POLYMORPHISM OF ATTACIN D GENE IN *Glossina pallidipes* TSETSE FLY POPULATIONS OF NGURUMAN AND BUSIA-TESO, KENYA

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DECLARATION AND RECOMMENDATION

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I hereby declare that this thesis is my original work and has not been presented for award of a degree in any institution of learning to the best of my knowledge.

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We confirm that this thesis was prepared under our supervision and has our approval to be presented for examination as per the Egerton University regulations.

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DEDICATION

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ABSTRACT

Tsetse flies (*Glossina* spp) that spread African trypanosomes express *Attacin*, an immune system protein, during trypanosome infection. The actual mechanism of protection by *Attacin* proteins in trypanosome-infected *Glossina pallidipes* is unknown. The objectives for this study was to assess polymorphism of Attacin gene in natural *G. pallidipes* populations isolated from Nguruman and Busia-Teso regions of Kenya (regions with different disease transmission levels and genetic diversity of tsetse flies) and correlate polymorphism of the gene to trypanosome infection in *G. pallidipes* from the study regions. Biconical traps and suitable attractants were used to lure and capture the *G. pallidipes* flies. Midguts were dissected from the captured flies and preserved in Trizol reagent. Trypanosome parasitemia in the salivary glands of the flies were determined through standard microscopy. PCR of Attacin D (AttD) gene was conducted on Genomic DNA (extracted from carcasses), and the product sequenced using automated sequencer. Trypanosome infections in the midgut tissues were detected by microscopy and their presence confirmed via nested RT-PCR using trypanosome species specific primers. A total of 586 non-teneral and 151 teneral *G. pallidipes* were sampled from Nguruman. In Busia – Teso, 17 and 16 non-teneral and teneral *G. palidipes* were sampled. A homologue of *G. m. morsitans* Attacin D in *G. pallidipes* was successfully identified from a region of 484 base pairs. Most polymorphism were mutations, deletions and substitutions but with predominance of mutation in coding sites. Two types of AttD were revealed, a conserved region and several introns in all individual flies' sequences in position 170 to 180. Attacin D polymorphism in *G. pallidipes* from Nguruman and Busia-Teso were predominant downstream from position 199 to position 298. Microcopy examination revealed putative presence of trypanosome in midgut and proboscis of two flies, one each from Nguruman and Busia–Teso, while PCR results revealed putative infections in two and four flies from respective towns. Putative *G. pallidipes* Attacin D homologous were identified only in *G. m. morsitans* and in *Aedes aegypti* among insect vectors of pathogens in *Glossina, Aedes* and *Rhodnius* genera. Interproscan analyses did not reveal any differences in domain architecture among the putative *G. pallidipes* attacin D sequences. The observed differences in polymorphism of Atttacin D populations in Nguruman and Busia-Teso may be responsible for the differences in Trypanosomiasis incidences in the two towns.

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LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
AttA	Attacin A
AttB	Attacin B
AttD	Attacin D
cDNA	Complementary Deoxyribonucleic Acid
CDS	Coding sequence
GmAttA	*Glossina morsitans* Attacin A
GPI	Glycophospholipid Inositol
GSPs	Gene Specific Primers
HAT	Human African Trypanosomiasis
Imd	Immunodeficiency
ITS	Internal transcribed Spacer
ORF	Open reading frame
PAMPs	pathogen-associated molecular patterns
PATTEC	Pan African Tsetse and Trypanosomiasis Eradication Campaign
PPO	Prophenoloxidase
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SIT	Sterile Insect Technique
SRS-EBI	Sequence Retrieval System -European Bioinformatics Institute

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Tsetse flies (Diptera: Glossinidae) are the only vectors of pathogenic African trypanosomes that are a potent and constant threat to humans and livestock over much of Sub-Saharan Africa (Aksoy *et al.,* 2005). World Health Organization statistics indicate that Africa is still recovering from an epidemic that resulted in 300,000 – 500,000 cases of Human African Trypanosomiasis (HAT) in 1998 (WHO,2005). On the other hand, Nagana in domestic animals causes losses to African agriculture of to the tune of US $4.5 billion per year (Jordan, 1986). High costs of trypanocidal drug therapies and increase in cases of drug resistance present tsetse control as an efficacious way of preventing humans and animals trypanosomiasis.

