95.8**	93.2	87.5	87.5	86.8	84.2
T229	79.4	**95.8**	93.2	87.5	87.5	86.8	84.2
T231	84.0	93.2	**99.7**	89.3	87.6	88.6	86.5
T253	79.7	**96.1**	93.5	87.8	87.8	87.1	84.5
T3	79.5	**95.2**	93.5	86.9	87.2	86.5	84.2
T30	81.0	**99.4**	92.5	89.0	88.4	89.0	86.7
T40	78.5	89.4	88.3	98.1	**100.0**	98.1	88.8
T49	81.6	92.9	**93.8**	87.4	87.7	87.1	85.4
T5	83.7	92.8	**99.3**	89.3	87.6	88.6	86.5
T51	79.7	**96.1**	93.5	87.8	87.8	87.1	85.4
T60	82.5	93.9	**98.4**	89.3	89.6	89.3	87.3
T7	80.3	**95.5**	93.8	87.7	87.7	87.1	83.8
T70	82.6	92.9	**93.8**	88.7	89.0	88.4	87.9
T75	78.2	89.0	88.0	97.8	**99.7**	97.8	88.5
T76	80.7	**95.5**	94.2	87.8	87.5	86.8	84.2
T78	**86.2**	72.6	73.4	72.6	72.2	72.3	74.1
T79	82.6	92.9	**93.8**	88.7	89.0	88.4	87.9
T8	80.4	**95.2**	93.8	87.5	87.1	86.5	83.8
T84	84.1	92.5	**99.0**	89.3	87.7	88.6	86.3

Bold numbers indicate % similarity of isolated DNA sequences as aligned by Clustal W method to known trypanosomes. These are also presumed to represent new isolates or strains having high score per row in relation to Nannomonas and Duttonella subgenus.

