Functional Analysis of a Midgut Proteolytic Gene in Tsetse Fly (*Glossina pallidipes*) by RNA Interference

By

Elisha Muchunga Mugo (BSc.)

Reg.No. I56/10099/2008

Department of Biochemistry and Biotechnology

A thesis submitted in partial fulfilment of the requirements for the award of the Degree of Master of Science in Biotechnology in the School of Pure and Applied Sciences of Kenyatta University

November 2011

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or any other award.

Elisha Muchunga Mugo (Bsc. Biochemistry)

I56/10099/2008

Signed: _____ Date: 15/12/2011

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

Dr. Joseph Ngeranwa

Department of Biochemistry and Biotechnology

Kenyatta University

Signed: _____ Date: 15/12/2011

Dr. Daniel Masiga

Molecular Biology and Biotechnology Department

International Centre of Insect Physiology and Ecology-ICIPE

Signed: _____ Date: 15/12/2011

DEDICATION

To my family and friends for their support during my studies.

ACKNOWLEDGEMENT

I give thanks and praises to the Almighty GOD for giving me the strength and ability to do my work to completion. My sincere gratitude to my supervisors, Dr. Daniel Masiga and Dr. Joseph Ngeranwa for steering me throughout the study. I particularly appreciate their kind supervision, valuable advice and continuous encouragement during the entire period of the study. I am also grateful to the authorities of the International Centre of Insect Physiology and Ecology (ICIPE) for awarding me the Dissertation Research Internship Program (DRIP) fellowship and for availing the use of the institutions facilities.

Special thanks to Joel Bargul, for introducing me to the work at ICIPE-MBBD, for his constant support and helpful scientific insights. I would like to thank Benedict Orindi for his tireless effort and patience in helping me with the statistical analysis of my experimental data. I am also grateful to Jeremiah Ojude and Jacob Opere for their technical support with the tsetse fly rearing and maintenance.

Many thanks to my fellow students and colleagues in the Molecular Biology and Biotechnology Department and, the staff at Capacity Building Unit, I.C.P.E. Special gratitude goes to Harrison Kibogo, Peter Kuria, David Omondi, Evans Rono, Martin Mbaya, Fiona Mumoki, Nelly Ndungu, Pamela Seda, David Wainaina and James Kabii for their friendship, generosity and sharing the happy time throughout my studying. Finally I would like to express my appreciation to my family, especially to my parents for their support and love throughout.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

aa	amino acids
bp	base pair
BSF	bloodstream form
cDNA	complementary deoxyribonucleic acid
DDT	Dichlorodiphenyltrichloroethane
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
GPL	*Glossina* proteolytic lectin
HAT	Huma African Trypanosomiasis
KD	Kilodalton
MEGA	molecular evolutionary genetics analysis
MDGs	millennium development goals
OD	odds ratio
ORF	open reading frame
PCF	procyclic
PM	peritrophic membrane
PBS	phosphate buffered saline
PAAT	Programme Against African Trypanosomiasis

RNA	ribonucleic acid
RISC	RNA-induced silencing complex
RNAi	RNA interference
SAS	statistical analysis system
siRNA	short interfering RNA
SIT	sterile insect technique
T.A.E	Tris EDTA
U.V	ultra violet light
VSG	variable surface glycoprotein
WHO	World Health Organisation
%	percentage
µ	Micro
µg	microgram
µl	microlitre
µM	micromolar
mM	millimolar
U.S	united states
hr	hour

ABSTRACT

Trypanosomiasis a devastating disease for human and animals in sub-Saharan Africa is transmitted via a tsetse fly (Genus: *Glossina*) bite. In absence of vaccine, effective and affordable drugs, the disease control relies heavily on vector control strategies because trypanosomes require an obligatory passage through the vector. Understanding the factors involved in tsetse-trypanosome interactions such as the midgut proteolytic genes that are known to be potential barriers to the initial trypanosome establishment stands to enhance vector control. However, very few *in vivo* studies have been undertaken to analyze such genes. The study describes functional analysis of a midgut *Glossina* proteolytic gene (GPL) by RNA interference to establish its influence on tsetse fly feeding success and survival. The coding region of the gene was amplified by RT-PCR and sequenced. The gene classified as a serine protease belonging to the trypsin family, with > 90% identity to the previously reported GPL genes from *G. austeni* and *G. f. fuscipes*. Sequence prediction showed it has a pi of 4.99, molecular weight of 29.47 KD and a hydrophobic leader peptide (16aa) indicating it is secretory in nature. When compared to other serine protease of *Diptera* origin, it exhibited close phylogenetic relatedness to those of other haematophagous insects. A reverse genetic approach to study the isolated gene function was limited by the short lived nature of the achieved knockdown as confirmed by semi quantitative RT-PCR. The highest transcript reduction (60%) lasted for two days, feeding success in this group was not significantly impaired ($p<0.05$) but, it improved with time as the transcripts level recovered. Also, the observed mortality was low and survival of the fly was normal when compared to the uninjected control group. The short lived GPL knockdown suggests it is crucial to tsetse fly physiology and may contribute to bloodmeal digestion. The study results through targeted transcript knockdown together with GPL dual role during trypanosome establishment could provide a foundation for the development of a genetic approach to control trypanosomiasis based on this gene.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Parasitic diseases continue to be a major challenge in sub-Saharan Africa with massive impact on the already overburdened economies of developing countries (Aksoy *et al.*, 2008). Among these diseases is African trypanosomiasis, a debilitating vector-borne disease of which the involved parasite and vector have been known for more than a century but the control still remains elusive. Efforts to develop preventive and/or protective vaccine are hampered by antigenic variation of the parasite, a feature that enables the parasite evade the host immunity (Vanderploeg *et al.*, 1982). The etiological agent of trypanosomiasis is a unicellular flagellated protozoan parasite known as trypanosome; belonging to the order *Kinetoplastida*, family *Trypanosomatidiae* and genus *Trypanosoma* (Hoare, 1972). Most of these parasites live partly in the vertebrate host and partly in the tsetse fly, *Glossina* species (*Diptera*; *Glossinidae*) that actively transmit the protozoan parasite between numerous vertebrate hosts (Bruce *et al.*, 1909).

The disease is restricted between latitudes 14° N and 29° S from Senegal in the West to the southern Somalia in the East, a region commonly referred to as the tsetse fly belt and is inhabited by the currently recognized twenty three species and eight subspecies of tsetse fly (Leak, 1999; Krafsur, 2009). The fly infestation in this area greatly hinders farming and a loss of ~4.5 billion U.S dollars in the agricultural sector is estimated to occur annually. Transmission of trypanosomiasis involves the interplay of three

organisms, the parasite, vector and host. Disease management measures targets one, two or all of the three but mainly have been oriented towards the elimination of the parasite from the host blood and/or the prevention of tsetse bite exposure through vector control. Use of drugs e.g, prophylactic (to treat animal form of the disease) and curative (for both human and animal forms) trypanocides has been an important component of such measures to curb infected cases.

The drug treatment approach has been hampered by numerous challenges including difficulties in accessing the target population who live mostly in rural areas, the toxicity of drugs used for chemotherapy in humans, their cost (Etchegorry *et al.*, 2001) and emerging resistance (Moloo and Kutuza, 1990; Mamman *et al.*, 1993; Matovu *et al.*, 2001; Nok, 2003). Furthermore, the phenomenon of antigenic variation severely hinders the development of reliable vaccine antigens (Donelson, 2003). While vector control remains the most promising approach to containing trypanosomiasis, methods currently in use such as trapping and insecticides have been met with varying success efforts given the problems associated with their maintenance and lack of sustainability overtime (Joja and Okoli, 2001).

Despite the arsenal of tools available over the years to control trypanosomiasis, the disease still remains a burden to the public health (60 million people at risk) and also causes huge losses in livestock production. The persistence of the disease could be related to several factors among which the complexity of the interactions between the

parasite and its insect vector tsetse fly. For the past few decades immense effort (using *in vivo* and/or *in vitro* approaches) have been made to try and understand the interactions between tsetse fly and the trypanosome that they cyclically transmit (Aksoy *et al.*, 2003). This has led to analysis of tsetse fly bloodmeal choices (Moloo, 1981; Mihok *et al.*, 1995; Nguu *et al.*, 1996; Muturi *et al.*, 2011), its immune response components as well as midgut factors that play a role in transmission cycle of trypanosome. Among such midgut factors are lectins, proteases, trypanolysins (Ibrahim *et al.*, 1984; Stiles *et al.*, 1990; Imbuga *et al.*, 1992; Welburn and Maudlin, 1999; Osir *et al.*, 1999) antimicrobial peptides and other yet uncharacterized molecules.

Lectins, trypanolysins and trypsins have been considered as major barriers to the development of the ingested bloodstream form trypanosome (Maudlin and Welburn, 1987; Welburn *et al.*, 1989; Imbuga *et al.*, 1992; Osir *et al.*, 1999). In *Glossina morsitans morsitans* a blood meal induced lectin was indentified (Abubakar *et al.*, 1995) and it has been associated with two roles; promoting establishment and transformation of bloodstream form trypanosome to procyclics forms as well as lysis of trypanosomes. The levels of lectin in the tsetse midgut determine whether it favours lysis (high levels) or establishment and transformation (low levels) (Maudlin and Welburn, 1988; Maudlin, 1991; Imbuga *et al.*, 1992; Osir *et al.*, 1993).

By using *in vivo* and *in vitro* systems initial experiments (Imbuga *et al.*, 1992; Osir *et al.*, 1993) were able to demonstrate the crucial role that lectins and trypsins play in the establishment and transformation of trypanosomes within the midgut of the tsetse fly

vector. This led to isolation, purification and characterization of a midgut proteolytic lectin (initially referred to as lectin-trypsin complex) from *Glossina longipennis* (Osir *et al.*, 1995), *G. fuscipes fuscipes* (Abubakar *et al.*, 2006), and *G. austeni* (Amin *et al.*, 2006). However, supporting data for the direct role of lectin in tsetse-trypanosome interactions are still limited since a trypanocidal lectin has not been purified yet from tsetse fly.

Major scientific advances pertinent to vector control have been made in recent years such as advances in molecular genetics, cell biology, immunology, and genomics that have resulted in the development of powerful technologies for laboratory study of the vector's biology to discover the weak links in disease transmission (Aksoy, 2003). In relation to this, recent discovery that RNA interference (RNAi) machinery exist in tsetse fly is providing crucial tools for the dissection of vector-parasite interactions and for the analysis of aspects of tsetse biology influencing the vectorial capacity. The genome resource now available for tsetse flies, their symbionts and trypanosomes have dramatically increased possible lines of scientific enquiry. In addition, the existence of a range of molecular tools such as, RNAi, proteomics, mutant trypanosomes lines and an *in vitro* culture system for *sodalis* offers a more integrated approach in research on tsetse-trypanosome interactions. (Walshe *et al.*, 2009b).

The development of effective RNAi knockdown procedures for tsetse flies has permitted detailed functional investigation of factors involved in tsetse trypanosome interactions.

For example, studies on tsetse immune responsive genes where knockdown of attacin (antimicrobial peptide) transcript resulted to significant increase in levels of trypanosome infection (Hu and Aksoy, 2006). The technique permits specific, post-transcriptional gene knockdown in whole organisms or cells by targeted mRNA degradation using double stranded RNAs (dsRNAs) (Napoli *et al.*, 1990; Fire *et al.*, 1998). Importantly, the general application of this gene-knockdown system to adult insects has enabled the *in vivo* study of key genes in insects which lack the sophisticated tool box available for model organisms such as *Drosophila* (Walshe *et al.*, 2009a).

1.2 Problem statement and justification

In the absence of vaccines and, effective and affordable drugs, trypanosomiasis control relies heavily on vector control. Reduction of tsetse populations by trapping, or by insecticide applications has been shown to be highly effective for disease control, but the sustainability of these tools by communities in endemic rural settings has been difficult. Several different control approaches are used with eventual impacts ranging from reduction of fly populations to total eradication. Despite the wide arsenal of vector control tools available, none of methods can be used separately without an integrated control strategy, a fact which is accepted by all tsetse fly researchers since it exploits all the weaknesses in the fly behavior. It is therefore necessary to improve on existing strategies or design alternative measures to combat the disease.

Recent research in vector-parasite interaction at the molecular level shows a potential for the development of novel control strategies aiming to inhibit the vector's capacity to transmit the parasite (Aksoy, 2003). A basic requirement to this approach is the identification of candidate molecules that could be used. One way to achieve this is by exploiting the expanding range of *Glossina* expressed sequence tags (http://www.genedb.org/genedb/glossina/index.jsp) as source of identifying genes coding for these molecules. One such molecule is the tsetse midgut proteolytic lectin which has been shown to play a crucial role in trypanosome establishment and transformation. Prior experiments have suggested a role for the blood meal-induced *Glossina* proteolytic lectin (GPL) in establishment of procyclic trypanosome forms in the midgut of tsetse flies. Furthermore, its expression after blood meal even in uninfected tsetse flies indicates it's of physiological importance to the tsetse fly. In order to determine the potential application of the midgut proteolytic lectin, further characterization as well as confirmation of its existence in other species of tsetse fly is required. In addition, the trypsin nature of this molecule and its role upon blood feeding in tsetse fly is less understood. To describe the role of GPL, this study applied a recently developed reverse genetic approach based on RNA interference (RNAi) to analyse its function in *G. pallidipes*. The findings would help to understand processes that either aid or retard trypanosome development, thereby providing a foundation for the development of a genetic approach to trypanosomiasis control.

Research questions

a) Does ablation of *Glossina* proteolytic lectin (GPL) function influence the feeding success in tsetse fly *G. pallidipes*?

b) Is *Glossina* proteolytic lectin (GPL) loss of function detrimental to the survival of tsetse fly?

1.3 Objectives

1.3.1 General objective

To undertake a functional study of a proteolytic gene from the midgut of *Glossina pallidipes,* in order to determine its role in feeding success and survival rates.

1.3.2 Specific objectives

a) To knockdown the activity of *Glossina* proteolytic lectin (GPL) gene in *Glossina pallidipes* by RNA Interference (RNAi), in order to assess function.

b) To investigate the effect of the knockdown of *Glossina* proteolytic lectin (GPL) activity on the feeding success and survival of tsetse fly.

CHAPTER TWO

LITERATURE REVIEW

2.1 Trypanosomiasis overview

Trypanosomiasis is a fatal disease affecting both human and many other mammals (both domesticated and wild). In human the disease is known as sleeping sickness (Human African trypanosomiasis, HAT) while the disease form in animals is referred to as nagana (African animal trypanosomiasis, AAT). Being a disease of the tropics, the variant in the new world is Chagas' disease. In human, trypanosomiasis has acute and chronic forms which vary depending on the species of the parasite. The human form of the disease is caused by two subspecies of *Trypanosoma brucei* namely *Trypanosoma brucei rhodesiense* (acute form) in East/southern Africa and *T. brucei gambiense* (chronic form) in west/central Africa. The diseases are commonly known as Rhodesian sleeping sickness and Gambian sleeping sickness respectively. Typically, the *T. b. rhodesiense* transmission involves wild and domestic animals, but intensified human to human transmission may occur during epidemics. The *T. b. gambiense* transmission cycle is mostly from human to human involving animals to a much less extent (Walshe *et al.*, 2009b).

Transmission of trypanosomiasis is by a hematophagous insect vector that belongs to genus *Glossina* commonly known as tsetse fly. Both male and female tsetse fly can successfully host and transmit trypanosome between numerous vertebrate hosts. The vector is distributed over a wide range of habitats covering about 10 million square

kilometers of potential grazing lands in 37 countries which are rendered unsuitable for livestock breeding and farming in Africa (Kuzoe, 1993).

Sleeping sickness (human African trypanosomiasis-HAT) is one of the most neglected diseases of this world and is always fatal if left untreated. The disease occurs in foci affecting poor communities with limited access to health services thus, the diseases is often left undiagnosed or mistaken for more common afflictions (Matemba *et al.*, 2010). Relaxation of surveillance and control measures led to resurge in HAT in early 1990s (Barrett, 2006). However, case detection and treatment have been increased and by 2009 the reported number of new HAT cases have dropped below 10,000 which probably equates to ~30,000 cases per year (Simarro *et al.*, 2011).

Other mammals apart from man are also affected by trypanosomes with several *Trypanosoma* species and subspecies being pathogenic to many wild and domesticated animals causing a disease known as nagana. In particular, *T. b. brucei, T. vivax* and *T. congolense* are the major causes of the animal form of trypanosomiasis. The severity of the disease is dependent on both the pathegonicity of the parasite strain and the genetics of the mammalian host (Courtin *et al.*, 2008). While most of the African wildlife is tolerant to the parasites, domesticated livestock are highly susceptible to the disease particularly if they originate from the European stock. As a result, animal trypanosomiasis (nagana) is an important constraint to the development of livestock production in sub-Saharan Africa, preventing full usage of land to feed the rapidly increasing populations. If not controlled, the disease can induce important losses by

limiting crop production due to less efficient nutrients cycling, reduced access to animal traction, lower income from meat and milk sales and reduced access to liquid capital (Swallow, 2000).

2.2 Impact of trypanosomiasis on animal and human health

The impact of trypanosome infection on animal and human health depends on various factors amongst which is the host susceptibility and the pathogenicity of the parasite involved in the infection. In susceptible domestic animals, the disease may be acute and highly devastating but chronic infections are more common. The host-parasite interaction produces extensive pathology, though the symptoms of trypanosomiasis may vary. Generally, it is characterized by an early fever stage when the parasites are mostly in the bloodstream and a later stage where it affects the nervous system. Clinically affected animals lose condition and become weak and unproductive. This affects all aspects of production including fertility, milk yields, growth and work output and finally the resulting mortality rate greatly reduces the herd size (Connor, 1994). The annual impact of nagana on African economy is estimated to be 4.5 billion US dollars due to losses in agriculture and livestock production (Walshe *et al.*, 2009b).

In humans, the two trypanosome species responsible for sleeping sickness are morphologically indistinguishable but have different epidemiological features. Both parasites of sleeping sickness affect the central nervous system. The typical East African form of trypanosomiasis is characterized by a rapid and acute development of the

disease, and if untreated it can lead to death within a few weeks or month of infection. The West Africa form of the disease is more chronic lasting for several years and accounts for more than 90% of reported sleeping sickness cases (Aksoy, 2011). The disease occurs in two stages (Priotto *et al.*, 2007), stage-1 (the haemolymphatic phase) characterized by non-specific symptoms like headache and bouts of fever. This phase generally goes undiagnosed without active sleeping sickness surveillance. The latter stage-2 (neurological phase) occurs when the parasite crosses the blood-brain barrier resulting to serious sleep cycle disruptions, paralysis, and progressive mental deterioration, with behavioral changes and, ultimately results to death without effective treatment (Barrett, 2010).

Almost more than any other disease affecting both people and livestock, trypanosomiasis straddles the ground between human health, livestock health and agricultural production and thus rural development. Therefore, according to the mandate of Programme Against African Trypanosomiasis (PAAT), dealing with the disease has the potential to impact on all eight millennium development goals (MDGs).

For the past few years (2000-2009), efforts by WHO, non-governmental organizations and public private partnership with pharmaceutical company Sanofi-Aventis and Bayer has led to decline of HAT cases reported annually to below 10,000 through free provision of drugs and funding for HAT control in the 37 countries in sub-Saharan Africa where HAT is endemic (Simarro *et al.*, 2011). This has improved the knowledge on HAT distribution and WHO estimated in 2006 the factor gap between cases reported

and infected to be three instead of ten as it was on 1995. However, the disease situation is not homogenous throughout the continent and in some foci, e.g, Uganda the merger of both *T .b. rhodesiense* and *T. b. gambiense* is a possibility (Aksoy, 2011) thus, active screening and control is highly recommended.

2.3 The parasite: *Trypanosoma spp*

Trypanosomes are unicellular, flagellated, elongated, and usually slightly curved protozoan parasites belonging to genus *Trypanosoma* (*Kinetoplastida*; *Trypanosomatidiae*). They are extracellular parasites mostly found in the blood circulation of their mammalian host. However, tissue localisations have been observed in some species such as *T. brucei* , *T. vivax* and *T. equiperdum* (Stephen, 1986). Some of the trypanosome unique features for the cyclically transmitted trypanosomes include the presence of long undulating flagellum and the existence of pleomorphic forms (Lumsden and Evans, 1979). Several species belonging to the genus *Trypanosoma* are known to date, and affect both human and other mammals. In addition to the two *T. brucei* subspecies that affect man (*T. b. gambiense* in west and central Africa, and *T. b rhodesiense* in East and Southern Africa) other species that are parasitic to other mammals include *T. congolense*, *T. b brucei*, *T. vivax*, *T. simae*, *T .b evansi* and *T. equiperdum* amongst others.

Trypanosoma spp. can be classified into sections (salivarian or stercorarian, shown on Fig 1) depending on the location where they complete their development and the means

of transmission. Tsetse transmitted trypanosome belong to the section of salivarian and based on the several criteria among which parasite's morphology and the site of development in the tsetse fly (Table 1), trypanosome are classified within this section, into four different subgenera and further into different species (Figure 1).

Table 1. Characterization of trypanosomes according to site of development in *Glossina spp* (after Aksoy, 2003,).