During human to human transmission by the tsetse vector, most parasites intimately interact with their invertebrate hosts, through significant biochemical and molecular modifications, including differential expressions of specific genes to survive, differentiate and multiply in the vectors (Lehane *et al.,* 2003). Such interactions are absent in the tsetse fly haemolymph due to presence of tsetse antimicrobial peptides (AMPs) wich confine the parasites to the midgut (Gillespie *et al.*, 2004). The AMPs include Cecropins, Defensin and Attacin families (Meister *et al.,* 1997), and are up-regulated in response to infection by trypanosmome (Dimopoulos, 2003). The Attacin based in the tsetse fat body are particularly more responsive to procyclic than to bloodstream forms of the parasite (Hao *et al.,* 2001), a process dependent on natural polymorphism of the gene in tsetse (Wang *et al.,* 2008). Those based in the midgut are responsive to RNA interference, as reflected in increases in midgut infection rates with silencing of the genes (Hu and Aksoy, 2006). This phenomenon suggests likelihood of transcriptional regulation of Attacin influencing tsetse infection phenotypic (Lazzaro *et al.*, 2004). The homolog of *Drosophila attA* has previously been described from the tsetse fly *Glossina morsitans morsitans* (Hao *et al.*, 2001).

The *Glossina pallidipes* from Nguruman in Kajiado have higher transmission rates, lower survivorship and are less susceptible to trypanosome infection than those from Busia in western Kenya (Okoth *et al.,* 2006). Factors responsible for these differences are still not well understood but may be linked to differences in Attacin polymorphisms in the regions. The *G. pallidipes* from

Nguruman have also genetically differentiated from those in Busia (Ouma and Krasfur, 2005). The study assessed polymorphism of the Attacin D gene in natural *G. pallidipes* populations in Busia-Teso and Nguruman regions of Kenya and correlated polymorphisms of *Attacin* D gene to infection rates in *G. pallidipes* from the study regions.

1.2 STATEMENT OF THE PROBLEM

Tsetse populations have differences in susceptibility to trypanosome infection. The differences influence local epidemiology of trypanosomiasis. Putative role of immune genes such as Attacin D in the local transmission has not been determined, despite potential application of such information in controlling the transmission. The Attacin based in the tsetse fat body are particularly more responsive to procyclic than to bloodstream forms of the parasite and a process dependent on natural polymorphism of the gene in tsetse.

1.3 OBJECTIVES

1.3.1 General objective

To determine polymorphisms of *Attacin* D immune gene and its putative effect on trypanosome infections in natural *G. pallidipes* populations of Nguruman and Busia Kenya.

1.3.2 Specific objectives

1 To determine polymorphisms of *Attacin* D immune gene in *G. pallidipes* populations of Nguruman and Busia-Teso in Kenya.

2 To determine infection of *G. pallidipes* populations of Nguruman and Busia-Teso in Kenya with trypanosomes in relation to polymorphisms of *Attacin* D immune genes in the respective flies.

3 To determine putative genetic differences based on Attacin D homologues between *G. pallidipes* and other haematophagous vectors.

1.4 HYPOTHESIS

1 There are no differences in polymorphisms of *Attacin* D immune gene in *G. pallidipes* populations of Nguruman and Busia-Teso Kenya.

2 There is no correlation between infection rates in *G. pallidipes* and *Attacin* D immune gene polymorphism.

3 There are no putative genetic differences based on Attacin D homologues between *G. pallidipes* and other haematophagous vectors.