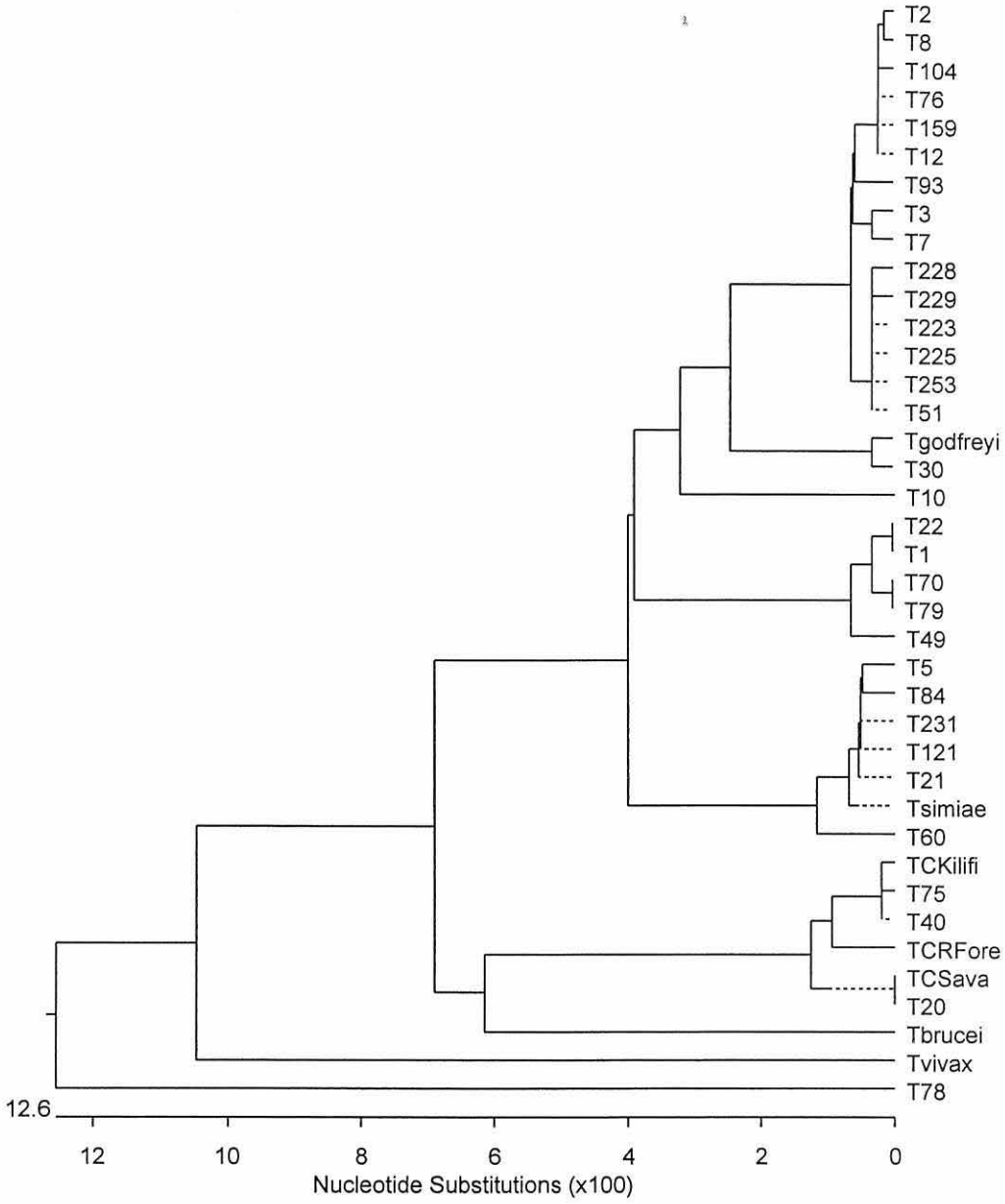

Figure 4:1 Phylogenetic tree of all trypanosomes isolated in this study and their relationship to *Nannomonas and Duttonella.* The sequences of the subgenus used were retrieved from the Genbank.

Figure 4:4 Clustal alignment of representative trypanosome sequences isolated in this study

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C C G C G G T A A T T C C A G C T C C A A A A G C G T A T A T T A A T G C T G T  Tvivax
C C G C G G T A A T T C C A G C T C C A A A A G C G T A T A T T A A T G C T G T  Tgodfreyi
C C G C G G T A A T T C C A G C T C C A A A A G C G T A T A T T A A T G C T G T  Tsimiae
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C C G C G G T A A T T C C A G C T C C A A A A G C G T A T A T T A A T G C T G T  TCKilifi
C C G C G G T A A T T C C A G C T C C A A A A G C G T A T A T T A A T G C T G T  TCRForest
C C G C G G T A A T T C C A G C T C C A A A A G C G T A T A T T A A T G C T G T  Tbrucei
C C G C G G T A A T T C C A G C T C C A A A A G C G T A T A T T A A T G C T G T  T104
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T G C T G T T A A A G G G T T C G T A G T T G A A C T G T G G G C G C G A - - -  Tsimiae
T G C T G T T A A A G G G T T C G T A G T T G A A C T G T G G G C G T T G T G -  TCSavannah
T G C T G T T A A A G G G T T C G T A G T T G A A C T G T G G G C G T T G T G -  TCKilifi
T G C T G T T A A A G G G T T C G T A G T T G A A C T G T G G G C G T T G T G -  TCRForest
T G C T G T T A A A G G G T T C G T A G T T G A A C T G T G G G C C A C G T A G  Tbrucei
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-CAGGTGCCGTGT-CCGTCCCACC-CGACTCGGGGGTGGT-  TCRForest
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C G G G C C - A C C G A T T C G G C T T T T - G T T G G    Tvivax
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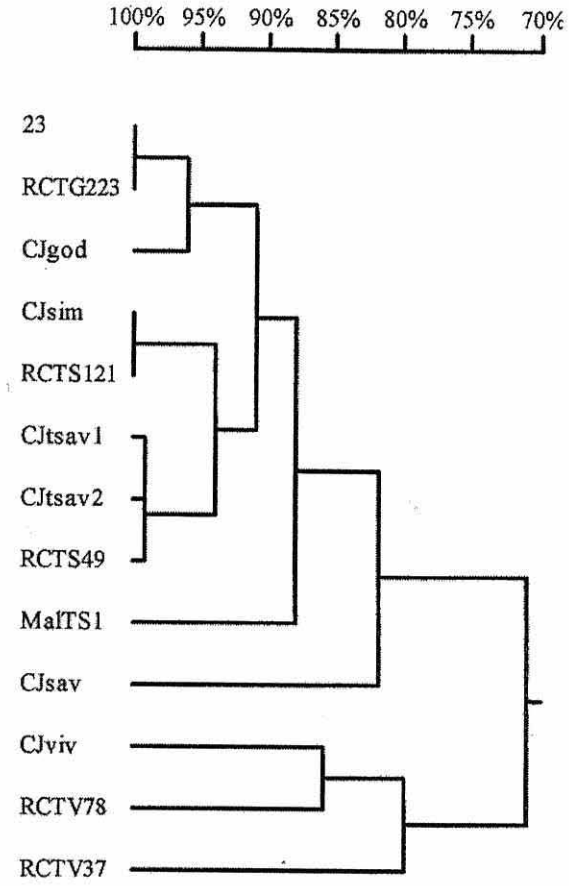