Subgenus	Species	Trypomastigote	Epimastigote	Metacyclic	Infective medium
Duttonella	*T. vivax*	proboscis	proboscis	proboscis	saliva
Nannomonnas	*T. congolense*	midgut	Proboscis	Proboscis	Saliva
	T. simiae	midgut	Proboscis	Proboscis	Saliva
	T. godfreyi	midgut	Proboscis	Proboscis	Saliva
Trypanozoon	*T. b. brucei*	midgut	Salivary glands	Salivary glands	Saliva
	T. b. rhodesiense	midgut	Salivary glands	Salivary glands	Saliva
	T. b. gambiense	midgut	Salivary glands	Salivary glands	Saliva

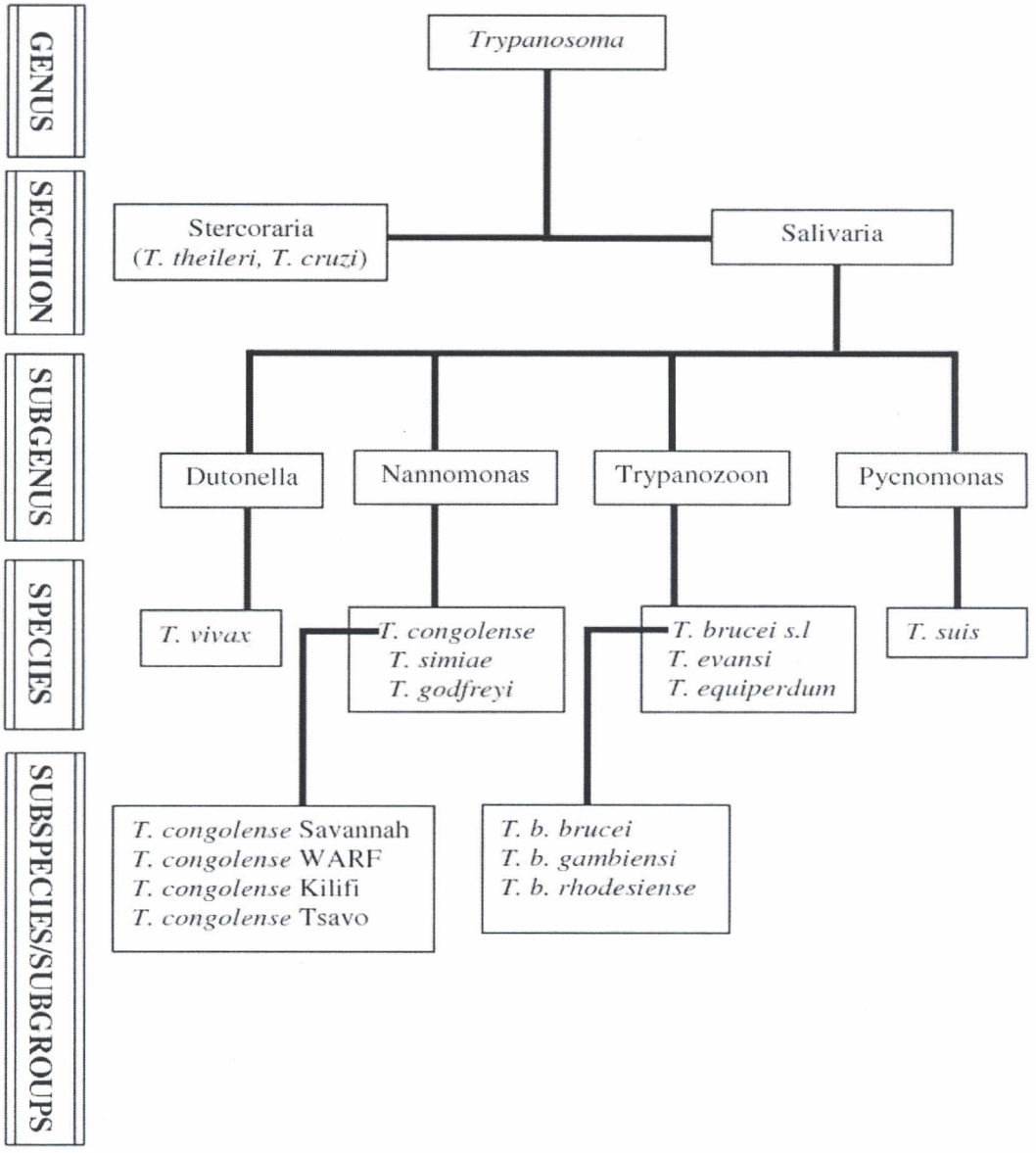


Figure 1. Classification of human and animal pathogenic tsetse-transmitted trypanosome species (Masumu Mulubu, 2006)

Salivarian trypanosomes are transmitted during feeding when an insect vector takes blood meal from an infected host. On the other hand, stercorarian trypanosome species such as *T. cruzi* develop in the hindgut of the insect vector and are transmitted via contaminations with the insect faeces. Tabanids, leeches and ticks form the predominant vectors of stercorarian trypanosomes (Walshe *et al.*, 2009b). Though salivarian and stercorarian mechanisms are the two major means by which trypanosomes are transmitted, some trypanosome species such as *T. evansi* and *T. vivax* can be transmitted mechanically by Tabanids, Stomoxys spp. or other biting flies (Hoare, 1972; Molyneux and Ashford, 1983). So far known, only *T. equiperdum* is transmitted sexually.

2.3.1 Trypanosome life cycle

T. brucei has the most complex, but perhaps the best characterized lifecycle of all African trypanosome (Figure **2**). Within the vertebrate bloodstream at least two different major forms of trypanosomes are found; a long slender form, which replicates by asexual division, and a short stumpy, non-replicating form (Figure **2**-1). The differentiation of the long slender bloodstream form (BSF) into the non-dividing stumpy BSF occurs in high density populations of long slender BSFs (Vassella *et al.*, 1997; Seed and Wenck, 2003). The switch to stumpy BSF involves changes in metabolism within the trypanosome, but the molecular signals involved are not clearly understood.

Transmission begins when short stumpy BSFs (believed to be pre-adapted for survival within the insect midgut due to the presence of a functional mitochondrion) are picked by

tsetse fly during feeding. For the first 3 days, trypanosomes are mostly contained within the bloodmeal as it is being digested. The critical events in parasite establishment appear to occur approximately 3 days after infection, when the relatively small proportion of surviving trypanosomes (~10%) either die or rapidly multiply in number (Gibson and Bailey, 2003). Trypanosomes in an established infection migrate to the ectoperitrophic space 3–5 days post-infection (Gibson and Bailey, 2003) (Figure **2**-3). It is believed that this occurs by direct penetration through the peritrophic membrane (Ellis and Evans, 1977; Gibson and Bailey, 2003) although an alternative but less likely, suggestion is that it occurs by circumnavigation around the open, posterior end of the peritrophic membrane (PM) in the hindgut. Whether trypanosomes penetrate the peritrophic membrane, or circumnavigate around its broken ends to get to the ectoperitrophic space is still subject of speculation (Walshe *et al.*, 2009b).

From 6 to 8 days post-infection, large numbers of trypanosomes congregate within the proventriculus (Van den Abbeele *et al.*, 1999; Gibson and Bailey, 2003; Sharma *et al.*, 2008) (Figure **2**-4). Here they appear to cease division, elongate to mesocyclic forms and later differentiate into long trypomastigotes (Figure **2**-4) (Van den Abbeele *et al.*, 1999). Trypanosomes then migrate back into the endoperitrophic space by actively penetrating the PM and move anteriorly in the lumen of the foregut to the opening of the hypopharynx at the tip of the proboscis. An alternative theory of migration involves the direct penetration of the tsetse salivary glands after trypanosomes have traversed the fly haemolymph (Mshelbwala, 1972). It is generally accepted that this is unlikely, as

trypanocidal factors known to be present in the haemolymph would act as a major barrier for trypanosomes attempting to traverse it (Croft *et al.*, 1982).

Asymmetric division of the proventricular epimastigote form generates both long and short parasites (Figure **2**-5) and it is either the asymmetrically dividing trypanosome or the short epimastigote that arrives at the salivary gland (Sharma *et al.*, 2008). Each tsetse fly has two salivary glands. Evidence suggests that each gland is invaded and colonized separately, with few epimastigotes constituting the founder populations (Peacock *et al.*, 2007). The short epimastigote forms are believed to attach to the salivary gland epithelium by interdigitation of their membranes (Figure **2**-6). Upon binding, the non-infective epimastigotes complete several rounds of replication and differentiate into the metacyclic form. Differentiation (metacyclogenesis) includes the appearance of a VSG surface coat. Metacyclic VSGs display a specific VSG repertoire subset and their expression is regulated differently to bloodstream VSGs (Barry *et al.*, 1998; Graham *et al.*, 1999). Mitochondrial changes also occur, including loss of mitochondrial cristae and Krebs cycle enzymes. The biochemical changes accompany the posterior migration of the kinetoplast before the parasite detaches into the lumen as a mature, free-form, infective metacyclic trypomastigote. At this point, each mature metacyclic parasite has undergone the transformation necessary for survival in a mammalian host.

Described above is an account of the most complex trypanosome life cycle completed in the tsetse fly and it should be noted that there are distinct differences between the

lifecycle of *T. brucei sp.* trypanosomes and other African trypanosome species. For example, *Trypanosoma vivax* has the simplest life cycle in the tsetse fly normally developing in the proboscis (Gardiner, 1989) and it takes about 5-13 days to complete (Leak, 1999), while *T. congolense* infections develops in the tsetse fly midgut and matures in the hypopharynx (as shown on Table 1).

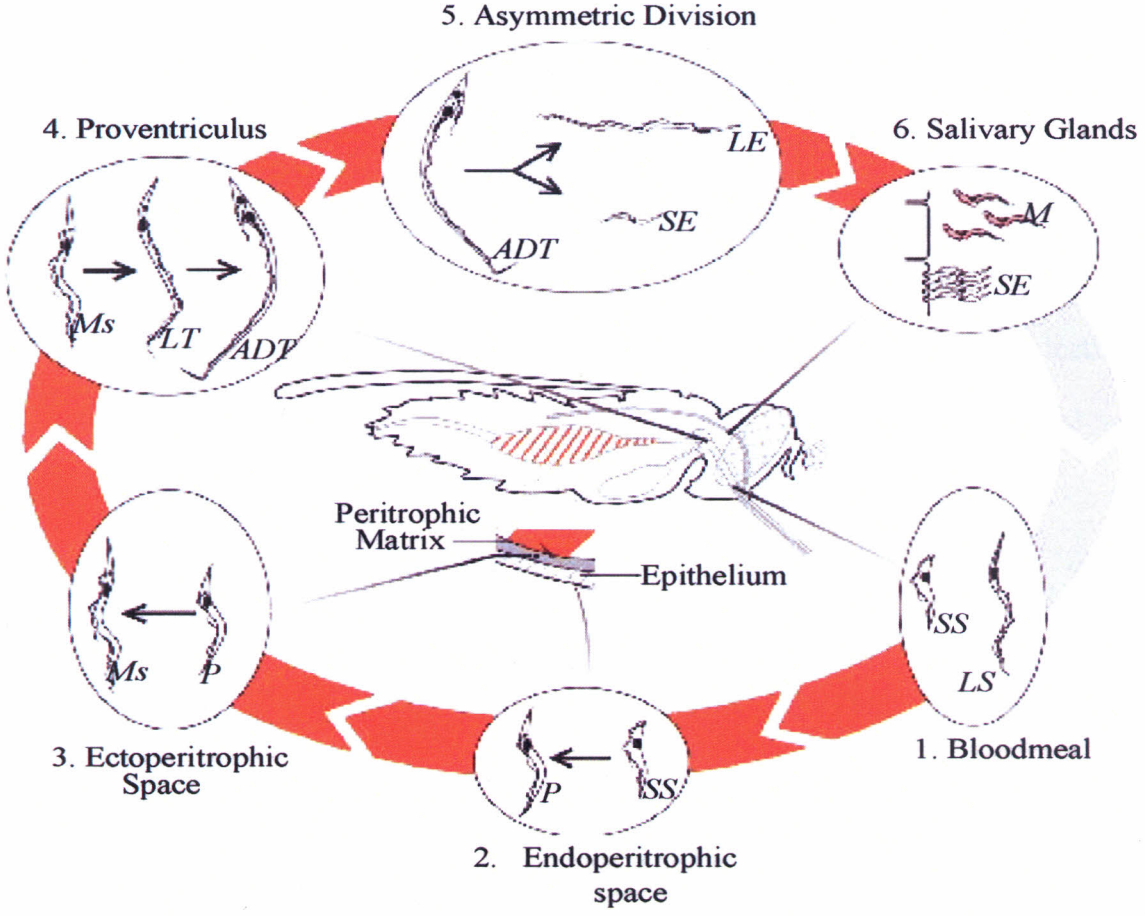


Figure **2**. Diagram of the life cycle stages of *T. b. brucei* within the tsetse fly (Walshe *et al.*, 2009b). Stages of the trypanosome life cycle within the tsetse are indicated within circles joined by the orange arrow; the grey arrow represents transit between insect and mammalian host. Abbreviations: (SS, Short stumpy; LS , long slender bloodstream forms (BSFs); P, procyclics; Ms, mesocyclics; LT, long trypomastigotes; ADT, asymmetrically dividing trypomastigotes; LE, long epimastigotes; SE , short epimastigotes; M, metacyclics).

2.4 The vector: *Glossina* spp

2.4.1 Morphological features and classification

Tsetse flies are obligate blood feeding insects with both male and female taking blood meals every two to five days contributing hence to trypanosome transmission. They belong to the order *Diptera*, family *Glossinidae* and genus *Glossina*. The genus *Glossina* consist of three groups and recently (Solano *et al.*, 2010) they were subdivided into three subgenera, namely the *fusca* group (subgenus *Austenina*), the *morsitans* group (subgenus *Glossina*) and the *palpalis* group (subgenus *Nermohina*). These are then subdivided into currently 31 species and subspecies of tsetse flies (Gooding and Krafsur, 2005; Krafsur, 2009). Morphologically, tsetse flies are rather dull in appearance, varying in colour from a light yellowish-brown to a dark blackish-brown. The abdomen may be uniformly colored or transversed by stripes according to species. Two visually distinctive characteristics that are apparent to the eye are the forward projecting proboscis and the unusual hatchet shaped cell formed by the wing venation (Walshe *et al.*, 2009b).

2.4.2 Life cycle

Tsetse flies reproduce by adenotrophic viviparity i.e. giving birth to live offspring nourished within the mother by secretion from highly modified accessory glands and born at an advanced stage of development. The female tsetse fly is inseminated only once during the course of its reproductive life and produces offspring at regular interval. It produces a single egg, which hatches into a first-stage larva in the uterus. After a period of development and moulting, a third instar larva is deposited on the ground. The

three larval stages develop within the mother. They are nourished with a milky secretion produced by the female accessory glands. Females produce one full-grown larva every 9-10 days, which then pupates within 1-2 hours. The adult fly will emerge after a puparial period that varies according to temperature but is about 30 days at 24°C (Leak, 1999). The newly emerged fly that has not taken its first blood meal is called teneral fly.

2.4.3 Distribution and habitat preference

Tsetse flies are found exclusively in about 10 million km^2 of sub-Saharan Africa. This area, ranging between 14°N and 29°S from Senegal in the West to Southern Somalia in the East, is infested with the 31 identified species/subspecies of tsetse. The distribution of different tsetse groups in the tsetse-infested belt is related to their habitat preferences (Figure 3). The *fusca* group species typically occur in the dense, lowland rain forests of West and West-central Africa. The species belonging to the *palpalis* group are also basically forest-dwellers, mostly found in the riverine vegetation of West Africa (Leak, 1999). The species of the *morsitans* group typically occur in the savannahs ranging from moist Savannahs or the margins of the forest to dry savannahs near the margins of the African desert. Only tsetse flies are biological vector of trypanosome i.e. capable of transmitting the parasites cyclically. However, other biting insects may mechanically transmit trypanosomes (Desquesnes and Dia, 2003).

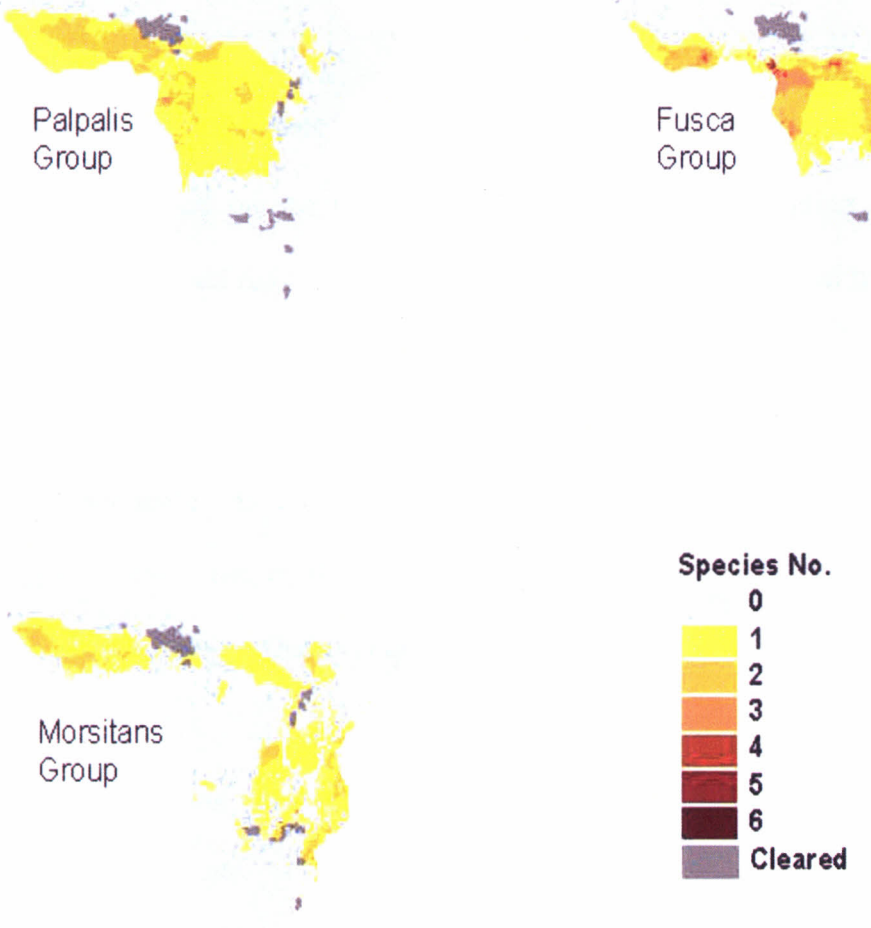


Figure 3. Distribution of the three groups of tsetse fly species, belonging to the *fusca*, *morsitans* and *palpalis* groups in Africa (http://ergodd.zoo.ox.ac.uk/livatl2/tsetse.htm; (Leak, 1999).

2.5 Trypanosomiasis control

In tsetse-infested areas, several strategies can be used to control trypanosomiasis. They are mainly orientated towards the elimination of the parasites from the host's blood and the prevention of tsetse bite through vector control.

2.5.1 Control of the parasite in the host

This control is based on the use of trypanocidal drugs. Depending on the control strategy, drugs are used for curative or preventive purposes. In animal trypanosomiasis, several compounds such as diminazene aceturate, isometamidium chloride have been used to combat the parasite. Isometamidium chloride has a prophylactic activity whilst diminazene aceturate has a short-term therapeutic activity. However, there has been widespread incorrect use of these drugs which has lead to the development of drug resistance by the parasite (Geerts *et al.*, 2001).

In the case of sleeping sickness, the most widely used drugs to treat the patients are suramin, pentamindine, melarsoprol and eflornithine (difluoromethylornithine) depending on the trypanosome species and the clinical stage of disease. A new treatment using a combination of nifurtimox-eflornithine is available for sleeping sickness (Priotto *et al.*, 2007; Priotto *et al.*, 2009). However, up to now, there is no vaccine available to control human or animal trypanosomiasis and the drugs available are not only toxic but some of them do not meet present day drug-safety standards (Atouguia and Costa, 1999; Baral, 2010). Furthermore, trypanosomiasis is often considered as a poor man's diseases

thus it has gained little interest by pharmaceutical companies to develop new drugs. Another component that makes management of trypanosomiasis more complicated is the need for patients to be hospitalized since treatment requires extensive and careful nursing, as well as follow-ups to check for relapses. The epidemiology of the disease, the technicality in diagnosis as well as the absence of an adequate number of well trained personnel in the affected areas are some of the other factors that contribute towards hampering the treatment of trypanosomiasis.

The poor input and insensitivity by the pharmaceutical industrial sector in the quest for trypanocides has led to seeking of alternative medicine to improve the fight against HAT based on documented ethno-pharmacology practices (Nok, 2005). The huge base of information is exploited by scientists for the isolation of active principles from medicinal plants and improving their efficacy via biotransformation or organic synthesis using the molecules as parent compounds. This approach is especially appealing considering the current trend of functional genomics and proteomics, whereby the binding efficacy of an identified ligand can be highly improved by computer-assisted drug design (*In silico* drug development).