1.5 JUSTIFICATION

Laboratory studies based on *Drosophila melanogaster* fruit fly and *Glossina morsitans morsitans* tsetse fly has established that the gene is immunogenic, evidenced by countering infection by bacteria and other microbes in the insects. Additionally, differences in transmission dynamics and vectorial capacity of *G. pallidipes* from Busia-Teso and Nguruman subpopulations have been observed, and *Attacin* D gene and other immune genes have been suspected to be responsible for the phenomenon. Attacin D gene will be evaluated in *G. pallidipes*, collected from two sites in Kenya, to assess the impact of polymorphisms in immune system genes in tsetse disease transmission. Since immune response genes may also be diverse according to the ecological or geographical location of tsetse distribution, this study proposed to determine the polymorphism in the *Attacin* D gene in *G. pallidipes* populations in two ecologically diverse study sites. The study will also establish putative genetic relationship between the Attacin gene in Busia-Teso and Nguruman subpopulation of *G. pallidipes* and those of other documented insects to determine their evolutionary relationships.

CHAPTER TWO

LITERATURE REVIEW

2.1. Background of the study

African trypanosomiasis, a set of diseases of humans and their domesticated animals, have devastated economies much of Sub-Shara Africa. The disease is exclusively and cyclically transmitted by Tsetse flies, *Glossina* species. The disease had virtually been controlled and almost disappeared by early 1960s (Steverding, 2008), but resurged, peaking in 1997 with about 450 000 human mortality cases cases (Barrett, 2006) and about 60 million people in 37 countries of sub-Saharan Africa exposed to the disease due to reduced surveillance and control measures (WHO, 2000). Some parasite species and sub-species of the *Trypanosoma* genus are infectious to wild and domestic animal species, thus creating not only unhealthy animal populations, but also establishing important parasite reservoirs. In particular, *T. b. brucei*, *T. congolense* and *T. vivax* are major causes of the animal form of trypanosomiasis. Disease severity is dependent on both the pathogenicity of the parasite strain and the genetics of the mammalian host (Courtin *et al.*, 2008). Despite implementation of many tsetse control strategies, nagana still has a large negative impact on agricultural and livestock production systems and land use. The disease has an estimated annual economic cost of approximately US$4.5 billion to the African economy due to losses in milk, meat and wool yields through adult mortality, calf mortality and subsequent depressed herd growth (Kristjanson *et al.*, 1999). Furthermore, nagana is a major restriction to the development of arable agricultural in sub- Saharan Africa, limiting the use of draught and pack animals and preventing the development of mixed agricultural practices (Jordan, 1986). Currently, nagana is managed predominantly by use of trypanotolerant breeds of cattle and use of chemoprophylactic and trypanocidal drugs (Miruk *et al.*, 2008).

2.2. Tsetse identification, distribution and control

Tsetse flies are easily distinguishable from other insects. They are light brown to black in colour and, dependant on the species, are roughly twice the size of a housefly. Characteristic aristae are present on the third antennal segment. In addition, the unique "hatchet" wing cell is found in the centre of each wing between the 4th and 5th veins. Also, tsetse flies adopt a characteristic resting attitude with their single pair of wings folded scissor-like over the dorsal

surface of the abdomen.Tsetse flies fall into a single genus, *Glossina*, and are restricted to sub-Saharan Africa except for two localities in the Arabian peninsula. Twenty three species and eight sub-species of tsetse fly are currently recognized (Leak, 1999; Krafsur, 2009).Tsetse are distributed in almost all of sub-Sahara Africa (Kettle, 1990; Wall and Shearer, 1997), infesting an area of 10 million square kilometres in 36 countries (FAO, 1988) and inflicting a direct loss of between US$600 million and US$1.2 billion every year from bovine trypanosomosis alone (FAO, 1998). The flies are obligate blood sucking Diptera in the family Glossinidae and genus Glossina. The 23 species in this genus belong to *Fusca, Palpalis* and *Morsitans* groups. Fourteen species, mainly inhabiting rainforest constitute the Fusca, while five species, predominantly inhabiting rainforests and savannah woodlands form the *Papalis* group. *Morsitans* group, consist of five species of which *Glossina morsitans morsitans*, *Glossina pallidipes, Glossina swynnertoni* and *Glossina longipalpis* (Colvin and Gibson, 1992) are restricted to the savannah while *Glossina austeni* occupy coastal forests. Within this group, *G. m. morsitans* and *G. pallidipes* are the most widespread and common vectors of livestock trypanosomosis (Jordan, 1986).