Figure 4:5. Alignments of some of the isolated trypanosomes in this study by Dr. W. Gibson.

RCT223 = T223, RCTV 78 = T78, RCT121 = T121, CJgod = *T. godfreyi*, CJsim = *T. simiae*, CJtSav 1, CJtSav 2, CjSav = *T. congolense* type, CJviv = *T. vivax*.

Appendix for frequently quoted Tables

Table I: Summary of blood meal analyses excluding human, domestic animals and blood meals not fully identified.

Habitat	**Host**	**%**
Lacustrine and riverine feeders		
Subgenus *Nemorhina*	Reptiles	56
	Bushbuck	22
	Remainder	22
Forest thicket or forest edge feeders		
Glossina G. austeni	Bushpig & forest hog	74
Austenina G. tabaniformis	Remainder	26
Glossina G. pallidipes	Bushbuck	61
Austenina G. fusca	Bushpig	12
	Remainder	27
Savannah feeders		
Glossina G. morsitans	Warthog	57
G. swynnertoni	Buffalo	21
	Giraffe, Kudu & remainder	22
Specialized East African *Austenina*		
Austenina G. brevipalpis	Elephant, Rhino, Hipp Buffalo	71
G. longipennis	Bushpig	18
	Remainder	11

Source: Ford (1971)

Table II: Hosts of *Glossina*

Fusca group: Bold type - important hosts (value > 10% of total). n,s. + not specified

Hosts	*G. fusca*	*G. fuscipleuris*	*G. brevipalpis*	*G. longipennis*
Primates	0	6 (1.4%)	0	2 (0.25%)
Suidae	9 (7.6%)	301 (70.7%)	32 (8.6%)	502 (60.6%)
Domestic pig	2	0	0	0
Warthog	1	4	10	48
Bushpig	0	**273**	10	**168**
Wild Suidae	0	0	1	51
n.s Suidae	6	24	11	**235**
Ruminants	100 (84%)	90 (21.1%)	24 (6.4%)	178 (21.5%)
Cattle	3	**60**	4	36
Small ruminant	0	1	0	3
Buffalo	1	5	4	70
Bushbuck	**70**	3	4	1
Duiker	2	0	0	4
Hartebeest	0	0	0	0
Impala	0	0	0	0
Waterbuck	0	0	7	0
Wild ruminant	24	14	5	55
n.s. ruminants	0	7	0	9
Other mammals	5 (4.2%)	28 (6.6%)	317 (85%)	129 (15.6%)
Canidae	4	4	1	32
Elephant	0	0	24	7
Felidae (excl. lion)	1	19	1	13
Lion	0	0	0	0
Hippopotamus	0	3	**291**	70
Rodentia	0	2	0	4
Camelidae	0	0	0	3
Equidae	0	0	0	0
Reptiles	4 (3.4%)	0	0	2 (0.25%)
Crocodile	0	0	0	0
Monitor lizard	0	0	0	0
n.s. reptiles	4	0	0	0
Avian	1 (0.8%)	1 (0.2%)	0	15 (1.8%)
Total	119	426	373	828