2.5.2 Vector control

In the absence of effective vaccines and affordable drugs, and because of wildlife reservoirs, disease control relies heavily on controlling the vector. Historically, attempts to control the vector were based on strategies such as game elimination, creation of fly

barriers and widespread bush clearing to destroy habitats for tsetse breeding and survival. Ground and aerial insecticide spraying with highly persistent and toxic chlorinated hydrocarbons such as Dichlorodiphenyltrichloroethane (DDT) have also been used with considerable success in south Africa, Nigeria, Cameroon, Zambia (Allsopp, 2001) and other African countries. However, due to environmental concerns associated with the methods mentioned above, other strategies were developed e.g. trapping techniques. Based on knowledge of tsetse behavior and ecology, different traps have been developed that targets specific species of tsetse (see Table 2 below).

Table 2. Different traps used to capture various species of tsetse.

Trap Type	Target species
Biconical	*G. pallidipes*; *G. palpalis; G. gambiensis*; *G. tachnoides*; *G. fuscipes* *G. longipennis*
Pyramidal	*G. palpalis ; G. gambiensis* ; *G. tachnoides* ; *G. fuscipes*
Vavoua	*G. palpalis ; G. gambiensis* ; *G. tachnoides* ; *G. fuscipes*
lancien	*G. fuscipes*
Epsilon	*G. morsitans* ; *G. pallidipes* ; *G. longipennis*
F3	*G. pallidipes*
H-trap	*G. austeni* ; *G. brevipalis*
Ngu	*G. pallidipes* ; *G. longipennis*
Sticky trap	*G. austeni* ; *G. brevipalis*

The efficiency of the trap can be greatly improved with the use of odour baited traps containing a tsetse attractant that can either be of natural (e.g. cow urine) or synthetic (e.g acetone) origin. Such an approach in combination with a synthetic repellent for tsetse is currently being employed by ICIPE scientists to control tsetse fly in various parts of Kenya, on a push-pull strategy (unpublished data). The strategy relies on the use of two different chemical compounds, one of which repels the tsetse from hosts (push) while the other compound attracts the flies towards a trap or target (impregnated with insecticide) where they can be killed (pull). A further modification of this has led to the inclusion of live baits (e.g. cattle) to which insecticide have been applied through spraying, dipping and pour-on (Bauer *et al.*, 1992; Shereni, 1997; Baylis and Stevenson, 1998). A major advantage of this live bait technique is that a single farmer can comfortably use it on his livestock. In addition, the insecticide now widely used is a synthetic pyrethroid (e.g. deltamethrin and cypermethrin) which has a lower toxicity to mammals.

Another environmental friendly vector control method is the sterile insect technique (SIT) which was successfully used to eradicated tsetse flies (*G. austeni*) from the island of Zanzibar (Vreysen *et al.*, 2000), but the choice of this technique largely depends on the isolation of the area, with the level of reinvasion from neighboring populations being a major problem. Apart from the above methods, the use of trypanotolerant animals (naturally tolerant to the disease e.g. N'Dama cattle) is also an alternative in the control of animal form trypanosomiasis.

2.5.3 Towards new approach for trypanosomiasis control

Current interventions for management and control of trypanosomiasis are focused predominantly on drug chemotherapy and tsetse fly control especially for the control of nagana (Torr *et al.*, 2005). While both these existing methods are successful, their implementation over extended time periods has not always been sustainable (Vale, 1982; Aksoy *et al.*, 2003). Additionally, concern over the development of resistance to available drugs and their toxicity is mounting, and the prospects for new drugs to treat HAT and nagana are bleak (Legros *et al.*, 2002; Fevre *et al.*, 2006). Consequently, new means of controlling HAT are urgently required. With the genome resources now available for tsetse flies (full annotated complete genome projected for 2011), their symbionts and the trypanosomes (Walshe *et al.*, 2009b), as well as development of paratransgenesis, researchers are seeking new ways of controlling trypanosomiasis based on genomic information. To exploit these resources to their fullest, molecular tools for studying gene function (e.g. RNA-interference) in tsetse fly are proving to be important in elucidation of genes involved with tsetse-trypanosome interactions.

2.6 Gene knockdown in *Glossina*

The very slow reproductive rate in tsetse (one offspring every 9 days; 40 days adult to adult) and the high costs of maintaining colonies have prevented maintenance of multiple mutant fly lines. In addition, the unusual, viviparous reproductive system of tsetse flies prevents the development of germ-line transgenesis. Gene knockdown through RNA interference (RNAi) (Fire *et al.*, 1998) has been of particular value in the study of tsetse

biology and gene function. RNAi is based on administration or induced synthesis of dsRNA into an organism leading to post-transcriptional knockdown of genes sharing specific sequence with the introduced dsRNA. In the current model of RNAi (Figure 4), two major steps are involved. The initiator phase involving recognition of dsRNA (that is expressed in or introduced into the cell) and cleavage into short interfering RNA (siRNA) fragments 21–23bp in length by the enzyme Dicer (Hammond, 2005). This is followed by an effector phase involving the incorporation of cleaved dsRNA into a multi-protein complex, known as the RNA-induced silencing complex (RISC), capable of silencing homologous mRNA transcripts (Siomi and Siomi, 2009).

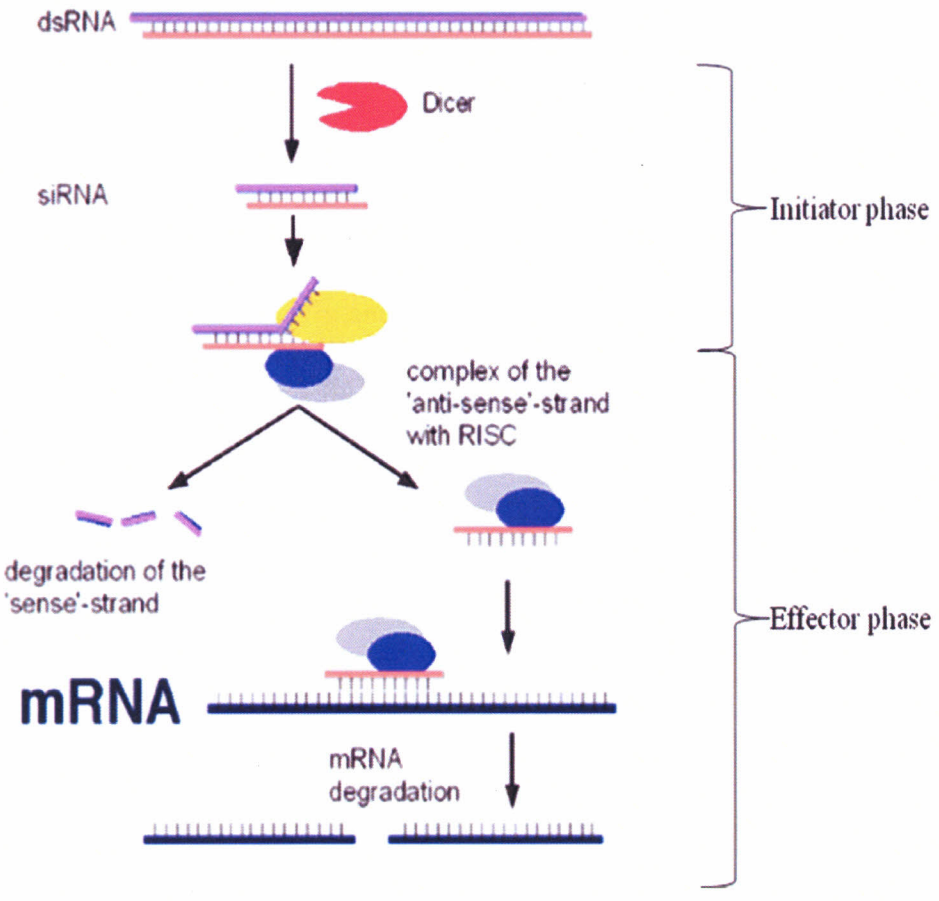


Figure 4. A schematic illustration of the steps involved in RNA interference (modified from, http://hades1.bioquant.uni-heidelberg.de/rnai.html). Abbreviations: (dsRNA, double stranded ribonucleic acid; siRNA, short interfering ribonucleic acid; RISC, RNA induced silencing complex)

One major limitation of insect RNAi system is finding a suitable delivery system of the dsRNA (Walshe *et al.*, 2009b). However, the most commonly used method for dsRNA delivery to insects is the direct injection of dsRNA into the haemocoel. This was first demonstrated in *A. gambiae* (Blandin *et al.*, 2002) where the host defence peptide (HDP) defensin was successfully knockdown. This technique has been successfully extended to the analysis of *Glossina* genes (Hu and Aksoy, 2006; Lehane *et al.*, 2008; Attardo *et al.*, 2008; Yang *et al.*, 2010) and to date three *G. m. morsitans* genes, a transferrin, an attacin and tsetseEP protein, have been implicated in tsetse-trypanosome interactions. Knockdown of these genes by dsRNA injection resulted in a statistically significant increase in trypanosome midgut prevalence (Hu and Aksoy, 2006; Lehane *et al.*, 2008; Haines *et al.*, 2010).

Although direct injection is widely used for gene function studies in tsetse fly, high mortality rates have been observed following injection (Walshe *et al.*, 2009a) due to the physical damage that it causes. This complicates the interpretation of experimental results because gene knockdown may have occurred to differing extents in the surviving and killed flies (Walshe *et al.*, 2009b; Haines *et al.*, 2010). Consequently, gene knockdown by direct injection of dsRNA into the tsetse haemocoel is not ideal when studying fly immunity. For example Hao *et al.*(2001) observed in the PBS injected control group sustained upregulation of defensin and attacin transcripts in tsetse fat body until 18-30 hours post injection indicating an immune response as a result of the physical injury from microinjection.

Recently, successful local gene knockdown in *Glossina* has been demonstrated by ingestion of dsRNA in a bloodmeal (Walshe *et al.*, 2009a). Thus, feeding dsRNA could be used as an alternative and preferable means of delivering dsRNA as oral administration is less invasive than dsRNA injection (Walshe *et al.*, 2009b).With the available functional analysis tool (RNAi) in tsetse fly and the greatly expanding genomic information resource, numerous efforts are being made towards understanding vector-parasite interaction that seems to be a complex affair. Of particular interest are the various factors known to modulate the vectorial capacity of tsetse fly. So far, tsetse immune components have been recognized as a major barrier to trypanosome development within the fly through functional studies of immune related genes (Hu and Aksoy, 2006). In addition, the tsetse fly midgut a crucial site for bloodmeal digestion and pathogen transmission has also been highly studied.

Various midgut factors associated to bloodmeal ingestion such as, formation of bilayered peritrophic membrane (Lehane and Msangi, 1991; Peter, 1992), secretion of proteases (Imbuga *et al.*, 1992), lectins (Stiles *et al.*, 1990; Maudlin, 1991), trypanolysins (Stiles *et al.*, 1990; Osir *et al.*, 1999) and other yet unknown factors, creates a hostile environment for the trypanosomes (Stiles *et al.*, 1990; Onyango, 1993; Abubakar *et al.*, 1995) eliminating most of the ingested trypanosomes. Consequently, successful establishment of trypanosome infections in tsetse greatly depends on them and there is always a crosstalk between tsetse fly and trypanosome that it hosts, with the parasite exploiting some of those factors to enhance its survival. Differentiation of bloodstream-form

trypanosomes into procyclic (midgut) is an important first step in the establishment of an infection within the tsetse fly. This complex process is mediated by a wide variety of factors, including those associated with the vector itself (Imbuga *et al.*, 1992), the trypanosome and the bloodmeal (Mihok *et al.*, 1995).

Initial evidence that parasitized blood when incubated with crude midgut homogenates stimulates transformation of blood stream form trypanosomes (Imbuga *et al.*, 1992), led to the hypothesis of involvement of trypsin or trypsin-like enzymes in the transformation of blood stream form trypanosomes into procyclic form. Further studies (Osir *et al.*, 1993) established a correlation between the trypsin-like enzymes and lectin activities, and the crucial role they play in the establishment and transformation of trypanosome in tsetse midgut. This led to purification and characterization of the midgut protein involved (Osir *et al.*, 1995) which had two non-covalently linked subunits (α and β) and both lectin and trypsin-like properties. The holoprotein (lectin-trypsin complex) was later shown to be capable of stimulating transformation of bloodstream form trypanosome *in vitro* (Abubakar *et al.*, 2003).

Consecutive studies (Abubakar *et al.*, 2006; Amin *et al.*, 2006) to characterize the genes coding for that protein, identified a gene named *Glossina* proteolytic lectin (GPL) from *G. fuscipes fuscipes* (Acc No. DQ060150) and *G. austeni* (Acc No. AF525314) respectively. The sequence when analysed with bioinformatics tools showed the cDNA encodes a putative mature polypeptide with 274 amino acid residues. The deduced

amino acid sequence contained a hydrophobic signal peptide and highly conserved N-terminal sequence motifs. The typical features associating it to the serine protease trypsin family included the His/Asp/Ser active site triad with the conserved residues surrounding it, three pairs of cysteine residues for disulphide bridges and an aspartate residue at the specificity pocket. When the gene was expressed in a bacteria (Abubakar *et al.*, 2006; Amin *et al.*, 2006) and baculovirus system (Amin *et al.*, 2006), it yielded a recombinant protein (GPL-M_r ~32500) that bound D(+) glucosamine, agglutinated bloodstream-form trypanosome and rabbit red blood cells in a similar manner to the protein purified from tsetse midgut homogenates highlighting its lectin properties (Abubakar *et al.*, 2006; Amin *et al.*, 2006). The protein was also found to be capable of inducing transformation of bloodstream trypanosomes into procyclic forms *in vitro* (Abubakar *et al.*, 2003; Abubakar *et al.*, 2006; Amin *et al.*, 2006). In addition, an expression profile analysis of GPL in several haematophagous insects showed the gene occurs only within the *Glossina* species and it is differentially expressed following trypanosome infections (Burugu *et al.*, 2007).

Lectins have been implicated with the refractoriness of tsetse fly. Based on the effects of plant lectin concanavalin A (Con A) on trypanosomes *in vitro*, lectin mediated killing of trypanosomes was believed to occur by a process termed proto-apoptosis (Welburn and Maudlin, 1999; Pearson *et al.*, 2000). Such factors within tsetse fly forms potential targets for disruption of trypanosome transmission as a novel approach to trypanosomiasis control involving modulation of vector competence through paratransgenesis (Aksoy *et*

al., 2003; Weiss *et al.*, 2008). Paratransgenesis involves the genetic transformation of symbiotic bacteria residing in the insect to produce anti-parasitic molecules into the midgut. The endosymbiont *Sodalis* is the target expression vector in the tsetse fly. This bacterium resides in the tsetse midgut as well as other body sites. Consequently, invading *brucei* and *congolense* group trypanosomes would encounter trypanocidal secretion products before the trypanosomes could establish in the midgut. With the development of *Sodalis in vitro* culture (Matthew *et al.*, 2005), genetic manipulation system (Beard *et al.*, 1993), as well as the ability to reintroduce by microinjection the recombinant *Sodalis* produced *in vitro* into the mother's haemolymph and subsequent passage *in utero* to the next progeny (Cheng and Aksoy, 1999), increases the potential for successful paratransgenesis.

However, several hurdles need to be overcome before this technology can be applied to disease control. For example, to replace the susceptible wild type tsetse population with the refractory population expressing trypanocidal molecules, a suitable biological drive system is required e.g. *Wolbachia* (Weiss *et al.*, 2007). Additionally, this approach would only be suitable for trypanosome species (*brucei* and *congolense* groups) that establish in the fly midgut. Therefore, another tactic would be required to control trypanosome species such as *T. vivax*, which establish in the mouthparts and would not be affected. Also, how rapidly trypanosome resistance would appear with such a system in place is unknown and a concern. Therefore, it would probably be advantageous to have a selection of effector molecules available for use in a managed control strategy (Walshe *et*

al., 2009b). To date, several candidate antitrypanosomal molecules have been identified which could be introduced into *Sodalis* to control trypanosome midgut establishment. So far, two candidates have been identified in tsetse, a tsetseEP (Haines *et al.*, 2005; Haines *et al.*, 2010) and attacin (Hu and Aksoy, 2006). Less obvious, but potentially more powerful candidate molecules are also under investigation. For example, BMAP-18 a truncated form of the bovine myeloid antimicrobial peptide-27 (BMAP-27). BMAP-27 (Skerlavaj *et al.*, 1996) is expressed by bovine neutrophils and exhibits low toxicity to mammalian cells, insect cells and *Sodalis*, yet causes rapid death to both BSF and PCF trypanosomes (Haines *et al.*, 2003).

In an attempt to fill in this gap, a functional genomic approach was used to study a midgut proteolytic lectin gene in *G. pallidipes* using dsRNA mediated RNA-interference. Up to date no trypanocidal lectin has been purified and, the supporting data for the direct role of lectins in the tsetse-trypanosome interaction are still limited.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Tsetse fly

Male teneral tsetse flies (*G. pallidipes*) were obtained from Animal Rearing and Containment Unit (ARCU) at ICIPE. These flies were maintained at 24–27°C, 65-80 % relative humidity and fed on rabbit blood (*in-vivo*) after every 48 hrs. For knockdown studies, three groups (n=30) of male teneral *G. pallidipes* flies (12-32hrs old) that had received a single bloodmeal were used. One group was injected with dsRNA (~4-6µg/µl); the second one was injected with water as a negative control while the third was uninjected to monitor the mortality rates. Prior to microinjection flies were chilled briefly (10-15 mins) at +4°C to immobilize them and 2µl of concentrated dsRNA (~4-6µg/µl) or nuclease free water microinjected in the dorsolateral surface of the thorax (scutum) under a dissecting microscope. The flies were allowed to recover for 24 hrs in the tsetse fly rearing unit before feeding. This was followed by subsequent feeding after every 48hrs for a period of two weeks during which knockdown and functional analysis was done.

3.2 Preparation of midgut homogenate

To isolate GPL gene and for knockdown analysis, midguts (2-4) were carefully dissected, washed in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH8.0) to remove any haemolymph and lipids then resuspended in 0.5ml ice cold PBS in 1.5 eppendorf tube. The midgut tissue was

homogenized using a plastic pestle and the ground tissue centrifuged at 12,000 rpm for 5 minutes at +4°C in a minifuge (Biofuge Heraeus, Germany). The resulting supernatant of the homogenate was then used for RNA extraction.

3.3 RNA isolation

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 250µl of the supernatant (above) was transferred into a clean RNAse free eppendorf tube containing 750µl of TRIzol LS reagent and let to stand at room temperature for 10 minutes. Then 200µl of chloroform was added to the sample and mixed, this was incubated at room temperature for 10 minutes and then centrifuged at 12,000 rpm for 10 minutes at +4°C. The resulting upper aqueous solution was carefully transferred into a clean RNASE free eppendorf tube to which 1µl of glycogen and 500µl isopropanol was added. The sample mixture was vortexed, incubated at room temperature for 10 minutes and then centrifuged at 12,000 rpm for 10 minutes at +4°C to pellet down the RNA. The supernatant was discarded away and the pellet washed by adding 500µl of 75% ethanol followed by centrifugation at +4°C for 2 minutes at 12,000 rpm. The ethanol was poured off, the pellet air dried briefly to remove any traces of ethanol and then resuspended in 15µl of nuclease free water. The total RNA was stored in -80°C for later use.

3.4 Total RNA analysis

Prior to cDNA synthesis the yield and the purity of total RNA was determined spectrophotometrically by reading the relative absorbance at 260 and 280nm (i.e., A_{260}/A_{280}). To check for genomic DNA contamination 3µl of the total RNA was heat denatured at 70°C for 5 minutes then immediately electrophoresed through 0.3% ethidium bromide stained agarose gel for 1.5 hr at 60V and visualized using a UV transilluminator.

3.5 cDNA Synthesis from total RNA

The total RNA was first treated with DNaseI (Fermentas, USA) to remove any genomic DNA and the enzyme heat inactivated at 65°C for 10 minutes in presence of 2.5 mM EDTA to prevent RNA hydrolysis. A RevertAid^{TM}H Minus first strand cDNA synthesis kit and protocol (Fermentas, USA) was used to prepare cDNA according to the manufacturer's instructions.

3.6 Synthesis of first strand complementary DNA (cDNA)

The enzyme employed in the kit is RevertAid^{TM}H Minus M-MuLV Reverse Transriptase, a point mutant of Moloney murine leukemia virus (M-MLV) reverse transriptase, which lacks RNaseH activity but has polymerase and terminal transferase activities. Additionally the RiboLock^{TM} RNase inhibitor provided in the kit effectively protects RNA degradation at temperature up to 55°C. Four microliters of DNase I treated total RNA (~1µg) was mixed with 5µM of oligo $(dT)_{18}$ primers and incubated at

65°C for 5 minutes in a MJ Research PTC 100 thermal cycler. The content were then cooled on ice and spun briefly before addition of 1X reaction buffer (250 mM Tris-HCL (pH8.3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 1 mM dNTPs, 1 unit of RiboLock™ RNase inhibitor (20U/µl) and RevertAid™ H Minus M-Mul V reverse transcriptase (200U/µl). The 20µl content was mixed gently and spun briefly before incubation at 42°C for 1 hour. At the end of incubation period the reaction was terminated by heating at 70°C for 5 minutes and the resulting cDNA used in a PCR.