Long individual adult life of tsetse and exclusive haematophagy in both sexes (Kettle, 1990; Wall & Shearer, 1997; FAO 1988), has led to widespread epidemic of the disease, forcing farmers and herdsmen to either abandon wide areas of land across Africa, covering different ecological habitats, or to maintain their herd under regular chemotherapy (Kettle, 1990; FAO, 1998). Chemotherapy has not been sustainable mainly due to widespread and increasing resistance of trypanosomes to existing drugs (Gray and Roberts, 1971; Jordan, 1986; Connor, 1994), high cost and sporadic availability of drugs in areas with high fly challenge (Jordan, 1986; Makumyaviri, 1998). Chemotherapy has also been jeopardized by permanent wildlife trypanosome reservoir (Jordan, 1986). Rearing of trypanotolerant livestock have been tried with minimal success (Allsopp, 1984). Tsetse control is a promising means of disease control and constitutes the corner stone in the disease suppression (Elliot *et al.*, 1978; Kettle, 1990). This control was affected initially by elimination of game animal hosts of tsetse (Allsopp, 1984) and destruction of tsetse preferred habitats (Nash, 1940).

Advent of modern insecticides ushered in a massive eradication campaign of the Savannah tsetse species (Allsopp, 1984). Discovery of toxicological properties of

Dichlorodiphenyltrichloroethane (DDT) to tsetse boosted the campaign, making tsetse control increasingly dependent upon insecticides (Allsopp, 1984). DDT was cheap, persistent and highly effective against tsetse (Vale, 1968). Several other insecticides such as dieldrin and endosulfan, were subsequently discovered, evaluated and adopted in tsetse control programs to various extents (Allsopp, 1984). The control was by both aerial and ground insecticide spraying (Shereni, 1990).

Duration of apparent reluctance to accept and expand the role of insecticides was later experienced since insecticides appeared to provide only a temporary solution to a permanent problem (Harley, 1978), manifested by tsetse re-infestation of cleared areas due to degradation of insecticide toxicity with time, among other factors (Allsopp, 1984). These shortcomings, coupled with environmental hazards posed by the insecticides, shifted the control emphasis in favour of non-chemical control methods. Despite intensive research into these alternatives, they are still far from providing a practical alternative to insecticide control (Allsopp, 1984). An exception is perhaps a four-year sterile insect release (SIT) campaign on the island of Zanzibar that achieved a historic breakthrough success in complete tsetse eradication from the island (FAO, 1998). However, the success was attributable to integration with other control methods and factors peculiar to the island such as geographical isolation of Zanzibar Island, which minimizes reinvasion once the knockdown effect talks place (FAO, 1998). Enhanced insecticide efficacy and relative low cost further strengthened insecticide position in tsetse control (Haynes, 1988).

Insecticide application to cattle for tsetse control was demonstrated in southern Africa in the mid 1980s, but seemed of limited use where the intention was to remove tsetse from vast invasion source or where cattle were absent or could not be introduced (Colvin and Gibson, 1992; Vale, Mutika, and Lovemore, 1999). Artificial baits technology in tsetse control has been applied in similar areas successfully (Vale, 1993). Relative low cost, community acceptability, ability to stem tsetse re-invasion from adjacent areas (Vale *et al.*, 1988; Green, 1994; Mangwiro *et al.*, 1999; Allsopp, 1984), high specificity and minimal environmental contamination, (Allsopp, 1984) have made the technology applicable for both riverine as well as Savannah tsetse control (Nagel, 1995). The technology, consisting of traps and targets, exploits visual and olfactory tsetse responses to their hosts, with shapes, movements, colours and odours playing significant role in tsetse attraction (Vale, 1993). The attractions are governed by long chain of distinct tsetse