Palpalis group: Bold type - important hosts (value > 10% of total). n.s. + not specified

Hosts	G. palpalis	G. fuscipes	G. tachnoides
Primates	**284 (18.2%)**	116 (8.9%)	54 (2.0%)
Suidae	685 (43.8%)	199 (15.3%)	16 (0.6%)
Domestic pig	**283**	23	0
Warthog	11	11	0
Bushpig	28	1	0
Wild Suidae	0	0	2
n.s Suidae	**363**	**164**	14
Ruminants	278 (17.8%)	298 (22.9%)	900 (33.6%)
Cattle	11	103	4
Small ruminant	0	0	15
Buffalo	0	0	17
Bushbuck	**157**	32	**428**
Duiker	7	3	29
Hartebeest	0	0	0
Impala	0	0	0
Waterbuck	2	4	38
Wild ruminant	70	**156**	206
n.s. ruminants	31	0	163
Other mammals	110 (7.0%)	85 (6.5%)	1333 (49.7%)
Canidae	25	39	18
Elephant	1	3	1
Felidae (excl. lion)	55	12	0
Lion	0	0	0
Hippopotamus	14	11	**1286**
Rodentia	14	20	20
Camelidae	0	0	0
Equidae	1	0	8
Reptiles	164 (10.5%)	547 (42.1%)	366 (13.7%)
Crocodile	13	20	29
Monitor lizard	151	**527**	**337**
n.s. reptiles	0	0	0
Avian	42 (2.7%)	56 (4.3%)	11 (0.4%)
Total	1563	1301	2680

Morsitans group: Bold type – important hosts (value > 10% of total). n.s. + not specified

Hosts	*G. morsitans*	*G. longipalpis*	*G. pallidipes*	*G. austeni*
Primates	50 (0.7%)	7 (2.5%)	28 (2.3%)	3 (5.2%)
Suidae	4047 (57.1%)	29 (10.2%)	449 (36.2%0	52 (89.7%)
Domestic pig	15	11	0	0
Warthog	**2627**	0	**135**	4
Bushpig	**824**	4	**179**	**12**
Wild Suidae	186	4	22	0
n.s Suidae	395	10	113	**36**
Ruminants	1491 (21%)	206 (72.8%)	645 (52.2%)	2 (3.4%)
Cattle	387	4	78	0
Small ruminant	7	0	3	0
Buffalo	159	0	**265**	1
Bushbuck	248	**109**	**201**	1
Duiker	76	15	8	0
Hartebeest	0	0	0	0
Impala	1	0	0	0
Waterbuck	37	1	7	0
Wild ruminant	528	**40**	39	0
n.s. ruminants	48	37	44	0
Other mammals	1467 (20.7%)	11 (3.9%)	101 (8.2%%%)	0
Canidae	13	2	3	0
Elephant	220	0	17	0
Felidae (excl. lion)	15	1	4	0
Lion	0	0	3	0
Hippopotamus	**1210**	5	50	0
Rodentia	3	3	5	0
Camelidae	0	0	8	0
Equidae	6	0	11	0
Reptiles	13 (0.2%)	27 (9.5%)	7 (0.6%)	0
Crocodile	3	1	0	0
Monitor lizard	10	26	7	0
Avian	17 (0.3%)	3 (1.1%)	6 (0.5%)	1 (1.7%)
Total	7085	283	1236	58

Source: Clausen *et al.*, (1998)

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