3.7 Design of PCR Primers

Primers were designed based on GPL full length cDNA sequences from *G. austeni* (Acc No. DQ060150) and *G. f. fuscipes* (Acc No. AF525314), available in the GenBank (www.ncbi.nlm.nih.gov/). This involved manual selection of primers amplifying the coding region of GPL for cloning primers and in the case of RNAi primers a web based tool (http://e-rnai.dkfz.de/) (Arziman *et al.*, 2005) was used to evaluate and design primers that would give an optimized dsRNA construct. In addition, potential gene cross-silencing was gauged using the DEQOR software (http://cluster-1.mpi-cbg.de/Deqor/deor.html) (Henschel *et al.*, 2004). T7 promoter sequence was added at the 5' end for RNAi primers which allowed dsRNA preparation from PCR products *in vitro*, alternatively the inclusion of restriction enzyme site permitted directional cloning into RNAi vector (pLL10). Analysis of manually designed primer was done using DNA calculator (http://www.sigma-genosys.com/calc/DNACalc.asp) to determine their

melting temperature (Tm), self compatibility and also hairpin formation. Primers were purchased from Bioneer Co. (http://www.bioneer.-co.kr/biomall/malloligo.jsp).

Table 3. Primer sequences for dsRNA construction, RT-PCR and cloning. The underline indicate restriction enzyme sites (GAATTC for *Eco*RI; AAGCTT for *Hind*III and GGATCC for *Bam*HI) and the portion in bold is the T7 promoter sequence. Abbreviations: F & R indicates the forward and reverse primers respectively.

Primer	5'-3' sequences	Product size (bp)
GPLT7. F	GAATTC**TAATACGACTCACTATAGGGA**GCCATTTCTGT-GGTGGTTCT	430
GPLT7. R	AAGCTT**TAATACGACTCACTATAGGGA**AGCGTTCTTG-ATTCTTCGT	-
GPL. F	TTTGGATCCATGAAGTTTGCAGTGTTCGC	830
GPL. R	CGGTAGTAAGCTTACAAAAGTTGCGCATAG	-
Lectizyme. F	AAGCTTATGAAGTTCTTTGCAGTG	830
Lectizyme. R	GGATCCTTACAAAAGTTGCGCATA	-
GAPDH. F	TAAAATGGGTGGATGGTGAGAGTC	380
GAPDH. R	CTACGATGAAATTAAGGCAAAAGT	-

3.8 Reverse transcriptase polymerase chain reaction (RT- PCR)

Amplification of GPL gene was done using different sets of primers (Table 3) that gave PCR products of varying sizes. However, to ensure sequence specificity GPL coding region (~830bp) was amplified from the cDNA, cloned into pGEM-T Easy vector, sequenced and the pGEM-T-GPL construct used as template in subsequent PCR to amplify different GPL fragments (413bp & 570bp) for RNAi. The PCR set-up was in a 20µl reaction volume containing H_2O, 1µl of the RT product (above) as PCR template, 1X phusion ^{TM} HF buffer, 0.5 mM dNTPs, 2 mM $MgCl_2$, 0.5µM of primers (forward and

reverse) and 0.02U/μL high fidelity phusion DNA polymerase. The amplification conditions involved 1 cycle at 98°C for 1 min; 35 cycles of 98°C for 30 secs, 50°C for 30 secs, 72°C for 45 secs and a final extension at 72°C for 8 mins in a 9800 fast thermal cycler (Applied Biosystem, Foster city, USA). An aliquot of the PCR product was analysed on 1% agarose gel.

3.9 DNA gel electrophoresis

DNA samples were analysed using 1% agarose gel electrophoresis. The agarose gel was prepared by melting 0.5 g of agarose in 50 ml of 1X TAE buffer [45 mM Tris Base and 1 mM EDTA] then cooled on a running tap water before addition of 0.5μg/ml ethidium bromide and pouring into the gel casting mould. The samples were mixed with 6X loading dye [25% (v/v) glycerol, 60 mM EDTA, and 0.25 (w/v) bromophenol blue] in a ratio of 5:1 and loaded along with DNA size markers into the prepared horizontal gel. The electrophoresis was carried out in TAE buffer at 80V for 1 hour and the gel visualized on a UV transilluminator. The gel image was recorded using a Kodak GL-200 gel documentation system (Eastman Kodak, USA).

3.10 DNA Purification

Two methods of PCR product purification were employed depending on the quality of amplification desired.

3.10.1 PCR product purification

Where clear distinct bands were observed purification was done using QuickClean 5M PCR Purification Kit (Genescript, New Jersey USA) according to manufacturer's instructions. Briefly, 2.5 volume of binding solution I was added to the tube containing the PCR product and mixed thoroughly by inversions. The mixture was transferred into a spin column, centrifuged at 12,000 rpm for 1 minute in a micro-centrifuge (Biofuge Heraeus, Germany) and the flow through discarded. Washing was done twice using 500µl of wash solution and centrifugation at 12,000 rpm for 30 secs, then any residue wash solution removed by additional centrifugation for 1 min at 12,000 rpm. The column was then transferred into a clean 1.5 eppendorf tube and 30µl of elution buffer (2.0 mM Tris-HCl pH8.5) added at the centre of the column, capped and allowed to stand for 2 minutes at room temperature then centrifuged at 12,000 rpm for 1 minute to elute and collect the DNA. An aliquot (3µl) of the purified product was analysed on agarose gel and the rest stored at -20°C for later use.

3.10.2 Gel extraction

In some instances, the total PCR product was loaded into 1% ethidium bromide stained agarose gel and the bands of appropriate size sliced out of the gel under low wavelength (302nm) UV transilluminator. Purification was then achieved using QuickClean 5M Gel Extraction Kit (Genescript, New Jersey USA) according to the manufacturer's instructions. Briefly, the sliced gel in a clean 1.5 eppendorf tubes was weighed and 3 volumes of binding solution II added based on the weight (100mg = 100µl). This was

followed by incubation in a water bath at 50°C for 10 minutes with occasional mixing by inversion of the tubes to melt the gel. To the solubilized gel one volume of isopropanol (with respect to the original weight) was added, mixed by inversion then transferred to a spin column and centrifuged at 12,000 rpm for 30 secs. The flow through was discarded and the column washed twice using 500µl of wash solution followed by centrifugation at 12,000 rpm for 1 minute, any residue wash solution was removed by additional centrifugation for 1 min at 12,000 rpm and the column transferred into a clean 1.5 eppendorf tube. 30µl of elution buffer (2.0 mM Tris-HCl pH8.5) was added at the center of the column, capped and allowed to stand for 2 minutes at room temperature then centrifuged at 12,000 rpm for 1 minute to elute and collect the DNA. To check the DNA recovery an aliquot (3µl) was analysed on 1% agarose gel and the rest stored at -20°C. To sequence the amplified GPL gene the fragments were cloned into pGEM-T Easy vector and later sub cloned into pLL10 (RNAi) vector for dsRNA preparation.

3.11 Molecular cloning

3.11.1 Ligation

Since a thermostable DNA polymerase with proof reading ability was used during PCR, the purified blunt ended PCR products had to be A-tailed prior to T/A cloning. This was done in a 10µl reaction volume containing 6µl of purified PCR product, 1X Taq DNA polymerase reaction buffer (with $MgCl_2$), 0.2 mM dATP, 5U Taq DNA polymerase and H_2O. The reaction mixture was incubated at 70°C for 30 minutes. 4µl of the A-tailed product was used in a ligation reaction consisting of 50ng of pGEM-T Easy vector

(Promega, USA), 5µl of 2X rapid ligation buffer and T4 DNA ligase (3 Weiss units/µl) in a 10µl reaction volume. The reaction mixture was gently tapped to mix, briefly spun down and incubated at +4°C overnight for maximum number of transformants. Later 4µl of the ligation product was used to transform competent *E.coli* DH5α cells.

3.11.2 Preparation of *E.coli* DH5α competent cell

A single bacterial colony was picked from the LB plate (no antibiotics) that had been incubated for 16-24 hours at 37°C. The colony was transferred into 5ml of SOB medium [2% tryptone (Difco), 0.5% yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl and 2M Mg^{2+} Solution] and incubated overnight at 37°C with shaking at 250 rpm. 2 ml of the overnight bacterial culture was diluted in 250ml of SOB broth and incubated at 18°C with moderate shaking at 200 rpm until the OD_{600} reached 0.5-0.6. The cell culture was transferred to five sterile 50 ml falcon tubes, chilled on ice for 10 minutes and the cell harvested by centrifugation (in a Beckman Aventis™ J-25 I, centrifuge, USA) for 10 minutes at +4°C and 3000 rpm. The media was discarded and any remaining drops dried from the tubes using paper towels. The cell pellet was resuspended in 20ml of ice cold TB buffer [10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, and 250 mM KCl], incubated on ice for 10 minutes and spun at +4°C and 3000 rpm for 10 minutes. Again the supernatant was discarded, the tubes blotted on paper towel and the pellet resuspended in 20ml of ice cold TB buffer. Then, dimethyl sulfoxide (DMSO) was added with gentle swirling to the final concentration of 10% and the cells incubated on ice for 10 minutes. The cell suspension was dispensed as 100µl aliquots into sterile microcentrifuge tubes,

snap closed and immersed in liquid nitrogen. The prepared competent cells were stored at -80°C for later use.

3.11.3 Transformation of *E. coli*

A volume of 4µl ligated product was mixed with 50µl of competent *E.coli* DH5α cells by gently tapping and the tubes incubated on ice for 20 minutes. The mixture was heat shocked at 42°C for 1 minute and immediately chilled on ice for 2 minutes. 900µl of SOC medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract, 1M NaCl, 1M KCl, 2M Mg^{2+} solution and 2M glucose] was added to the mixture and incubated at 37°C for 2 hours in a water bath with occasional tapping of the tubes to mix. To increase the number of colonies the cells were pelleted by centrifugation at 5000 rpm for 5 minutes. 800µl of the supernatant (SOC media) was discarded and the pellet resuspended in the remaining 100µl of SOC media. Transformed cells were plated by spreading on LB agar plates containing 0.5 mM IPTG, 80µg/ml X-Gal and 100µg/ml ampicillin. The agar plates were incubated at 37°C for 16-24 hours.

3.11.4 Screening of positive colonies by PCR

Positive colonies initially identified by blue-white colour screening were further screened for the presence of the correct insert by colony PCR. Using a sterile small pipette tip a small portion of each individual colony (~10 colonies per plate) was picked and, resuspended in an individual PCR reaction volume such that each PCR reaction tube contained an individual colony lysate, 1X PCR buffer, 0.2 mM dNTPs, 0.5µM

vector specific primers (both M13 forward and reverse) and 0.25U of *Taq* DNA polymerase in a 20µl final volume. A blue colony was used as a negative control. The PCR cycling condition involved initial denaturation at 94°C for 2 mins followed by 35 cycles of (94°C for 30 secs, 56°C for 1 min, 72°C for 1 min) and a final extension at 72°C for 8 mins in a 9800 fast thermal cycler (Applied Biosystem, Foster city, USA). An aliquot of the PCR product was analysed on 1% agarose gel as described in section 3.9. A few (2-3) positive colonies identified by PCR were picked from the plates using sterile pipette tip, this was inoculated into 5 ml of SOB broth containing 100µg/ml ampicillin and incubated at 37°C overnight with shaking at 200 rpm in an orbital shaker (Environ-shaker 3597-1, Labline Instrument Inc., USA).

3.11.5 Plasmid purification

Overnight cultures were used for recombinant plasmid isolation using Quickclean 5M Miniprep Kit (Genescript, New Jersey USA) according to manufacturer instructions. Briefly, recombinant cells were harvested by centrifuging 3ml of the overnight culture at 12,000 rpm for 30 secs and then the pellet resuspended in 200µl of solution I. To the mixture, 400µl of solution II was added, mixed gently by inverting the tube 4-6 times after which 600µl of solution III was added and again mixed by inversion (4-6 times). The reaction mixture was centrifuged at 12,000 rpm for 5 minutes and the supernatant transferred into a spin column and spun for 30 seconds at 12,000 rpm. The flow through was discarded and the column washed twice using 500µl of wash solution then centrifugation at 12,000 rpm for 30 seconds. An additional spin at 12,000 rpm for 1

minute was done to remove any residual wash solution and the column placed in a clean 1.5ml micro-centrifuge tube. 50µl of the elution solution was added at the centre of the membrane, let to stand for 1 minute at room temperature and the DNA eluted by centrifugation at 12,000 rpm for 1 minute. An aliquot of the purified plasmid was separated by gel electrophoresis (see section 3.9) against an empty plasmid to check the mobility shifts and the rest was kept at -20°C for later use.

3.11.6 Restriction enzyme digestion analysis of the recombinant clones

After purifying the recombinant plasmids, the size, and presence of the insert was further confirmed by restriction enzyme mapping. The enzyme *Bam*HI and *Hind*III were used since their restriction sites were introduced by the primers used (see Table 3). 30ng of recombinant plasmid DNA was double digested using *Bam*HI and *Hind*III restriction enzyme in a 10µl reaction volume containing 1 unit of each restriction enzyme, 1X NEB buffer 2 supplemented with 100µg/ml bovine serum albumin (BSA). The reaction mixture was incubated at 37°C for 3-4 hours and the size of the digested product analysed on 1% agarose gel as described in section 3.9.

3.12 Sequencing

Aliquots of 10µl of the purified recombinant plasmids were sent for sequencing (Sequencing Platform BecANet, Kenya). Complete sequence determination was done bidirectionally using M13 vector specific primers on ABI 3730 sequencer.

3.12.1 Sequence analysis

Editing and base scoring was done using BioEdit (Version 7.0.5.3, Carlsbad CA) software to generate a consensus sequence after which restriction sites introduced by the primers were removed manually and the resulting sequence screened using VecScreen (NCBI) tool to remove vector portions. The edited sequences were used to carry out BLAST searches in Gene Bank. Sequences showing high similarity to the cloned GPL gene were picked from the database to perform multiple sequence alignment as well as phylogenetic analysis using CLUSTALW and MEGA 4 software's respectively. Further, conserved motifs within the sequenced GPL were determined using a ProSite Scan (under ExPASy) tool and the presence of a signal peptide predicted using SignalP 3.0.

3.13 GPL dsRNA design

Two different GPL dsRNA were designed based on GPL full length cDNA (Amin *et al.*, 2006; Abubakar *et al.*, 2006), with the primer sets (their design described in section 3.7) used amplifying 413bp and 570bp fragments of GPL. For the small fragment (413bp) the primers had a T7 promoter region at the 5' end (see Table 3) thus the dsRNA was prepared using PCR product as template. In contrast, the larger fragment (570bp) had to be T/A cloned into pGEM-T vector (described in section 3.11) then directionally subcloned into pLL10 vector (Figure 5).

3.13.1 Plasmid construction for dsRNA preparation

To generate dsRNA for the 570bp GPL fragment, the fragment had to be cloned between the two T7 promoters of pLL10 vector (Figure 5). To achieve this, GPL fragment was cloned into pGEM-T Easy vector as described in section 3.11 and then subcloned into the *Bam*H1- *Hind*III region of pLL10 vector.

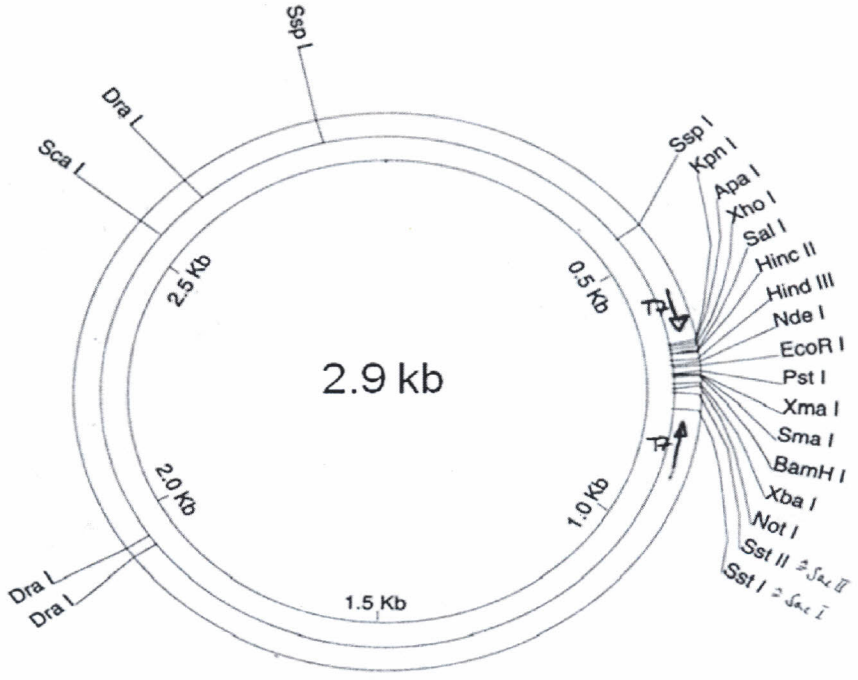


Figure 5. A schematic representation of pLL10 vector map indicating the multiple cloning sites that is flanked by the dual opposing T7 promoters for in-vitro transcription of cloned inserts (Blandin *et al.*, 2002).

3.13.2 Sub-cloning of GPL fragment into pLL10 vector

The recombinant pGEM-T GPL construct (having the 570bp fragment) was double digested with *Bam*HI and *Hind*III restriction enzyme to release the insert. Similarly, pLL10 vector (Figure 5) was also digested using the same enzymes. Both digest

reactions were set in a 30µl reaction volume consisting of 10µl of vector DNA, 1X NEB buffer 2 (New England Biolabs, UK), 1µl of acetylated Bovine Serum Albumin [BSA] (New England Biolabs, UK), 1-3U of *Bam*HI and *Hind*III restriction enzymes (New England Biolabs, UK) and H_2O to top up the volume. The reaction mixture was incubated at 37°C for 3-4 hours. The double digested (*Bam*HI and *Hind*III) GPL fragment and pLL10 vector were separated on a 1% agarose gel as outlined in section 3.9 and the insert and plasmid DNA recovered from the gel using QuickClean 5M Gel Extraction Kit (Genescript, New Jersey USA) as described in section 3.10.2.

The purified digested (*Bam*HI/*Hind*III) insert and vector DNA were ligated together using T4 DNA ligase (promega, USA) in a 1:3 vector to insert ratio. The following components were assembled in a microcentrifuge tube: 5µl of 2X rapid ligation buffer, 1.5µl linearized plasmid DNA, 4.5µl of GPL fragment, 1µl T4 DNA ligase (3 Weiss units/µl) and nuclease free water to a final volume of 15µl. The ligation reaction was incubated overnight at +4°C for maximum number of transformants. 4µl of the ligation product was used to transform competent *E.coli* DH5α cells. Transformation was performed as in section 3.11.3 and positive clones identified by colony PCR as described in section 3.11.4. A few of positive colonies were grown in liquid media and the recombinant plasmid purified as described in section 3.11.5. Confirmation of the insert size was further done by restriction mapping as outlined in section 3.11.6. Positive clones were sequenced and the sequences analysed as described in sections 3.12 and 3.12.1.

3.14 dsRNA preparation

Two GPL dsRNA that differed in size (413bp and 570bp) were prepared. For the small fragment, dsRNA was synthesized from a purified PCR product while with the longer fragment a plasmid construct was used as the template for *in vitro* transcription reaction. The reaction assembly was the same for both once the plasmid construct had been linearized (*Xho*I and *Xba*I restriction enzyme) and purified prior to *in vitro* transcription reaction.

3.14.1 *In vitro* transcription

3.14.1.1 Preparation of template DNA

For maximum dsRNA yields a template DNA (plasmid or PCR product) free of contaminating proteins and RNA was used. In addition, for the plasmid template DNA complete linearization downstream of the insert to be transcribed was highly required.

3.14.1.1.1 Plasmid template

The pLL10-GPL construct was singly digested with either *Xho*I or *Xba*I for the synthesis of sense and antisense strands respectively. In a 20µl reaction volume the following were added: 6µl of recombinant plasmid, 1X NEB buffer 4 (New England Biolabs, UK), 1µl of acetylated Bovine Serum Albumin [BSA] (New England Biolabs, UK), 1-3U of either *Xho*I or *Xba*I restriction enzymes (New England Biolabs, UK) and H_2O to top up the volume. The reaction mixture was incubated at 37°C for 2-3 hours and the linearized

plasmids confirmed by gel electrophoresis (see section 3.9) was gel purified as described in section 3.10.2.

3.14.1.1.2 PCR template

PCR products were generated by two separate PCR reactions with a single T7 promoter-containing PCR primer in each reaction for the synthesis of either the sense or antisense strands. PCR was performed as described in section 3.8 with the difference being only in the primers (with T7 at the 5' end) set and the annealing temperature that was raised to 55°C. The right size of the amplicon was confirmed by gel electrophoresis (see section 3.9) and the PCR products purified as described in section 3.10.1.

3.14.1.2 *In vitro* transcription reaction assembly

The Megascript® RNAi kit (Ambion, Applied Biosystems, Foster City, CA, USA) was used for the *in vitro* transcription reaction as described by the manufacturer. Briefly, the reaction was assembled at room temperature with the thawed ribonucleotide kept on ice while the reaction buffer was left at room temperature. In a 20µl reaction volume the following was added: nuclease free water to 20µl, 1-2µg of template (PCR product or linearized plasmid), 2µl of each NTP, 2µL 10X reaction buffer, and 2µl of enzyme mix. The reaction mixture was mixed gently by tapping, briefly centrifuged and then incubated overnight at 37°C. Since the sense and antisense strands were synthesized in separate tube, the entire content of one of the reaction (20µl each) was added up in the other tube containing the complementary strand (amounting to 40µl) and annealed

together by incubation in boiling water for 5 minutes. The beaker containing the hot water and the tubes with reaction mixture was covered with an aluminium foil and left on the bench to cool to room temperature with the ssRNA annealing to form dsRNA as the water cooled. An aliquot (of 1:100 dilution) was electrophoresed on 1% agarose gel (see section 3.9) to check the integrity and efficiency of the duplex formation.