behavioral responses (Vale, 1993). Peak responsiveness to baits occur early in the morning and late in the afternoon (Groenendijk, 1996). Odours effect long distance attraction of tsetse to the baits, with visual stimuli required only for final orientation (Hargrove and Vale, 1978). These stimuli are detected by tsetse both in flight and while resting (Vale 1980). Strongest attraction and landing responses in *G. pallidipes* and *G .m. morsitans* is exhibited by blue and black targets respectively (Vale, 1982; Green, 1986; FAO, 1988).

2.3. Trypanosome (T. brucei spp.) life cycle: development and differentiation

Trypanosoma brucei has the most complex, but perhaps the best characterized, life cycle of all African trypanosome species. The trypanosome life cycle was first described in detail by Muriel Robertson who described the successive stages of parasite establishment and maturation within the insect and mammalian hosts, demonstrated the migration of parasites through the fly midgut and proved that only salivary gland forms were capable of producing a mammalian infection (Robertson, 1913). Since then, a more complete understanding of trypanosome development has been achieved, with an agreed parasite nomenclature adopted (Roditi and Clayton, 1999) and a consensus achieved on many of the barriers present in the fly that the trypanosome must overcome to survive and develop in order to complete cyclical transmission.

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 (Vickerman, 1969; Barry and McCulloch, 2001; Barry *et al.*, 2005). Differentiation of the long slender bloodstream forms (BSF) into the non-dividing stumpy BSF occurs in high density populations of long slender BSFs (Vassella *et al.*, 1997; Seed, 2003). The short stumpy BSF are believed to be pre-adapted for survival within the insect midgut due to the presence of a functional mitochondrion (Maudlin *et al.*, 2004).

Differentiation of the BSF to the procyclic forms (i.e. the insect midgut-adapted form), involves replacement of surface VSGs by procyclins and occurs rapidly after blood meal ingestion by the fly (Acosta-Serrano *et al.*, 2001; Vassella *et al.*, 2001; Gibson and Bailey, 2003). Most flies successfully kill all invading trypanosomes in a process termed self-cure. For the first three days, trypanosomes are mostly contained within the bloodmeal as it is being digested. The critical events in parasite establishment appear to occur approximately three days after infection,

when the relatively small proportion of surviving trypanosomes (~10%) either die or rapidly multiply in number (Gibson and Bailey, 2003).

Typically, from eight days after the infected bloodmeal, dissected flies can be confidently divided into two groups; the first in which most flies will have self-cured (having completed the clearing of ingested trypanosomes from their midguts) and the second, which have established midgut infections. Trypanosomes in an established infection migrate to the ectoperitrophic space three to five days post infection (Gibson and Bailey, 2003). It is believed that this occurs by direct penetration through the PM (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 PM in the hindgut. Typically the midgut population in an established infection reaches approximately 5 x 105 trypanosomes (Van den Abbeele *et al.*, 1999; Gibson and Bailey, 2003). From six to eight 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). Here they appear to cease division, elongate to mesocyclic forms and later differentiate into long trypomastigotes (Figure 1.3(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 (Croft *et al.*, 1982) would act as a major barrier for trypanosomes attempting to traverse it. Early positioning of trypanosomes in the anterior midgut and proventriculus should also favor passage along the foregut to the salivary glands (Peacock *et al.*, 2007).