3.14.1.3 Removal of DNA, ssRNA and purification of dsRNA

To remove the template DNA and any ssRNA (that did not anneal) the annealed dsRNA was treated with DNaseI and RNase in a 50µl reaction volume consisting of 20µl annealed dsRNA, 21µl of nuclease free water, 5µl 10X digestion buffer, 2µl of DNase and 2µl of RNase enzyme. The reaction mixture was incubated at 37°C for 1 hour and dsRNA purified using MEGAclear kit (Ambion, Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instruction. In brief, the DNA and ssRNA free dsRNA was purified by assembling the dsRNA binding mix consisting of 100µl dsRNA sample, 350µl binding solution concentrate and 250µl of 100 % ethanol in a clean 1.5 microcentifuge tube. The reaction mixture was mixed gently by pipetting and the entire sample mixture transferred onto a filter in the filter cartridge that was inserted in a collection tube. The binding mix was drawn through the filter by centrifugation at 13,000 rpm for 1 minute, the flow through was discarded and the filter washed twice using 500µl wash solution and centrifugation at 13,000 rpm for a minute followed by a dry spin for 1 minute at 13,000 rpm to remove any traces of the wash solution. The filter cartridge was then transferred into a clean collection tube and 50µl of pre-heated elution

solution applied at the centre of the filter. The tube was snap closed then centrifuged at 13,000 rpm for 1 minute to elute the dsRNA. For maximum dsRNA recovery, the elution step was repeated with another 50 µl of pre-heated elution solution and the eluate collected in the same tube.

3.14.2 Analysis of the dsRNA

To check the integrity of the purified dsRNA, an aliquot (1:100 dilution) of the pure dsRNA was electrophoresed on 1% agarose gel (see section 3.9). Also, to determine the concentration a 1:10 dilution of the pure dsRNA in nuclease free water was made and the dsRNA quantified by reading the absorbance at 260nm in a UV spectrophotometer (BioSpecmini Shimadzu, Japan). The concentration of the RNA in µg/ml was calculated using the formula (A_{260} X dilution factor X 40 = µg/ml RNA). The rest of the purified dsRNA was stored at -20°C for later use.

3.14.3 Concentration of dsRNA

The dsRNA prepared from 6-8 reactions as described above was pooled together and precipitated to reach the concentration (10-15µg) required to achieve gene knockdown (Lehane *et al.*, 2008) using ammonium acetate/ethanol precipitation method. In brief, to the purified dsRNA, 1:10 volume of 5M ammonium acetate (NH_4Ac) and 2.5 volumes of 100% ethanol was added, mixed well and incubated at -20°C for 1 hour. The reaction mixture was then centrifuged at 13,000 rpm for 15 minutes at room temperature and the supernatant carefully discarded not to lose the pellet. The pellet was washed with 70%

ice cold ethanol and the ethanol poured off after centrifugation at 13,000 rpm for 15 minutes. To remove the last traces of ethanol the tubes were briefly spun and any residue fluid aspirated with a fine tipped pipette followed by air drying for a few minutes. Finally, the pellet was resuspended gently in the right volume of water to give a final concentration of 10-15µg based on the initial concentration values of the purified dsRNA . In addition, an aliquot of the concentrated dsRNA was analysed as described in section 3.14.2 and the rest of the concentrated dsRNA stored at -20°C for later use in the microinjections experiment.

3.15 Microinjection of dsRNA

All injections were performed as described by Walshe *et al.*(2009a). This entailed three tsetse flies groups (~n=30) of same species, age and sex. In this case male *G. pallidipes* flies (12-32 hrs old) that had received a single bloodmeal were used, they were grouped into three; the test (treated with dsRNA), negative control (treated with water) and the uninjected groups. Prior to microinjection the flies had to be immobilized by chilling at +4°C. The microinjection needles used were made from borosilicate glass capillaries (2.00 mM outside diameter) pulled using a needle puller to an approximate external tip diameter of 45µm. Injections was carried out with great care under a dissecting microscope and, to avoid any damage to the organ in the ventral half of the thorax the needle was angled horizontally or slightly dorsally. The volume injected in the immobilized flies was 2µl of dsRNA or nuclease free water for the test and control groups respectively while, the third group was only chilled at +4°C. The injected flies

were put into cages covered with dump paper towel and, after 24 hrs recovery period they were fed then maintained at 24–27°C, 65-80% humidity and fed on rabbit blood offered after every 48 hrs. The mortality rates and the feeding success in all the groups were recorded throughout the experiment, with the dead, weak and not fully engorged flies being removed from the experiment. The fed flies were easily identified as those with fully engorged abdomen and the bloodmeal colour was red, unfed ones could have clear empty abdomen or had bloodmeal that had darkened indicating incomplete digestion from the previous feed.

3.16 Knockdown screening

Monitoring for knockdown was started on the 1st day post injection and RT-PCR was carried out using cDNA pools from 2-4 tsetse fly midguts per group. The groups were monitored for knockdown for up to 14 days (done daily or on alternating days) post injection and semi-quantitative RT-PCR used to determine GPL transcript levels with glyceraldehyde 3 phosphate (GAPDH) as the internal control. The total RNA (isolation procedure described in section 3.2-3.4) was treated with DNaseI (Fermentas, USA) and quantified using a spectrophotometer (BioSpecmini Shimadzu, Japan) prior to first strand cDNA synthesis as outlined in section 3.6. The RT-PCR was performed as described in section 3.8 but, using 25 cycles and GPL primer set amplifying a larger fragment (to avoid overlap with the injected dsRNA) than the designed dsRNA. Similar conditions were used to amplify GAPDH but with an annealing temperature of 55°C. The PCR products were separated on 1% ethidium bromide stained agarose gel for 1.5

hours at 80V and the gel visualized on a UV transilluminator using a Kodak GL-200 gel documentation system (Eastman Kodak, USA). The digitized gel images were stored and the band intensities quantified using ImageJ software (National Institute of Health: http://rsbweb.nih.gov/ij/).

3.17 Densitometric image analysis

Gel images saved as *TIFF* files was used to determine signal intensity from PCR data obtained during knockdown screening using ImageJ software (NIH). The images (in grayscale) opened with ImageJ software were transformed into 8-bit-type images and the rectangle tool used to draw the area for measurement around the PCR band, the same rectangle tool was moved to each band on the gel to obtain densitometric data from equal areas and the peak area under the curve for each DNA band was recorded. In all cases, the corresponding GAPDH band was used to normalize the gel loading.

3.18 Data analysis

Data entry and management was done using Microsoft Excel (Version 2007). To establish the relative values for GPL reduction, the intensities of the GPL mRNA bands of both the test and the control group were divided by that of GAPDH specific PCR product densities. To obtain the transcript change after dsRNA treatment, the test and control group relative levels were divided by the average value from the control group for normalization of the control group to 1 and the reduction obtained reported as percentage knockdown.

The GPL dsRNA treatment effect on the feeding success of tsetse fly was analysed by logistic regression model since the response of the treatment was categorical (fed or unfed); the analysis was done using SAS software version 9.2 (SAS Institute Inc, 2002-2008, Cary, NC, USA). The model was fitted on the linear effect of treatment group and day of monitoring and the level of significance tested using the standard Pearson chi-squared analysis. In addition, the survival of the flies in the treatment groups was analysed in relation to the uninjected control group.

CHAPTER FOUR

RESULTS

4.1 Molecular cloning of GPL gene

Total RNA isolated from a pool of 3 midguts of G. *pallidipes* was analysed on agarose gel electrophoresis to check the RNA integrity. The absence of smearing and the heavy weighted genomic DNA near the gel wells (Figure 6) indicated absence of genomic DNA contamination. In addition, the presence of 28S and 18S rRNA in a 2:1 ratio confirmed the RNA was intact. However, prior to cDNA synthesis the total RNA had to be DNAse I treated to ensure a DNA free RNA was used.

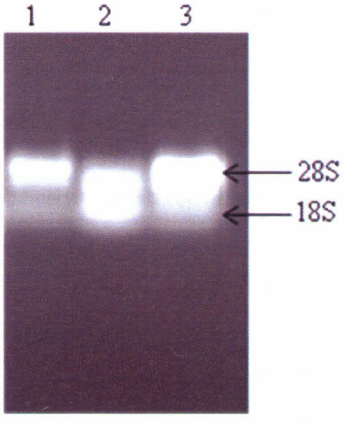


Figure 6. Agarose gel electrophoresis of isolated total RNA (lanes 1-3) that was heat denatured before loading on a 0.3% agarose gel stained with ethidium bromide. Lanes 1-3 represent total RNA from 3 midguts per sample.

Using oligo dT primed cDNA, GPL gene transcript was isolated from the midgut mRNA pool using GPL gene specific primers amplifying the coding region (~830bp) of the GPL gene. To ensure high sequence specificity, amplification was achieved using a high fidelity enzyme (Phusion polymerase) that has a proofreading ability as well as a low

error rate. The amplified GPL gene (Figure 7) was gel extracted and cloned into pGEM-T Easy vector.

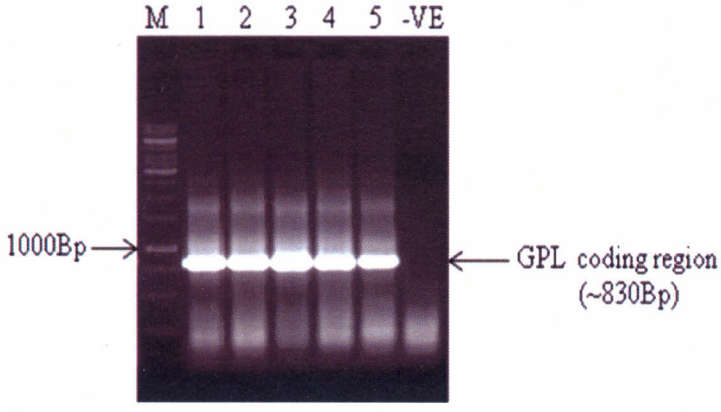
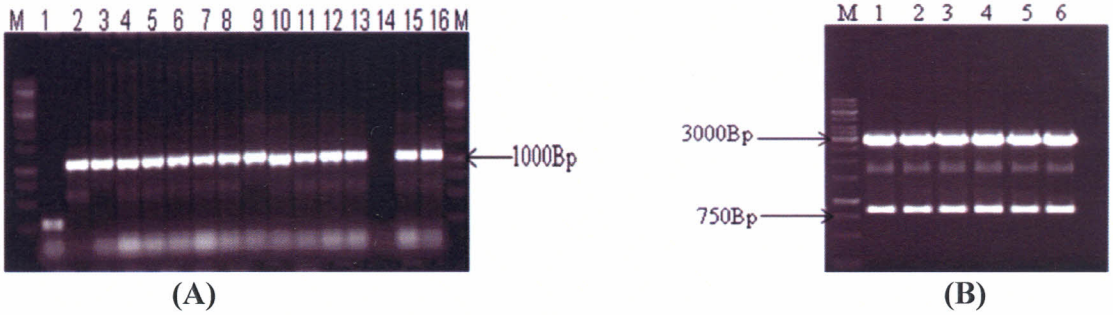


Figure 7. A 1 % agarose gel illustrating PCR product of the amplified GPL gene (~830bp) together with a 1 Kb (M) DNA ladder (Fermentas,USA). Lanes 1-5 are PCR replicates and the last lane (-ve) is a minus RT negative control.

Positive colonies identified by colony PCR (Figure 8A) were grown for plasmid minipreps and the presence of the insert further confirmed by restriction mapping (Figure 8B) using *Bam*HI and *Hind*III enzymes whose restriction sites were introduced via the primers. An aliquot (10µl) of the purified pGEM-T-GPL plasmids which showed positive colony PCR and restriction mapping were sequenced using vector specific M13 primers. GPL fragments designed for RNAi experiments were amplified using the sequenced pGEM-T-GPL clones as the templates. However, one of the GPL fragment for dsRNA preparation had to be T/A cloned then subcloned into pLL10 vector for *in-vitro* transcription, the 2nd GPL fragment did not require cloning since the primers used had been designed to introduce T7 promoter sequences at the 5' ends enhancing its transcription from a PCR product.



(A) (B)

Figure 8. (A) 1 % agarose gel electrophoresis showing colony PCR products of sixteen colonies screened with M13 vector primers. Lane 1 shows an empty plasmid, lane 14 is a no template negative control while the rest of the lanes are positive colonies. The plasmids purified from a few of these positive colonies were restriction digested (see panel **(B)**; lanes 1-6) to release the insert (the lower band ~830bp) and a pGEM-T backbone of 3000 bp. M is a 1Kb DNA ladder (Fermentas,USA).

4.1.1 Subcloning of GPL fragment into pLL10 vector

A 570bp GPL fragment was amplified (Figure 9) from a pGEM-T-GPL construct that contained the GPL coding region (~830bp) using primers with *Bam*HI and *Hind*III restriction sites to allow directional cloning into pLL10 vector.

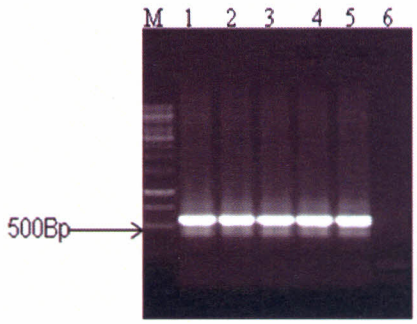


Figure 9. 1% ethidium bromide stained agarose gel electrophoresis illustrating amplified GPL fragment (~570bp). M: is 1 Kb DNA ladder (Fermentas, USA), lanes 1-5 are PCR replicates and lane 6 is a no template negative control.

The purified amplicon (~570bp) was first T/A cloned into pGEM-T Easy vector because the restriction site introduced by the primers lacked a 5' overhang upstream of the restriction site making it hard to generate sticky ends by digesting the PCR products directly. Cloning into pGEM-T improved restriction enzyme (*Bam*H1 and *Hind*III) efficiency and was done to prevent having the restriction sites too close to the terminal. The double digested GPL fragment was directionally cloned into pLL10 vector (Figure 5) for *in vitro* transcription. Positive colonies were identified via colony PCR (Figure 10B) using M13 vector specific primers and the purified plasmid restriction mapped using *Xba*I and *Xho*I (Figure 10C) to further confirm the presence of the insert.

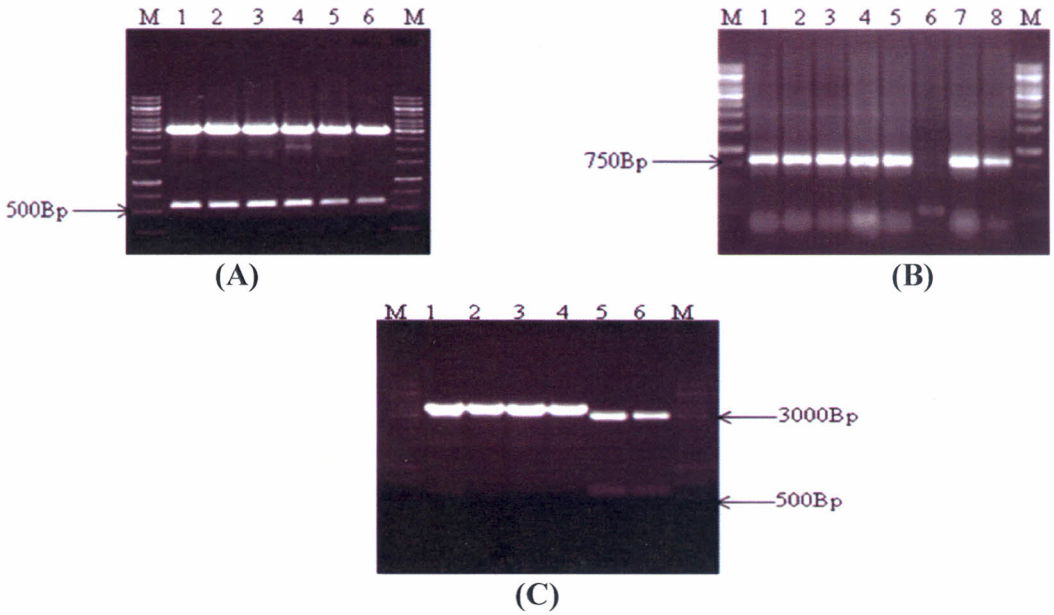


Figure 10. 1 % agarose gel electrophoresis showing: **(A)** lanes 1-6; GPL fragment (to be subcloned) digested out of the pGEM-T clone to release the fragment (~570bp) and the plasmid backbone (~3000 bp). **(B)** Colony PCR after subcloning into pLL10 vector, lane 6 indicates an empty plasmid while the rest of the lanes are positive colonies. **(C)** Restriction mapping of the purified pLL10 recombinant plasmid with single enzyme, lanes 1-2 for *Xba*I and lanes 3-4 for *Xho*I while lanes 5-6 are double digested plasmids. M: is 1 Kb DNA ladder (Fermentas, USA).

4.2 GPL sequence analysis

The coding region of GPL gene was successfully amplified from the midguts of *G. pallidipes* yielding a product of ~830bp (Figure 11). The DNA sequences results in form of chromatograms were viewed, edited and assembled into consensus sequences using BioEdit software (Hall, 1999). Sequence analysis using BlastN search revealed high similarity to other GPL genes in Gene Bank (http://www.ncbi.nlm.nih.gov/) among which there was an identity of 92% to *G. austeni* (Accession No.DQ060150.1) and 91% to *G. f. fuscipes* (Accession No AF525314.1) proteolytic lectin genes. Other BlastN hits were 96% to *Glossina morsitans morsitans* chymotrypsin-like serine protease precursor (Accession No. AF252868.1) and 93% to *Glossina morsitans morsitans* salivary gland trypsin (Accession No. EZ422113.1).

In silico analysis for motifs conserved within the translated protein (276 amino acids) using bioinformatics tools predicted that the deduced amino acid sequence contains a signal peptide region and the signature motifs for the serine protease trypsin family (Figure 12) including, the His/Asp/Ser active site triad with the conserved residues surrounding it, three pairs of cysteine residues for disulfide bridge formation as well as an aspartate residue at the specificity pocket. These features were all observed to be present when the sequenced *G. pallidipes* gene was aligned (Figure 12) with the previously reported proteolytic lectin genes from *G. austeni* (Amin *et al.*, 2006) and *G. f. fuscipes* (Abubakar *et al.*, 2006).

```
atgaagttctttgcagtgttcgctttatttgtggctagtgtgagtgcggcaaacttg 57
M  K  F  F  A  V  F  A  L  F  V  A  S  V  S  A  A  N  L
cgtgctattgccaaaccaggtttcccggaaggacgcattattaacggtcatgaggcc 114
R  A  I  A  K  P  G  F  P  E  G  R**I  I  N  G  H  E  A
gagaaaggtgaagctccttttattgtttctttaaaaaccactggccatttctgtggt 171
E  K  G  E  A  P  F  I  V  S  L  K  T  T  G  H  F  C  G
ggttctatcattgctgagaactgggttttgactgctggacactgcttgatcttcgat 228
G  S  I  I  A  E  N  W  V  L  T  A  G  H  C  L  I  F  D
gaattcgaaattgtagctggattacactcgcgtagtgatgagtctgacgttcaaatt 285
E  F  E  I  V  A  G  L  H  S  R  S  D  E  S  D  V  Q  I
cgcaaggttactggtaaacatcaacaaattgtccatgaaaaatatggcggtggcgtt 342
R  K  V  T  G  K  H  Q  Q  I  V  H  E  K  Y  G  G  G  V
ggtcccaacgacattggtctcatttacgtggacaaaccattcaatttgaatgcctta 392
G  P  N  D  I  G  L  I  Y  V  D  K  P  F  N  L  N  A  L
actcgtgacggaacagctgcagtggccaaggtcaatttgccaaccggcaaatatgag 456
T  R  D  G  T  A  A  V  A  K  V  N  L  P  T  G  K  Y  E
tctactggcgagggcaaattgtatggctggggagtagataactccggattcttacct 513
S  T  G  E  G  K  L  Y  G  W  G  V  D  N  S  G  F  L  P
aacgttctgaacactttggatgtaaacattattggatacgaagaatgcaaaaaggct 570
N  V  L  N  T  L  D  V  N  I  I  G  Y  E  E  C  K  K  A
ctgcccagcgatgctcctttagaccctgtcaatatctgttctcacaaagctgacgct 627
L  P  S  D  A  P  L  D  P  V  N  I  C  S  H  K  A  D  A
attgatggctcctgcagtggcgattccggtggtccaatggtgcgtatcactcctgac 684
I  D  G  S  C  S  G  D  S  G  G  P  M  V  R  I  T  P  D
ggtactgaactagttggcattgtatcttggggttacataccttgcgacagtgcaaca 741
G  T  E  L  V  G  I  V  S  W  G  Y  I  P  C  D  S  A  T
acgccatctgtttatacctggactgctgctttcgagaaatggattgaagagagtatc 798
T  P  S  V  Y  T  W  T  A  A  F  E  K  W  I  E  E  S  I
gagaactatgtcgtccctgcgcaacttttgtaa831
E  N  Y  V  V  P  A  Q  L  L  STOP
```

Figure 11. The nucleotide sequence of GPL isolated from *G. pallidipes* and its deduced amino acid sequences. The putative signal peptide is underlined and the cleavage site of the mature peptide are shown by (**). The amino acids highlighted in bold (black and blue) indicates the region targeted by the dsRNA, with the small dsRNA (413bp) shown in blue while the longer dsRNA (570bp) included this region plus the N-terminal shown in bold.

```
GPL-G.aus     MKFFAVFALCVASVSAANLDAIAKPGFPAGRIINGHEAEKGEAPFIVSLKAGKG 54
GPL-G.fusc    MKFFAVFALCVASVSAANLDAIAKPGFPAGRIINGHEADKGEAPFIVSLKAGKG 54
GPL-G.pal     MKSFAVFALFVASVSAANLRAIAKPGFPEGRIINGHEAEKGEAPFIVSLKT-TG 53
              ** ****** ********* ******** *********:***********: .*

GPL-G.aus     HFCGGSIIAENWVLTAGHCLIFDEFEIVAGLHSRNDESDVQIRKVTGKHQQIVH 108
GPL-G.fusc    HFCGGSIIAENWVLTAGHCLIFDEFEIVAGLHSRNDESDVQIRKVTGKHQQIVH 108
GPL-G.pal     HFCGGSIIAENWVLTAGHCLIFDEFEIVAGLHSRSDESDVQIRKVTGKHQQIVH 107
              **********************************.******************

GPL-G.aus     EKYGGGVGPNDIGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYG 162
GPL-G.fusc    EKYGGGVGPNDIGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGKGKLYG 162
GPL-G.pal     EKYGGGVGPNDIGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYG 161
              **********************************************:*****

GPL-G.aus     WGLDNSGFSPNILNTLDVNIIGYEECKNALNSDAPLDPVNICSYTAGAIDGACN 216
GPL-G.fusc    WGLDNSGFSPNILNTLDVDIIGYEECKNALNSDDPLDPVNICSYTAGAIDGACN 216
GPL-G.pal     WGVDNSGFLPNVLNTLDVNIIGYEECKKALPSDAPLDPVNICSHKADAIDGSCS 215
              **:***** **:******:********:** ** *********:.*.****:*.

GPL-G.aus     GDSGGPMVRITPDGTELVGIVSWGYQPCASTTMPSVYTWTSAFDKWIEDSIENY 270
GPL-G.fusc    GDSGGPMVRITPDGTELVGIVSWGYQPCASTTMPSVYTWTSAFDKWIEDSIKNY 270
GPL-G.pal     GDSGGPMVRITPDGTELVGIVSWGYIPCDSATTPSVYTWTAAFEKWIEESIENY 269
              ************************ ** *:* *******:**:****:**:**

GPL-G.aus     ---AQLL 274
GPL-G.fusc    ---AQLL 274
GPL-G.pal     VVPAQLL 276
              ...****
```

Figure 12. CLUSTALW 2.0.12 multiple sequence alignment of the translated proteolytic lectin genes from *G. austeni* (protein ID. AAY59001.1), *G. f. fuscipes* (Protein ID. AAM82602.1) and the one sequenced (in this study) from *G. pallidipes*. The six conserved cysteine residues are in green, the red residues are the His/Asp/Ser catalytic triad and the conserved surrounding region in grey. A putative signal peptide was predicted using SignalP (shown as underlined bold section).

Further, using the translated GPL sequence the non-redundant protein database as well as the *Glossina morsitans* geneDB (http://www.genedb.org/genedb/glossina/index.jsp) was searched using BlastP. The sequences showing high identity to the predicted *G. pallidipes* GPL protein were selected from Gene Bank (http://www.ncbi.nlm.nih.gov/) then multiple aligned using ClustalW. Phylogenetic analysis conducted in Mega 4 software (Tamura *et al.*, 2007) using the neighbour-joining method (Saitou and Nei, 1987) and 10,000 replications generated a dendogram (Figure 13) supported by high

boostrap values (≥80%) and the sequenced *G. pallidipes* proteolytic lectin was observed to clade together with other midgut serine proteases belonging to the *Glossina* genus.

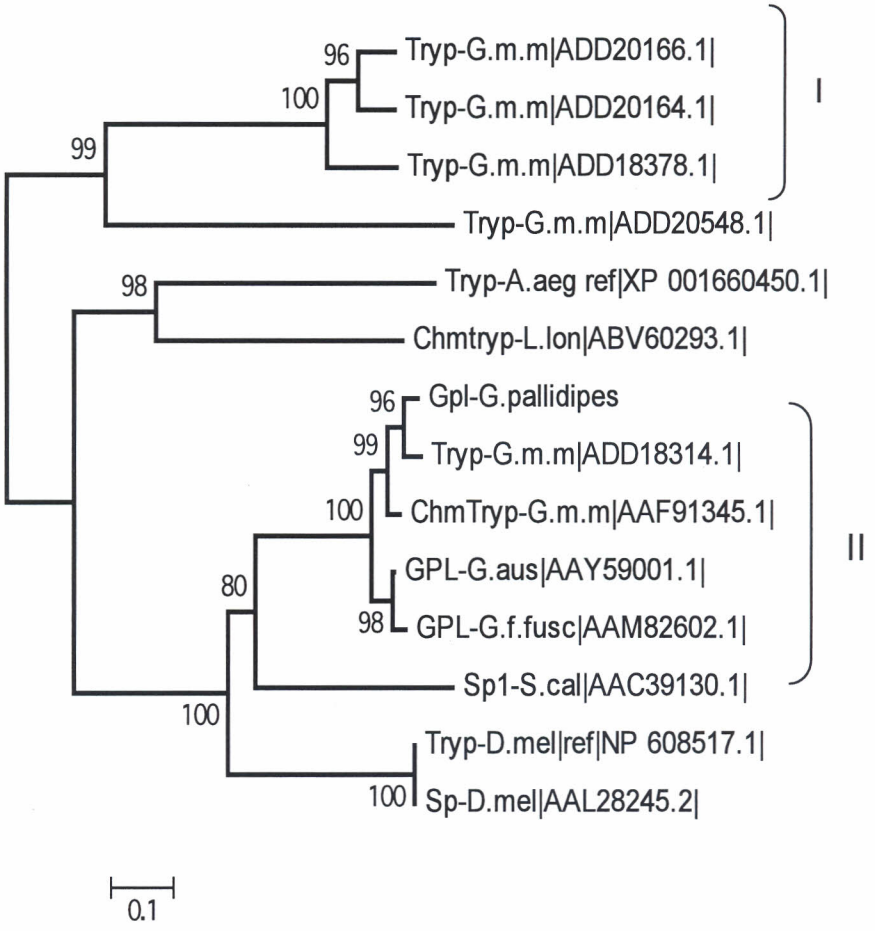


Figure 13. A dedongram illustrating phylogenetic relationship of serine protease from several *Glossina* species (*G. austeni* as G.aus; *G. morsitans morsitans* as G.m.m & *G. fuscipes fuscipes* as G.f.fusc) and other insects (*Drosophilla melanogaster* as D.mel; *Stomoxy calcitran* as S.cal; *Lutzomyia longipalpis* as L.lon & *Aedes aegypti* as A.ae). Indicated at the branches are the protein accession numbers; the brackets highlights the two *Glossina* EST libraries (I-salivary tissue & II-midgut) searched with BlastP. The bar 0.1 indicates amino acid substitution per site and the numbers at the branches are bootstrap support values. Abbreviations: Tryp-trypsin; Sp-serineprotease; ChmTryp-chymotrypsin; GPL-*Glossina* proteolytic lectin.

4.3 dsRNA preparation.

Purified dsRNA template which was either a PCR product (Figure 14A) or a linearized plasmid construct (Figure 15A) was used to synthesis a 413bp (Figure 14B) and 570bp (Figure 15B) GPL dsRNA respectively.

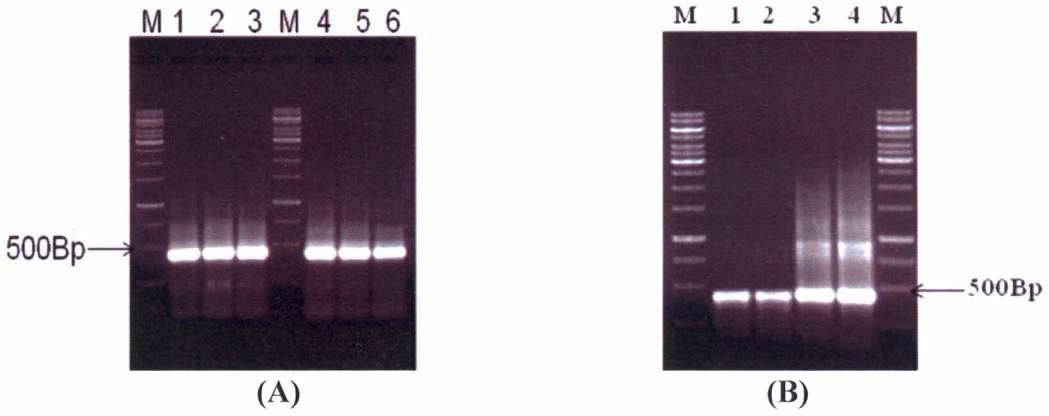
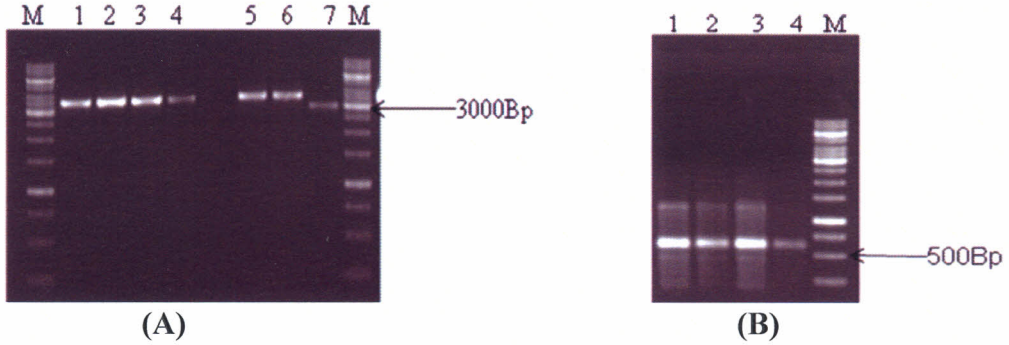


Figure 14. 1% agarose gel electrophoresis showing: **(A)** PCR amplicons with T7 promoter region at the 5' ends for both sense (lanes 1-3; ~430bp) and antisense (lanes 4-6; ~430bp) strand of the dsRNA; **(B)** Purified 1:100 diluted dsRNA (Lanes 1&2; ~430bp) and the unpurified ones (Lanes 3&4; ~430bp). M is 1Kb ladder Fermentas, USA.

Both templates (Figure 14A; and Figure 15A) were sequenced and confirmed to have the 23bp T7 promoter region flanking the GPL fragment on 5' ends. Upon transcription, the *in vitro* synthesised sense and antisense RNA strands were annealed and gave the expected sizes of the GPL dsRNA as shown on Figure 14B; and Figure 15B .



(A) (B)

Figure 15. 1 % agarose gel electrophoresis showing: **(A)** Purified *Xba*I (lanes 1-3), *Xho*I (Lanes 4-6) linearized pLL10 recombinant plasmid separated against an empty linearized pLL10 plasmid (lane 7) indicating the difference in mobility shift due to the GPL (570 bp) insert. **(B)** The purified 1:100 diluted dsRNA (lanes 1-4 ~570bp); the second band twice the size of the expected dsRNA is as result of secondary structure formation after annealing to form the dsRNA. M: is 1 Kb DNA ladder (Fermentas, USA).

To reach the range of dsRNA concentration (4-15µg in nuclease free water) that have been established to induce knockdown of different *Glossina* genes, several reactions for dsRNA synthesis were setup and after purification, a portion of the final elute was used to calculate the RNA yield and agarose gel electrophoresis used to validate the dsRNA size and integrity (Figure 14B & Figure 15B). The remainder was ethanol precipitated using sodium acetate and the recovered dsRNA resuspended in nuclease free water to a final concentration range of 4-7µg/µl.

4.4 Microinjection and knockdown analysis

4.4.1 Optimization of GPL gene knockdown.

Injection with 8µg of a 413bp dsRNA GPL fragment that was designed *in silico* using a Web based tool (Arziman *et al.*, 2005) resulted to indication of no knockdown of the

midgut *G. pallidipes* GPL when monitored using semi-quantitative RT-PCR (Figure 16). The amplified GPL cDNA obtained from the dsRNA treated and the nuclease free water injected control groups were normalized using GAPDH in a multiplex RT-PCR and when analysed using gel electrophoresis (Figure 16) showed no difference in transcript levels when the two groups were compared.

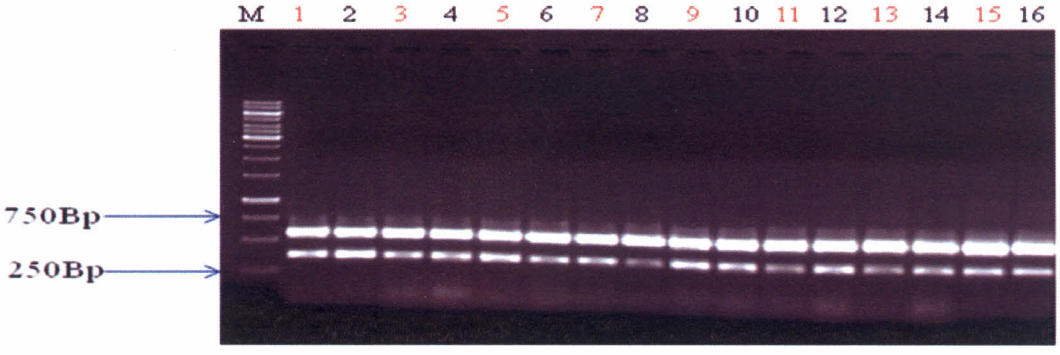
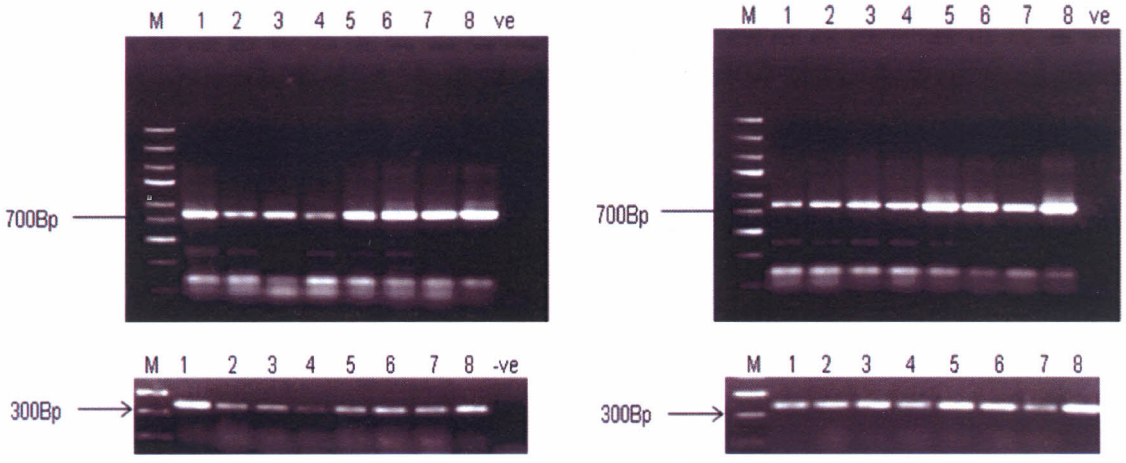


Figure 16. 1% agarose gel electrophoresis illustrating GPL levels obtained after microinjection with 8µg of 413bp dsRNA. Monitoring was done after 2 days for a period of 14 days; test and control samples from the same days were loaded adjacent. The lanes with (red) odd numbers are test group samples; the (black) even numbers indicates the control group samples. The upper band (~570bp) is the GPL fragment while lower band (~370bp) is the corresponding GAPDH from the same sample that was used as an internal control. M: is 1 Kb DNA ladder (Fermentas, USA).

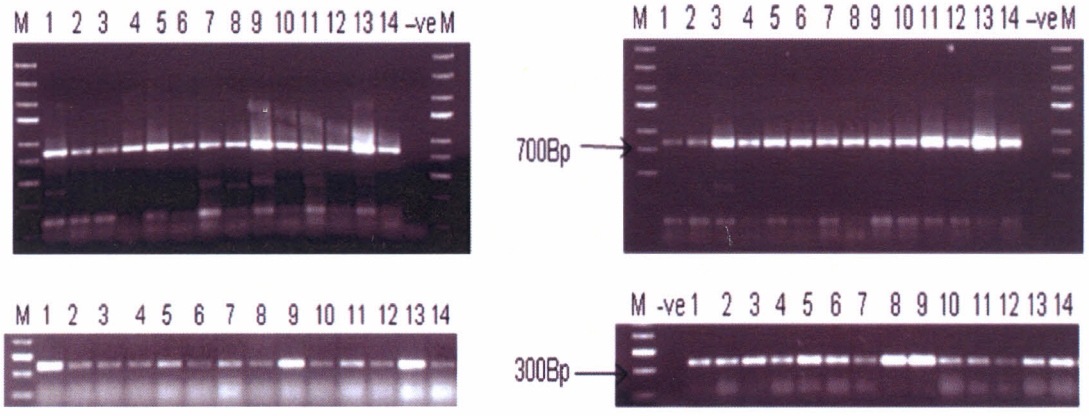
Increase in the concentration of the dsRNA to ~12µg was attempted using the same 413bp GPL fragment; this resulted to a slight decline in transcript only for day 2 post injection (Figure 17) indicating that the GPL knockdown occurs after around 48 hours post injections. However, the observed reduced GPL levels didn't have a significant effect ($P=0.7735$) on the feeding success of the tsetse flies in the treatment group when compared to the control that was mock injected with nuclease free water. Also, no mortality was recorded in this case.



a) Control group **b)** Test group

Figure 17. 1% agarose gel electrophoresis showing semi-quantitative RT-PCR analysis following microinjection with 12µg of a 413bp GPL dsRNA. The control group is labeled **a)** and test group is shown as **(b).** The amplified GPL fragment (~830bp) is on the upper gel while the corresponding GAPDH internal control (~370bp) is shown on the lower gel. Lanes 1-8 indicates the samples from knockdown screening after 2 days for a period of 14 days. M: is O'GeneRuler^{TM} Express DNA ladder (Fermentas, USA); -ve is minus RT enzyme negative control.

When there was no detectable knockdown using the 413bp GPL fragment, another dsRNA construct (~570bp) that included the N-terminal region of the GPL protein was designed. As with the previous, ~12µg of a 570bp GPL dsRNA was injected and monitoring was followed daily instead of alternating days. The results (Figure 18) were consistence to those observed previously where decline in transcript was observed as from day two post injection after which the levels started to increase.



a) Control group **b)** Test group

Figure 18. 1% agarose gel electrophoresis showing knockdown verification using RT-PCR. **a)** Is the nuclease free water injected control group; **b)** is the test group injected with ~12µg of a 570bp GPL dsRNA. The upper gels are screens done using GPL specific (~830 bp product) primers, the lower gels are the corresponding GAPDH (~370bp product) as the internal controls for both the test (A) and the control groups (B). Lanes 1-14 indicates the samples from knockdown screening; done daily for a period of 14 days. M: is a O' GeneRuler^{TM} Express DNA ladder (Fermentas, USA), -ve is a minus reverse transcriptase negative control.

Decline of GPL transcripts levels observed on day two and three post dsRNA treatment reached an average of 60% (**Table 4**) before it begun to go up as from day three. This indicated recovery from knockdown by the presence of a band of increasing intensity (Figure 18) for the rest of the fourteen days monitored.

Table 4. The observed change in *Glossina* proteolytic lectin (GPL) gene on day 2 and 3 post dsRNA treatment. The values are normalized transcript levels obtained after semiquantitative RT-PCR and densitometric quantification of DNA bands intensities using Imagej software.

	GPL transcript levels day 2 & 3 post Treatment		
Groups	**day2**	**day3**	**change**
Control	0.66	1.34	1.00
Test	0.28	0.52	0.40
			0.60

Analysis of the total reduction in GPL transcript levels observed for fourteen days was only 20% indicating how the achieved GPL knockdown was short lived. Though this hindered some functional analysis, the trypsin nature of the GPL gene as identified during in-silico sequence analysis was assayed to determine if the observed GPL knockdown had any significant influence on the feeding success and survival of tsetse fly *G. pallidipes*.

4.5 Functional analysis of GPL

4.5.1 Feeding success

The feeding success and mortality was monitored from day one post treatment in all the three groups. Successful feeding (see Table 5) in the dsRNA treated group was low on day one post treatment. Out of 31 flies injected with dsRNA, 7 flies failed to feed despite prolonged periods of feeding. This was not the case in the water injected control group with only 2 flies failing to feed which was normal case when compared to the uninjected group. In all the other days monitored the number of flies unable to feed remained high in the dsRNA treated group when compared to the controls despite the fact that two flies had to be randomly sampled from the groups for verification of knockdown.