2.4. Tsetse midgut interactions

It appears from the established literature that all tsetse species are susceptible, to some degree at least, to trypanosome infections. In general, the *Palpalis* group species tend to be poor vectors of congolense-type trypanosomes compared to the *Morsitans* group flies (Harley and Wilson, 1968; Moloo and Kutuza, 1988a; Ndegwa *et al.*, 1992). Conversely, tsetses of the Morsitans group are poorer vectors of *T. b. gambiense* than the *Palpalis* group (Richner *et al.*,

1988). Care needs to be taken with much of the data on susceptibility, as often fly and trypanosome strains used in experiments are from widely divergent geographical origins. Many factors influence fly susceptibility to trypanosome infection. Understanding of these factors and their underlying mechanisms is still rudimentary.

2.5. Tsetse immune system

Despite their obvious efficiency in maintaining large burdens of trypanosome based disease in Africa, tsetse flies exhibit a considerable level of refractoriness to trypanosome infection. Even under optimal laboratory conditions, where flies are fed at regular intervals, only a proportion of flies will establish midgut infections and the number decreases dramatically after the adult fly has taken three to four bloodmeals (Distelmans *et al.*, 1982; Welburn and Maudlin, 1992; Kubi *et al.*, 2006). Furthermore, less than half of the infections that become established in the midgut will mature (Van den Abbeele *et al.*, 1999; Gibson and Bailey, 2003; Peacock *et al.*, 2006). A key factor in this refractoriness is the fly immune system (Hao *et al.*, 2001). Immune stimulation, by injection of live *E. coli* or lipopolysaccharide (LPS) into the haemocoel of the fly prior to feeding an infective blood meal, leads to a statistically significant decrease in trypanosome midgut infection rates (Hao *et al.*, 2001). Identification of the tsetse immune molecule(s) responsible for conferring resistance to trypanosome infection has been hampered by the lack of an annotated *Glossina* genome (scheduled for completion in 2011). However, the sequencing and annotation of EST libraries from several tissue sources, including the major immunoresponsive tissues of midgut (Lehane *et al.*, 2003) and fat body (Attardo *et al.*, 2006), has provided the foundation for more extensive studies of the *Glossina* innate immune system at a molecular level (http://www.genedb.org/genedb/glossina/index.jsp).

Based largely on work on *Drosophila melanogaster*, it is known that insects possess a complex, interacting, innate immune system. This system is comprised of physical barriers (such as the cuticle and the PM), cellular responses (such as encapsulation and phagocytosis), and humoral responses, such as the generation of host defence peptides (HDP, previously called antimicrobial peptides), reactive oxygen species (ROS) and melanisation by the phenoloxidase pathway (Lemaitre and Hoffmann, 2007). The immune response depends not only on the nature of the immune stimulus, but also the point of entry of the antigenic molecule/organism, with quite

distinct epithelial (ingestion) and systemic immune (wounding) response profiles generated to the same pathogen (Hao *et al.*, 2001). Clearly, in tsetse trypanosome interactions, it is the epithelial immune responses of the alimentary canal and salivary gland tissues that are likely to be of major importance, as trypanosomes involved in the natural life cycle are exposed only to epithelial surfaces throughout the parasite life cycle. Trypanosomes have been reported in the tsetse haemocoel (Mshelbwala, 1972; Otieno *et al.*, 1976), but these are almost certainly not important to the completion of the normal parasite life cycle. Those trypanosomes that do traverse the midgut epithelium are rapidly killed by an unidentified systemic immune response (Croft *et al.*, 1982), which effectively confines the trypanosomes to the lumen of the alimentary canal.

The HDPs are evolutionarily conserved effector molecules of the humoral defence system and are found throughout nature. They exhibit a broad spectrum of activity against bacteria, fungi, viruses and transformed cells (Lemaitre and Hoffmann, 2007). In addition, the anti-parasite activity of HDP has been illustrated in several vector-parasite systems (Durvasula *et al.*, 1997; Shahabuddin *et al.*, 1998; Boulanger *et al.*, 2002b) including a tsetse-trypanosome system (Hu and Aksoy, 2005; Hu and Aksoy, 2006). Many HDPs target pathogens by disturbing the pathogen membrane potential or by disrupting internal cell functioning leading to cell death by apoptosis or necrosis. Early research into these immune mediators in *G. m. morsitans* identified four HDPs: an attacin (AttA1), a cecropin, a defensin and a diptericin (Hao *et al.*, 2001; Boulanger *et al.*, 2002a).

More recently, characterization of the *G. m. morsitans* attacin loci has recognized that attacin genes are organized in three clusters encoding three different attacins: attA, attB and attD. The amino acid sequences of AttA and AttB are almost identical while AttD is only 69% identical to the AttA/B form. These genes are differentially regulated (Wang *et al.*, 2008). Mature *Attacin* peptides are typically around 190 amino acids in length and adopt a "random coil" structure in solution (Gunne *et al.*, 1990). This loose, flexible structure is devoid of disulfide bonds and does not take a rigid conformational shape. This lack of strict structural constraint may allow relatively free amino acid substitution, explaining the low level of amino acid identity between *Attacin* homologs between distant taxa. There is, however, conservation of general structure and functional activity (Lazzaro and Clark, 2001). Analysis of Attacin cDNA sequences

shows polymorphisms that could arise either from allelic variations or from the presence of additional *Attacin* genomic loci (Wang *et al.*, 2008).

Interestingly, two additional HDP, one with anti-Gram-negative activity and the other with anti-Gram-positive activity, have also been identified following trypanosome challenge (Boulanger *et al.*, 2002a). These HDP remain as yet uncharacterized. *In vivo* analysis of HDP transcript expression during trypanosome infection has indicated that these peptides are differentially regulated in the haemolymph and in the major immunoresponsive tissues of midgut, fat body and proventiculus (Hao *et al.*, 2001; 2003; Hu and Aksoy, 2006). Early in the infection process, the presence of trypanosomes in the midgut or haemolymph does not lead to activation or increased transcription of midgut or fat body HDP genes (Hao *et al.*, 2001). However, by day 6, as parasite numbers increase, attacin (AttA/B and AttD) and defensin transcript expression is high in the fat body (Hao *et al.*, 2001; Hao *et al.*, 2003; Wang *et al.*, 2008). In self-cured flies HDP transcript expression levels fall, but in flies with established midgut infections expression levels remain high in the fat body and proventriculus (Hao *et al.*, 2001; Hao *et al.*, 2003). This does not appear to affect the viability of the parasite population within the midgut, although it remains to be seen if individual parasites are affected, thus resulting in a change in the nature of the parasite population. It is possible that trypanosomes exhibit a stage-specific sensitivity to particular immune molecules, with procyclics exhibiting higher resistance to the trypanocidal activity of HDP than BSF trypanosomes as was observed *in vitro* by Haines *et al.* (2003). Alternatively, fat body synthesised peptides circulating in the fly haemolymph may fail to reach parasites located in the midgut environment.

The trypanocidal activity of HDP has been recognised. Stomoxyn, isolated from the facultative hematophagous fly *Stomoxys calcitrans*, exhibits trypanocidal activity (Boulanger *et al.*, 2002b). In addition, there is direct evidence of trypanosome killing by *Glossina* HDP themselves (Hu and Aksoy, 2005; Hu and Aksoy, 2006; Nayduch and Aksoy, 2007). Recombinant attacin (AttA1) inhibited both BSF and PCF growth *in vitro* (Hu and Aksoy, 2005). *In vivo*, gene knockdown of the AttA1 peptide, or its transcriptional regulator Relish, led to a statistically significant increase in midgut and mature salivary gland trypanosome infection rates (Hu and Aksoy, 2006). Relish also regulates cecropin expression in *Glossina*, but whether cecropin possesses trypanocidal properties is yet to be directly investigated. More recently,