Table 5. The feeding success and mortality rates of *G. pallidipes* observed in the dsRNA, nuclease free water injected and un-injected groups. Two flies were randomly sampled per day from the dsRNA treated and the mock injected group.

Treatment	Days post treatment	Fed	Failed to feed	Sampled	Dead
dsRNA injected N=31	1	24	7		0
	2			2	1
	3	25	3	2	0
	4			2	0
	5	20	3	2	1
	6			2	0
	7	18	0	2	1
	8			2	0
	9	13	1	2	0
	10			2	0
	11	9	1	2	0
	12			2	0
	13	6	0	2	0
	14			2	0
	15	2	0	2	0
Total					3
Treatment	**Days post treatment**	**Fed**	**Failed to feed**	**Sampled**	**Dead**
Nuclease free water injected control N=32	1	30	2		0
	2			2	0
	3	28	2	2	0
	4			2	1
	5	23	2	2	0
	6			2	0
	7	21	0	2	0
	8			2	0
	9	16	1	2	0
	10			2	0
	11	12	1	2	0
	12			2	0
	13	9	0	2	0
	14			2	0
	15	5	0	2	0
Total					1

Treatment	Days post treatment	Fed	Failed to feed	Dead
Un-injected control N=30	1	28	2	0
	2			0
	3	29	1	0
	4			0
	5	30	0	0
	6			0
	7	30	0	0
	8			0
	9	30	0	0
	10			0
	11	30	0	0
	12			0
	13	30	0	0
	14			0
	15	30	0	0
Total				0

When the logistic regression model was fitted to the proportions of the flies that failed to feed and the effect of treatment and the day of monitoring analysed (results on Table 6), the parameter estimate for the treatment had an odds ratio (OR) of 2.20 [95% CI: 0.9, 5.4] indicating no significant difference (p=0.0855). However, the effect of the day of monitoring was found to be significant (p=0.0381). The significant day effect indicated that given the dsRNA treated group, the risk of not feeding decreased significantly as the days went, OR=0.865 [95% CI: 0.75, 0.99].

Table 6. Maximum Likelihood estimates and standard errors from the logistic regression model.

Parameter	Estimate	Standard error	*p*-value
Intercept	-2.18	0.46	<.0001
Treatment	0.79	0.46	0.0855
Day	-0.15	0.07	0.0381

4.5.2 Survival rates

The recovery of the microinjected tsetse flies 1-24 hours post treatment was 100% in dsRNA treated, the mock injected (with water) control and the uninjected (only chilled at +4°C) group indicating that the tsetse fly could survive despite the cold treatment and the physical damage during the microinjection procedure. Mortality (see Table 7) was observed, in the dsRNA treated group on days 2, 5 and 7 with a total of 3 flies' dead and, in mock injected group on day 4 with only 1 fly dead. As expected there was no mortality observed in the uninjected control group. Thus, based on when mortality was observed in the mock injected control group, death arising from the physical damage implicated by microinjection could occur within the first 4 days post injection.

By examining the colour of the bloodmeal most of the fly's digestion ability in the three groups was normal, it was observed that flies (dsRNA treated) that died on day 2 and 5 had their bloodmeal dark in colour indicating successful digestion ability as it was the case to the one that died in the control group. In contrast, the fly that died on day 7 in the

dsRNA treated group, retained the characteristic bright red colour indicative of undigested blood despite an interval of more than 24 hours from the last feed.

Table 7. The number of flies dead and the days when it was observed in the three groups post treatment.

	Mortality	
Groups	**Total dead**	**Day observed**
Test- dsRNA injected	3	2, 5 & 7
Control - water injected	1	4
Un-injected control	0	

CHAPTER FIVE

5.1 Discussion

Trypsin and chymotrypsin serine proteases are the main digestive proteases in *Diptera* midguts and are also involved in many aspects of the vector-parasite relationship. In tsetse flies, trypsin has been shown to be potential barrier to trypanosome growth and development within the midgut. So far, several tsetse fly proteolytic enzymes have been characterized, including trypsin, carboxypeptidase A, carboxypeptidase B, amino peptidase, a trypsin-like enzyme and chymotrypsin-like enzymes (Yan *et al.*, 2001).

In this study a proteolytic gene from *G. pallidipes* was successfully isolated by RT-PCR and analysis of the amplified cDNA sequence (~830bp) showed extensive similarity to *Glossina* proteolytic lectin gene previously isolated from *G. austeni* and *G. fuscipes fuscipes* as well as *G. m. morsitans* trypsin and chymotrypsin genes. According to *in silico* analysis the sequence has a potential trypsin specific activation site indicating it is expressed as a prepro peptide, a histidine (position 67-72; LTAGHC) and serine (position 212-223; GSCSGDSGGPMV) active sites classifying it as a serine protease that belongs to the trypsin family. Signal peptide prediction showed the gene product had a 16 amino acid hydrophobic leader peptide at the N-terminal indicating it is secretory in nature. Based on this putative activation site as the start of mature protein, the gene isolated codes for a mature protein of 245 amino acid residues; the predicted pi is 4.99 and a molecular weight of 29.47 KDa. However, its open reading frame (276aa) was two amino acids (located at the C-terminal end) longer than the one predicted

(274aa) from the *G. austeni* and *G. f .fuscipes,* those additional residues were observed in all the independent clones sequenced in the study. This could be related to species variation and, the implication of such a change to the functionality of the protein is unknown.

Comparative protein sequence analysis with the *G. austeni* and *G. fuscipes fuscipes* proteolytic lectin genes showed over 90% sequence identity. The alignment of the three amino acid sequences indicated that they have structural requirements of serine proteases; all harbor the His, Asp and Ser catalytic triad and the six highly conserved cysteine residues that form three cysteine bonds characteristic of invertebrate serine proteases. In addition to the conserved protease motives, the sequences when compared to other serine protease of *Diptera* origin exhibited close phylogenetic relatedness to serine proteases from haematophagous insects, e.g. stomoxy, mosquito, and sand fly. Other *Glossina* proteins, a midgut chymotrypsin, the two midgut proteolytic lectin protein and one salivary gland trypsin did clade together indicating their close relatedness. Prior study (Yan *et al.*, 2001) on *Glossina* midgut proteases reported that trypsin and chymotrypsin transcripts can be found in other tsetse fly tissues, e.g. the fat bodies and salivary gland. The gene products of such transcripts whose activity was not detected in those tissues might be translocated to the midgut where there is high requirement of trypsin and chymotrypsin activity for bloodmeal digestion.

While the digestive functions of the gut tissue are central to the insects' viability, it is a complex biological process that is diverse among the insect species. In the midgut of tsetse fly several serine protease genes have been identified, some of them are known to reach their peak levels during the bloodmeal digestion cycle and are considered as major barriers to trypanosome establishment (Burugu *et al.*, 2007; Yan *et al.*, 2001). Most tsetse-trypanosome interaction studies (*in-vivo*) targeting such genes are based on inhibitory molecules e.g soya bean trypsin inhibitors and D-glucosamine. The trypsin inhibitor is non gene specific and, the sugar have multiple effects such as the bloodmeal size increase, slowed movement of bloodmeal in the gut and causes fly mortality (Peacock *et al.*, 2006). Therefore, to block the midgut proteolytic gene isolated in this study, a dsRNA was used in a reverse genetic based approach to determine the influence of the gene on tsetse fly feeding success and survival. The right dsRNA concentration for this work had to be determined empirically since knockdown success varies with gene under study based on its initial level of abundance, turnover rate and its location.

In *Glossina* the optimal length of the dsRNA required to establish sufficient knockdown is not known but, in most cases it is gene specific (Haines *et al.*, 2010). Functional analyses of a tsetseEP gene with as low as 4µg of a 39bp long dsRNA have been reported to induce prolonged gene knockdown for up to two weeks (Haines *et al.*, 2010). However, knockdown of GPL could not be achieved (Figure 16) with 8µg of a 413bp dsRNA and, only slight decline in transcripts level was observed with 12µg of the same dsRNA. Even then, it was followed by a band of increasing intensity when analysed

indicating the quick recovery of the GPL transcripts. Although tsetseEP and GPL are from the midgut, the optimal amount and size of the dsRNA required to induce RNAi vary considerably. The differences in RNAi efficiency could be due to different gene expression profiles as well as mRNA turnover rates of the two genes. The knockdown observed was greatly improved when the length of the dsRNA was increased to 570bp and included the N-terminal of the gene. The highest knockdown observed lasted only two days with about 60% reduction in transcript levels that was followed by transcript recovery for the 14 days monitored. However, the average 14 days transcript reduction attained was only 20% demonstrating the short-lived nature of the knockdown. The target gene being a bloodmeal induced gene, occasional feeding after every 48hrs could have induced gene expression that overwhelmed the effect caused by the introduced dsRNA resulting to a very short-lived GPL knockdown when dsRNA treated flies were compared to the water injected control group.

The percentage knockdown levels required to cause a phenotypic change varies with genes and species. Not all genes show significant differences at transcriptional level and in some cases, e.g., in *A. gambiae*, 20% transcriptional reduction of a gene (cactus) has been reported to be sufficient to trigger a prominent phenotype (Frolet *et al.*, 2006). Although different response systems exist between mosquito and tsetse fly, the achieved GPL knockdown was used to assay for phenotypic change related to tsetse feeding success and survival. However, the infection assay was not possible since a more prolonged knockdown is required to study the effect of a gene on trypanosome midgut

establishment (Haines *et al.*, 2010), an experiment that requires about 7-10 days to be sure of the infection status in the midgut of tsetse fly.

To investigate the importance of the proteolytic nature of GPL and its role during bloodmeal digestion, flies that exhibited knockdown were also monitored for their feeding success with time. It was anticipated that for the days when GPL transcripts was low, the number of flies unable to feed in the dsRNA treated group would be high than in the control group. According to the parameter estimates obtained from a logistic regression analysis, the effect of the day of monitoring was found to be significant ($p=0.0381$), demonstrating that the inability of the flies to feed declined with time. This effect could be associated to the period when GPL knockdown was observed. However, when the model was fitted to the effect of the treatment while controlling for the day of monitoring, the result was not significant ($p=0.0855$). Consequently, RNAi of GPL has no major influence in tsetse fly feeding success. Many midgut proteolytic enzymes are secreted upon acquisition of a bloodmeal, reaching their peak levels at about 48-72 hrs post feeding. This could have provided a feedback system that complemented the reduced levels of GPL as supported by the phylogenetic analysis of multiple serine protease genes identified in *Glossina*. In addition, the knockdown achieved was not 100% and the residual transcript levels observed after semi quantitative RT-PCR could have been sufficient to allow normal functioning of the gene.

Subjecting tsetse fly to microinjection causes wounding to the fly and it can compromise their survival. This makes it difficult to determine the mortality related to gene silencing and the one arising from the physical damage. In this study, 100% survival was observed

24hrs post injection. The initial two experiments that resulted to no or slight decline in GPL transcript levels had no mortality and 100% survival was recorded in all the three groups. However, in the third trial where the highest transcript reduction was observed for two days, three flies died (one per day on days 2, 5 and 7) in the dsRNA treated group, while only one died on day 4 in the nuclease free water injected control. All flies survived in the uninjected control group. A similar study in *Glossina morsitans morsitans* (Walshe *et al.*, 2009a) reported high mortality rates within the first five days that was predicted to arise from the physical damage of microinjection. The improved RNAi efficiency could have resulted to the observed mortality past the fifth day although the number was quite low. Apart from the physical damage, high dsRNA concentration may give a high concentration pressure that causes a different physiological condition from the normal uninjected insect. Indeed exceeding concentration have been reported not to result to more silencing but rather cause side effect such as high mortality (Shakesby *et al.*, 2009).

By checking the bloodmeal colour in the gut after every 24hrs post feeding where the bright red colour represented undigested and darkened blood was recorded as incomplete digestion, comparison between the three groups showed normal blood digestive ability like other flies in the colony where darkening occurs upon 24hrs after the bloodmeal. Only one unique case in the dsRNA treated group was observed (day7) where the fly died with the characteristic bright red colour indicative of undigested blood. Such similar observation has been reported in *G. m. morsitans* fed on a bloodmeal

supplemented with high concentration (100 & 200 mM) of D-glucosamine to inhibit midgut trypsin activity (Osir *et al.*, 1993) and, mortality occurred within 24-48 hrs post feeding. Although this characteristic was observed on day seven rather than on day 2 and 3 or days close to this period when the highest GPL knockdown was achieved, this could have indicated different silencing efficiency existed among individual flies and the flies that survived was as a result of incomplete gene knockdown and also, quick recovery from the achieved knockdown.

5.2 Conclusion

A proteolytic gene from the midgut of *G. pallidipes* was isolated and classified as a serine protease belonging to the trypsin family and, with > 90% identity to the previously reported *Glossina* proteolytic lectin gene (GPL) from *G. austeni* and *G. f. fuscipes*.The study reported here did not include a study of trypanolytic activity, as was done with the other tsetse GPL genes.

A reverse genetic approach to study the isolated gene function was limited by the short lived nature of the achieved knockdown, different GPL dsRNA concentrations and constructs had varied RNAi efficiency. The highest transcript reduction (60%) lasted only for two days and was followed by a transcript recovery period (14 days monitored) with the overall transcript reduction of 20% reported in the dsRNA treated group. The feeding success in this group was not significantly impaired indicating that either a feedback system existed to counter the reduced GPL levels or the residual mRNA was sufficient for normal gene function since the knockdown was not 100%. However, the feeding ability improved with time and resumed to normal by the fourth feed.

Majority of the flies survived the cold chilling and microinjection with low mortality being recorded. This was observed within the early days after microinjection and it was hard to differentiate the ones related to physical injury or gene knockdown. Only one phenotypic case was observed from the dsRNA treated group where the fly died (on day 7) with undigested blood characteristic of trypsin activity inhibition. Therefore,

disruption of GPL doesn't significantly affect the ability of tsetse fly to feed successfully and thus its survival. Also, its resistance to RNAi suggests it is a crictical gene in tsetse fly physiology.

Recommendations

- The approach employed relied only on transcript reduction levels for functional analysis, it is also important to check if similar reduction in protein levels is achieved through western blot analysis and enzyme activity assays.

- Since GPL knockdown improved with increasing size of dsRNA, there is a need to attempt silencing using a longer or full length GPL dsRNA (~830bp).

- To overcome the challenge of mortality due to microinjection, tsetse flies can be fed on bloodmeal containing dsRNA which is a less invasive approach.

- Further studies of the isolated proteolytic gene are needed to establish other key roles of the gene product by molecular and biochemical characterization to assess its trypanolytic activity.

REFERENCES

Abubakar, L., Osir, E. O. and Imbuga, M. O. 1995. Properties of a blood-meal-induced midgut lectin from the tsetse-fly *Glossina morsitans*. *Parasitology Research,* **81:** 271-275.

Abubakar, L. U., Bulimo, W. D., Mulaa, F. J. and Osir, E. O. 2006. Molecular characterization of a tsetse fly midgut proteolytic lectin that mediates differentiation of African trypanosomes. *Insect Biochemistry and Molecular Biology,* **36:** 344-352.

Abubakar, L. U., Zimba, G., Wells, C., Mulaa, F. and Osir, E. O. 2003. Evidence for the involvement of a tsetse midgut lectin-trypsin complex in differentiation of bloodstream-form trypanosomes. *Insect Science and its Application,* **23:** 197-205.

Aksoy, S. 2003. Control of tsetse flies and trypanosomes using molecular genetics. *Veterinary Parasitology,* **115:** 125-145.

Aksoy, S. 2011. Sleeping sickness elimination in sight: time to celebrate and reflect, but not relax. Art. No. E1008. *Plos Neglected Tropical Diseases,* **5:** E1008-E1008.

Aksoy, S., Gibson, W. C. and Lehane, M. J. 2003. Interactions between tsetse and trypanosomes with implications for the control of *trypanosomiasis*. *Advances in Parasitology,* **53:** 1-83.

Aksoy, S., Weiss, B. and Attardo, G. 2008. Paratransgenesis applied for control of tsetse transmitted sleeping sickness. *Transgenesis and the Management of Vector-Borne Disease,* **627:** 35-48.

Allsopp, R. 2001. Options for vector control against *trypanosomiasis* in Africa. *Trends in Parasitology,* **17:** 15-19.

Amin, D. N., Kamita, S. G., Muluvi, G. M., Machuka, J., Hammock, B. D. and Osir, E. O. 2006. *Glossina* proteolytic lectin does not require a carbohydrate moiety for enzymatic or trypanosome-transforming activities. *Journal of Medical Entomology,* **43:** 301-308.

Arziman, Z., Horn, T. and Boutros, M. 2005. E-RNAi: a web application to design optimized RNAi constructs. *Nucleic Acids Research,* **33:** W582-8.

Atouguia, J. and Costa, J. 1999. Therapy of human african *trypanosomiasis*: current situation. *Memorias Do Instituto Oswaldo Cruz,* **94:** 221-224.

Attardo, G. M., Lohs, C., Heddi, A., Alam, U. H., Yildirim, S. and Aksoy, S. 2008. Analysis of milk gland structure and function in *Glossina morsitans*: Milk protein production, symbiont populations and fecundity. *Journal of Insect Physiology,* **54:** 1236-1242.

Baral, T. N. 2010. Immunobiology of African trypanosomes: need of alternative interventions. *Journal of Biomedicine and Biotechnology,* **2010,** 389153.

Barrett, M. P. 2006. The rise and fall of sleeping sickness. *Lancet,* **367:** 1377-1378.

Barrett, M. P. 2010. Potential new drugs for human african *trypanosomiasis*: Some progress at last. *Current Opinion in Infectious Diseases,* **23:** 603-8.

Barry, J. D., Graham, S. V., Fotheringham, M., Graham, V. S., Kobryn, K. and Wymer, B. 1998. VSG gene control and infectivity strategy of metacyclic stage *Trypanosoma brucei. Molecular and Biochemical Parasitology,* **91:** 93-105.

Bauer, B., Kabore, I., Liebisch, A., Meyer, F. and Petrichbauer, J. 1992. Simultaneous control of ticks and tsetse-flies in Satiri, Burkina-faso, by the use of flumethrin pour on for cattle. *Tropical Medicine and Parasitology,* **43:** 41-46.

Baylis, M. and Stevenson, P. 1998. *Trypanosomiasis* and tsetse control with insecticidal pour-ons - fact and fiction? *Parasitology Today,* **14:** 77-82.

Beard, C. B., Oneill, S. L., Tesh, R. B., Richards, F. F. and Aksoy, S. 1993. Modification of arthropod vector competence via symbiotic bacteria. *Parasitology Today,* **9:** 179-183.

Blandin, S., Moita, L. F., Kocher, T., Wilm, M., Kafatos, F. C. and Levashina, E. A. 2002. Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the defensin gene. *Embo Reports,* **3:** 852-856.

Bruce, D., Hamerton, A. E., Bateman, H. R. and Mackie, F. P. 1909. The development of *Trypanosoma gambiense* in *Glossina palpalis*. *Proceedings of the Royal Society of London. Series B,* containing papers of a biological character, **81:** 405-414.

Burugu, M. W., Mbatia, B. N., Osir, E. O., Kenya, E. U. and Abubakar, L. U. 2007. The *Glossina* proteolytic lectin (GPL) gene is expressed only in members of *Glossina* species. *International Journal of Tropical Insect Science,* **27:** 145-151.

Cheng, Q. and Aksoy, S. 1999. Tissue tropism, transmission and expression of foreign genes *in vivo* in midgut symbionts of tsetse flies. *Insect Molecular Biology,* **8:** 125-132.

Connor, R. J. 1994. The impact of nagana. *Onderstepoort Journal of Veterinary Research,* **61:** 379-383.

Courtin, D., Berthier, D., Thevenon, S., Dayo, G. K., Garcia, A. and Bucheton, B. 2008. Host genetics in african *trypanosomiasis*. *Infection Genetics and Evolution,* **8:** 229-238.

Croft, S. L., East, J. S. and Molyneux, D. H. 1982. Anti-trypanosomal factor in the haemolymph of Glossina. *Acta Tropica,* **39:** 293-302.

Desquesnes, M. and Dia, M. L. 2003. Mechanical transmission of *Trypanosoma congolense* in cattle by the African tabanid *Atylotus agrestis*. *Experimental Parasitology,* **105:** 226-231.

Donelson, J. E. 2003. Antigenic variation and the African trypanosome genome. *Acta Tropica,* **85:** 391-404.

Ellis, D. S. and Evans, D. A. 1977. Passage of *Trypanosoma brucei-rhodesiense* through peritrophic membrane of *Glossina morsitans morsitans*. *Nature,* **267:** 834-835.

Etchegorry, M. G., Helenport, J. P., Pecoul, B., Jannin, J. and Legros, D. 2001. Availability and affordability of treatment for human African *trypanosomiasis*. *Tropical Medicine and International Health,* **6:** 957-959.

Fevre, E. M., Picozzi, K., Jannin, J., Welburn, S. C. and Maudlin, I. 2006. Human African *trypanosomiasis*: epidemiology and control. *Advances in Parasitology, Vol 61,* **61:** 167.

Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature,* **391:** 806-811.

Frolet, C., Thoma, M., Blandin, S., Hoffmann, J. A. and Levashina, E. A. 2006. Boosting NF-KappaB-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. *Immunity,* **25:** 677-85.

Gardiner, P. R. 1989. Recent studies of the biology of *Trypanosoma vivax*. *In:* Baker, J. R. and Muller, R. (Eds.) *Advances in Parasitology.* Academic Press.

Geerts, S., Holmes, P. H., Diall, O. and Eisler, M. C. 2001. African bovine *trypanosomiasis*: the problem of drug resistance. *Trends In Parasitology,* **17:** 25-28.

Gibson, W. and Bailey, M. 2003. The development of *Trypanosoma brucei* within the tsetse fly midgut observed using green fluorescent trypanosomes. *Kinetoplastid Biology and Disisease,* **2:** 1.

Gooding, R. H. and Krafsur, E. S. 2005. Tsetse genetics: contributions to biology, systematics, and control of tsetse flies. *Annual Review of Entomology,* **50:** 101-123.

Graham, S. V., Terry, S. and Barry, J. D. 1999. A structural and transcription pattern for variant surface glycoprotein gene expression sites used in metacyclic stage *Trypanosoma brucei*. *Molecular and Biochemical Parasitology,* **103:** 141-154.

Haines, L. R., Hancock, R. E. W. and Pearson, T. W. 2003. Cationic antimicrobial peptide killing of African trypanosomes and *Sodalis glossinidius*, a bacterial symbiont of the insect vector of sleeping sickness. *Vector Borne and Zoonotic Diseases,* **3:** 175-186.

Haines, L. R., Jackson, A. M., Lehane, M. J., Thomas, J. M., Yamaguchi, A. Y., Haddow, J. D. and Pearson, T. W. 2005. Increased expression of unusual EP repeat-containing proteins in the midgut of the tsetse fly (*Glossina*) after bacterial challenge. *Insect Biochemistry and Molecular Biology,* **35:** 413-23.

Haines, L. R., Lehane, S. M., Pearson, T. W. and Lehane, M. J. 2010. Tsetse EP protein protects the fly midgut from trypanosome establishment. *Plos Pathogens,* **6:** E1000793.

Hall, T. A. 1999. Bioedit: A user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series,* **41:** 95-98.

Hammond, S. M. 2005. Dicing and slicing - The core machinery of the RNA interference pathway. *Febs Letters,* **579:** 5822-5829.

Hao, Z., Kasumba, I., Lehane, M. J., Gibson, W. C., Kwon, J. and Aksoy, S. 2001. Tsetse immune responses and trypanosome transmission: Implications for the development of tsetse-based strategies to reduce *trypanosomiasis*. *Proceedings of the National Academy of Sciences of the United States of America,* **98:** 12648-53.

Henschel, A., Buchholz, F. and Habermann, B. 2004. Deqor: a web-based tool for the design and quality control of siRNAs. *Nucleic Acids Research,* **32:** W113-20.

Hoare, C. A. 1972. *The trypanosomes of mammals.* A zoological monograph, Blackwell Scientific Publications, 5 Alfred Street, Oxford.

Hu, C. and Aksoy, S. 2006. Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans*. *Molecular Microbiology,* **60:** 1194-204.

Ibrahim, E. A., Ingram, G. A. and Molyneux, D. H. 1984. Haemagglutinins and parasite agglutinins in haemolymph and gut of *Glossina*. *Tropical Medicine and Parasitology,* **35:** 151-6.

Imbuga, M. O., Osir, E. O., Labongo, V. L., Darji, N. and Otieno, L. H. 1992. Studies on tsetse midgut factors that induce differentiation of blood-stream *Trypanosoma brucei brucei in vitro*. *Parasitology Research,* **78:** 10-5.

Joja, L. L. and Okoli, U. A. 2001. Trapping the vector: Community action to curb sleeping sickness in Southern Sudan. *American Journal of Public Health,* **91:** 1583-1585.

Krafsur, E. S. 2009. Tsetse flies: genetics, evolution, and role as vectors. *Infection Genetics and Evolution,* **9:** 124-141.

Kuzoe, F. A. S. 1993. Current situation of African *trypanosomiasis*. *Acta Tropica,* **54:** 153-162.

Leak, S. G. A. 1999. Tsetse biology and ecology: Their role in the epidemiology and control of *trypanosomiasis*., Cabi Publising, Wallingford.

Legros, D., Ollivier, G., Gastellu-Etchegorry, M., Paquet, C., Burri, C., Jannin, J. and Buscher, P. 2002. Treatment of human african *trypanosomiasis* - present situation and needs for research and development. *Lancet Infectious Diseases,* **2:** 437-440.

Lehane, M. J., Gibson, W. and Lehane, S. M. 2008. Differential expression of fat body genes in *Glossina morsitans morsitans* following infection with *Trypanosoma brucei brucei. International Journal for Parasitology,* **38:** 93-101.

Lehane, M. J. and Msangi, A. R. 1991. Lectin and peritrophic membrane-development in the gut of *Glossina morsitans morsitans* and a discussion of their role in protecting the fly against trypanosome infection. *Medical and Veterinary Entomology,* **5:** 495-501.

Lumsden, W. H. R. and Evans, D. A. 1979. Chapter 7. In biology of the kinetoplastida. , Academic Press London.

Mamman, M., Katende, J., Moloo, S. K. and Peregrine, A. S. 1993. Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats. *Veterinary Parasitology,* **50:** 1-14.

Masumu Mulubu, J. 2006. The importance of *Trypanosoma congolense* strain diversity in the epidemiology of bovine. Phd Thesis., Universiteit Gent. Gent.

Matemba, L. E., Fèvre, E. M., Kibona, S. N., Picozzi, K., Cleaveland, S., Shaw, A. P. and Welburn, S. C. 2010. Quantifying the burden of *rhodesiense* sleeping sickness in Urambo District, Tanzania. *Plos Neglected Tropical Diseases,* **4:** E868.

Matovu, E., Enyaru, J. C. K., Legros, D., Schmid, C., Seebeck, T. and Kaminsky, R. 2001. Melarsoprol refractory *T. b. gambiense* from Omugo, North-Western Uganda. *Tropical Medicine and International Health,* **6:** 407-411.

Matthew, C. Z., Darby, A. C., Young, S. A., Hume, L. H. and Welburn, S. C. 2005. The rapid isolation and growth dynamics of the tsetse symbiont *Sodalis glossinidius. Fems Microbiology Letters,* **248:** 69-74.

Maudlin, I. 1991. Transmission of African *trypanosomiasis*:interactions among tsetse immune system, symbionts, and parasites. *Advances in Disease Vector Research,* **7:** 117 - 148.

Maudlin, I. and Welburn, S. C. 1987. Lectin mediated establishment of midgut infections of *Trypanosoma congolense* and *Trypanosoma brucei* in *Glossina morsitans. Tropical Medicine and Parasitology,* **38:** 167-170.

Maudlin, I. and Welburn, S. C. 1988. The role of lectins and trypanosome genotype in the maturation of midgut infections in *Glossina morsitans. Tropical Medicine and Parasitology,* **39:** 56-58.

Mihok, S., Machika, C., Darji, N., Kang'ethe, E. K. and Otieno, L. H. 1995. Relationships between host blood factors and proteases in *Glossina morsitans* subspecies infected with *Trypanosoma congolense. Medical Veterinary and Entomology,* **9:** 155-60.

Moloo, S. K. 1981. Effects of maintaining *Glossina morsitans morsitans* on different hosts upon the vectors subsequent infection-rates with pathogenic trypanosomes. *Acta Tropica,* **38:** 125-136.

Moloo, S. K. and Kutuza, S. B. 1990. Expression of resistance to isometamidium and diminazene in *Trypanosoma congolense* in boran cattle infected by *Glossina morsitans centralis. Acta Tropica,* **47:** 79-89.

Molyneux, D. H. and Ashford, R. W. 1983. The biology of Trypanosoma and Leishmania, parasites of man and domestic animals., London, Taylor and Francis Ltd. London. 294pp.

Mshelbwala, A. S. 1972. *Trypanosoma brucei* infection in the haemocoel of tsetse flies. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **66:** 637-643.

Muturi, C. N., Ouma, J. O., Malele, Ii, Ngure, R. M., Rutto, J. J., Mithofer, K. M., Enyaru, J. and Masiga, D. K. 2011. Tracking the feeding patterns of tsetse flies (*Glossina* genus) by analysis of bloodmeals using mitochondrial cytochromes genes. *Plos One,* **6:** E17284.

Napoli, C., Lemieux, C. and Jorgensen, R. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell,* **2:** 279-289.

Nguu, E. K., Osir, E. O., Imbuga, M. O. and Olembo, N. K. 1996. The effect of host blood in the *in vitro* transformation of bloodstream trypanosomes by tsetse midgut homogenates. *Medical and Veterinary Entomology,* **10:** 317-322.

Nok, A. J. 2003. Arsenicals (melarsoprol), pentamidine and suramin in the treatment of human african *trypanosomiasis*. *Parasitology Research,* **90:** 71-79.

Nok, A. J. 2005. Effective measures for controlling *trypanosomiasis*. *Expert Opinion on Pharmacotherapy,* **6:** 2645-2653.

Onyango, P. 1993. Relationship between lectins and trypsins in the midgut of tsetse fly, *Glossina morsitans morsitans.* M.Sc. Thesis University Of Nairobi.

Osir, E. O., Abubakar, L. and Imbuga, M. O. 1995. Purification and characterization of a midgut lectin-trypsin complex from the tsetse-fly *Glossina longipennis*. *Parasitology Research,* **81:** 276-281.

Osir, E. O., Abubakar, M. and Abubakar, L. 1999. The role of trypanolysin in the development of trypanosome in tsetse. In proceedings 25th meeting of the international scientific council for *trypanosomiasis* research and control. 27th sep- 1st oct 1999. Nairobi,Kenya: Oau/Strc.

Osir, E. O., Imbuga, M. O. and Onyango, P. 1993. Inhibition of *Glossina morsitans* midgut trypsin activity by D-glucosamine. *Parasitology Research,* **79:** 93-97.

Peacock, L., Ferris, V., Bailey, M. and Gibson, W. 2006. Multiple effects of the lectin-inhibitory sugars D-glucosamine and N-acetyl-glucosamine on tsetse-trypanosome interactions. *Parasitology,* **132:** 651-8.

Peacock, L., Ferris, V., Bailey, M. and Gibson, W. 2007. Dynamics of infection and competition between two strains of *Trypanosoma brucei brucei* in the tsetse fly observed using fluorescent markers. *Kinetoplastid Biology and Disease,* **6:** 4.

Pearson, T. W., Beecroft, R. P., Welburn, S. C., Ruepp, S., Roditi, I., Hwa, K. Y., Englund, P. T., Wells, C. W. and Murphy, N. B. 2000. The major cell surface glycoprotein procyclin is a receptor for induction of a novel form of cell death in African trypanosomes *in vitro*. *Molecular and Biochemical Parasitology,* **111:** 333-349.

Peter, W. 1992. In peritrophic membranes: Zoophysiology, *Vol. 30.* Springer, Berlin.

Priotto, G., Kasparian, S., Ngouama, D., Ghorashian, S., Arnold, U., Ghabri, S. and Karunakara, U. 2007. Nifurtimox-eflornithine combination therapy for second-stage *Trypanosoma brucei gambiense* sleeping sickness: a randomized clinical trial in congo. *Clinical Infectious Diseases,* **45:** 1435-1442.

Priotto, G., Kasparion, S., Mutombo, W., Ngouama, D., Ghorashian, S., Arnold, U., Ghabri, S., Baudin, E., Buard, V., Kazadi-Kyanza, S., Ilunga, M., Mutangala, W., Pohlig, G., Schmid, C., Karunakara, U., Torreele, E. and Kande, V. 2009. Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei gambiense trypanosomiasis*: a multicentre, randomised, phase III, non-inferiority trial. *Lancet,* **374:** 56-64.

Rawlings, N. D. and Barrett, A. J. 1994. Families of serine peptidases. *Methods Enzymology,* **244:** 19-61.

Saitou, N. and Nei, M. 1987. The neighbor-joining method - a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* **4:** 406-425.

Seed, J. and Wenck, M. 2003. Role of the long slender to short stumpy transition in the life cycle of the African trypanosomes. *Kinetoplastid Biology and Disease,* **2:** 3.

Shakesby, A. J., Wallace, I. S., Isaacs, H. V., Pritchard, J., Roberts, D. M. and Douglas, A. E. 2009. A water-specific aquaporin involved in aphid osmoregulation. *Insect Biochemistry and Molecular Biology,* **39:** 1-10.

Sharma, R., Peacock, L., Gluenz, E., Gull, K., Gibson, W. and Carrington, M. 2008. Asymmetric cell division as a route to reduction in cell length and change in cell morphology in trypanosomes. *Protist,* **159:** 137-151.

Shereni, W. 1997. Integrated use of baits techniques for tsetse control in Zimbabwe. In proceedings of the 23rd meeting of the ISCTRC, Banjul, Gambia. OAU/STRC. Nairobi, Kenya.

Simarro, P. P., Diarra, A., Ruiz Postigo, J. A., Franco, J. R. and Jannin, J. G. 2011. The human African *trypanosomiasis* control and surveillance programme of the World Health Organization 2000–2009: The way forward. *Plos Neglected Tropical Disease,* **5:** E1007.

Siomi, H. and Siomi, M. C. 2009. On the road to reading the RNA-interference code. *Nature,* **457:** 396-404.

Skerlavaj, B., Gennaro, R., Bagella, L., Merluzzi, L., Risso, A. and Zanetti, M. 1996. Biological characterization of two novel cathelicidin-derived peptides and identification of structural requirements for their antimicrobial and cell lytic activities. *Journal of Biological Chemistry,* **271:** 28375-28381.

Solano, P., Ravel, S. and De Meeûs, T. 2010. How can tsetse population genetics contribute to African *trypanosomiasis* control? *Trends in Parasitology,* **26:** 255-263.

Stephen, L. E. 1986. *Trypanosomiasis*: A veterinary perspective. Pergamon Press Inc. New York. 551pp

Stiles, J. K., Ingram, G. A., Wallbanks, K. R., Molyneux, D. H., Maudlin, I. and Welburn, S. 1990. Identification of midgut trypanolysin and trypanoagglutinin in *Glossina palpalis* sspp (Diptera, Glossinidae). *Parasitology,* **101:** 369-376.

Swallow, B. M. 2000. Impacts of *trypanosomiasis* on African agriculture. PAAT technical and scientific series. . Rome: Fao.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. Mega4: Molecular Evolutionary Genetics Analysis (mega) software version 4.0. *Molecular Biology and Evolution,* **24:** 1596-1599.

Torr, S. J., Hargrove, J. W. and Vale, G. A. 2005. Towards a rational policy for dealing with tsetse. *Trends in Parasitology,* **21:** 537-541.

Vale, G. A. 1982. The improvement of traps for tsetse flies (Diptera: Glossinidae). *Bulletin of Entomological Research,* **72:** 95-106.

Van Den Abbeele, J., Claes, Y., Van Bockstaele, D., Le Ray, D. and Coosemans, M. 1999. *Trypanosoma brucei Spp.* Development in the tsetse fly: Characterization of the post-mesocyclic stages in the foregut and proboscis. *Parasitology,* **118:** 469-478.

Vanderploeg, L. H. T., Liu, A. Y. C., Michels, P. A. M., Delange, T., Borst, P., Majumder, H. K., Weber, H., Veeneman, G. H. and Vanboom, J. 1982. RNA splicing is required to make the messenger-RNA for a variant surface-antigen in trypanosomes. *Nucleic Acids Research,* **10:** 3591-3604.

Vassella, E., Reuner, B., Yutzy, B. and Boshart, M. 1997. Differentiation of African trypanosomes is controlled by a density sensing mechanism which signals cell cycle arrest via the cAMP pathway. *Journal of Cell Science,* **110:** 2661-2671.

Vreysen, M. J. B., Saleh, K. M., Ali, M. Y., Abdulla, A. M., Zhu, Z. R., Juma, K. G., Dyck, V. A., Msangi, A. R., Mkonyi, P. A. and Feldmann, H. U. 2000. *Glossina austeni* (Diptera:: Glossinidae) eradicated on the island of Unguja, Zanzibar, using the sterile insect technique. *Journal of Economic Entomology,* **93:** 123-135.

Walshe, D. P., Lehane, S. M., Lehane, M. J. and Haines, L. R. 2009a. Prolonged gene knockdown in the tsetse fly *Glossina* by feeding double stranded RNA. *Insect Molecular Biology,* **18:** 11-9.

Walshe, D. P., Ooi, C. P., Lehane, M. J. and Haines, L. R. 2009b. Chapter 3 The enemy within: Interactions between tsetse, trypanosomes and symbionts. In: Stephen, J. S. and Jeacuterocircme, C. (Eds.) *Advances in Insect Physiology.* Academic Press.

Weiss, B. L., Attardo, G. M., Pais, R., Wang, J. and Aksoy, S. 2007. Novel strategies targeting pathogen transmission reduction in insect vectors: Tsetse-transmitted *trypanosomiasis* control. *Entomological Research,* **37:** 231-237.

Weiss, B. L., Wu, Y., Schwank, J. J., Tolwinski, N. S. and Aksoy, S. 2008. An insect symbiosis is influenced by bacterium-specific polymorphisms in outer-membrane protein A. *Proceedings of the National Academy of Sciences of the United States of America,* **105:** 15088-93.

Welburn, S. C. and Maudlin, I. 1999. Tsetse-trypanosome interactions: Rites of Passage. *Parasitology Today,* **15:** 399-403.

Welburn, S. C., Maudlin, I. and Ellis, D. S. 1989. Rate of trypanosome killing by lectins in midguts of different species and strains of *Glossina. Medical and Veterinary Entomology,* **3:** 77-82.

Yan, J., Cheng, Q. Y., Li, B. B. and Aksoy, S. 2001. Molecular characterization of two serine proteases expressed in gut tissue of the African trypanosome vector, *Glossina morsitans morsitans. Insect Molecular Biology,* **10:** 47-56.

Yang, G., Attardo, G. M., Lohs, C. and Aksoy, S. 2010. Molecular characterization of two novel milk proteins in the tsetse fly (*Glossina morsitans morsitans*). *Insect Molecular Biology,* **19:** 253-62.

APPENDICES

APPENDIX 1: SOME FORMULATIONS

PBS (phosphate buffered saline), pH 7.4

8.0g NaCl

0.2g KCl

1.44g Na_2HPO_4

0.24g KH_2PO_4

Dissolve the salts in 600ml of deionized distilled water, adjust pH to 7.4 and make volume to 1L.

TAE buffer, pH 8.0

0.04M Tris-HCL

0.001M EDTA

Adjust to pH 8.0 with HCL

DNA sample buffer, pH 8.0

5% Glycerol

0.025% bromophenol blue

0.1M EDTA

Adjust to pH 8.0 with HCL

2M Mg^{2+} stock

20.33g $MgCl_2.6H_2O$

24.65g $MgSO_4.7H_2O$

Add distilled water to 100ml, filter sterilize.

2M Glucose (100ml)

36g D(+)Glucose

Filter and store at 4°C

SOC medium (100ml)

2.0g Bacto tryptone

0.5g Bacto Yeast extract

1ml 1M NaCl

0.25ml 1M KCl

1ml 2M Mg^{2+} stock-Filter sterilized

1ml 2M glucose-Filter sterilized

Preparation of SOC medium: Add tryptone, yeast extract,NaCl and KCl to 97ml of dH_2O. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20 mM. Bring to 100ml with sterile dH_2O.Filter the complete medium through a 0.2μm filter unit. The final pH should be 7.0.

IPTG stock solution (0.1M)

1.2g IPTG, add water to final volume. Filter sterilize and store at 4°C.

X-Gal (2ml)

100mg 5-bromo-4chloro-3-indoyl-β-D-galactoside. Dissolve in 2ml N,N'-dimethyl-formamide. Cover with aluminium foil and store at -20°C.

LB Broth (1L)

10g bacto tryptone

5g yeast extract

5g NaCl

Adjust Ph to 7.0 with NaOH. Autoclave.

LB plates (1L)

10g bacto tryptone

5g yeast extract

5g NaCl

Adjust Ph to 7.0 with NaOH. Add 15g of agar and outoclave. Allow the medium to cool to 50°C. Pour into petri dish (30-35ml/plate) after adding antibiotics (if necessary). Let the agar harden and store the seal plates at 4°C for 1 month.

LB plates with ampicilin/IPTG/X-Gal

Prepare LB agar, autoclave, allow to cool to 50°C. Add ampicillin to a final concentration of 100µg/ml; also, supplement with 0.5 mM IPTG and 80µg/ml of X-Gal and pour the plate. Alternatively, spread 100µl of 100 mM IPTG and 20µl of 50mg/ml X-Gal over the surface of an LB-ampicillin plate and allow to absorb at 37°C prior to use.

Transformation Buffer (TB) (1L)

3.0g PIPES

2.2g $CaCl_2.2H_2O$

18.6g KCl

Dissolve in 975 ml of ddH_2O, adjust the pH to 6.7-6.8 using 5M KOH (the white precipitate will not go into solution until the pH is close to 6.0). Then add;

10.9g $MnCl_2.4H_2O$

Top up with ddH_2O to 1 liter, filter sterilize (0.22µm filter) and store at 4°C for months.