differential expression of AttA1 transcripts in a trypanosome susceptible species (*G. m. morsitans*) and two comparatively trypanosome-refractory species (*G. pallidipes* and *G. p. palpalis*) has been reported (Nayduch and Aksoy, 2007). Refractory species showed higher attacin transcript expression in fat body (systemic) and proventiculus/ midgut (local) tissues in comparison to susceptible flies in both teneral and blood-fed states. Knockdown of attacin expression in *G. pallidipes* led to increased trypanosome susceptibility, although the possible confounding effects of high mortality rate, starvation, wounding and low sample number in this study should be noted (Nayduch and Aksoy, 2007). Nevertheless, the evidence suggests that attacin may be an important regulator of tsetse-trypanosome interactions. TsetseEP protein may also play a role in tsetse-trypanosome interactions. This immunoresponsive protein is expressed strongly in the midgut of *Glossina* (Chandra *et al.*, 2004) and is up regulated in response to immune stimulation with Gram-negative bacteria (Haines *et al.*, 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Determination of *Attacin* D immune gene polymorphisms in *G. pallidipes* populations of Nguruman and Busia-Teso in Kenya

3.1.1. Study sites

Tsetses were sampled from Busia-Teso and Nguruman in Kenya, a natural habitat of the flies in Kenya (Fig 1). Busia-Teso (0°36'South and 0°North and longitude 33°54' East and 34°25'24''West) lies within the Lake Victoria basin. The area is infested by *Glossina fuscipes* and *G. pallidipes,* with the latter having a patchy distribution, associated with woods hill side vegetation (Ford, 1971). However, most natural vegetation has been cleared for agriculture, leaving less breeding ground for the vector (Okoth, 2007). Human population density is 230/km^2 and the population of domestic animals in the district is approximately 150,000 zebus, 25,000 sheep and 45,000 goats. The area lies within the only sleeping sickness focus found around the Lake Victoria, Kenya. In addition, village livestock have been reported as potential reservoirs of sleeping sickness with monthly prevalence rates of *Trypanosoma vivax*, *Trypanosoma brucei* and *Trypanosoma congolense* varying between 3% and 37% in indigenous livestock (Okoth, 2007).

Nguruman (latitude 1°55'South and longitude 35°25' East), lies on the floor of the Rift Valley in south-eastern Kenya, between the Nguruman escarpment to the west and lake Magadi to the East. Nguruman has an arid climate with generally low agriculture potential and mean annual rainfall of 500 mm occurring in the months of November - May. This climate favors breeding of tsetse vector hence a higher vector presence than Busia (Bourn *et al.,* 2001). The area is infected by *Glossina pallidipes* and *Glossina longipennis* within the wood land *Glossina swynnertoni* on the adjoin escarpment (Brightwell *et al.*, 1997). However, *G. swynnertoni* has not been caught in Nguruman for the last 10 years, raising questions of its existence there. The incidence of trypanosomiasis in cattle varies from 0 to 50% depending on the mode of herd management breed, season and year (Wellde *et al.*, 1989).

Tsetse flies were sampled in Teso North and South from, Bunyala (Budubusi, Funyula) in Busia, and in Nguruman, Kenya by methods Okoth *et al.*, (2006)(Fig 1).

3.1.2. Collection and dissection of Tsetse fly samples

Tsetse flies were sampled using biconical traps baited with acetone and phenol. The traps were set up and emptied at 24 h intervals during the period November 25 -29, 2009 for Nguruman and April 21-26, 2010 in Busia-Teso. The flies were transported to field laboratories stations in cool box (at 4°C) for identification .They where identified to species level using taxonomic keys and separated into teneral and non-teneral categories. The non-teneral flies were individually placed in sterile saline solution on a glass microscope slide and their mid guts and fat bodies dissected out using forceps and a sterile needle under a dissecting microscope. The individual mid gut and fat body tissues was preserved for subsequent trypanosome infection analyses and/or separately stored in 75% ethanol at -80°C or Trizol reagent (Invitrogen life technologies) for subsequent molecular analyses. The carcases were similarly preserved in the Trizol reagent (Invitrogen life technologies) according to the manufacturer's instructions.

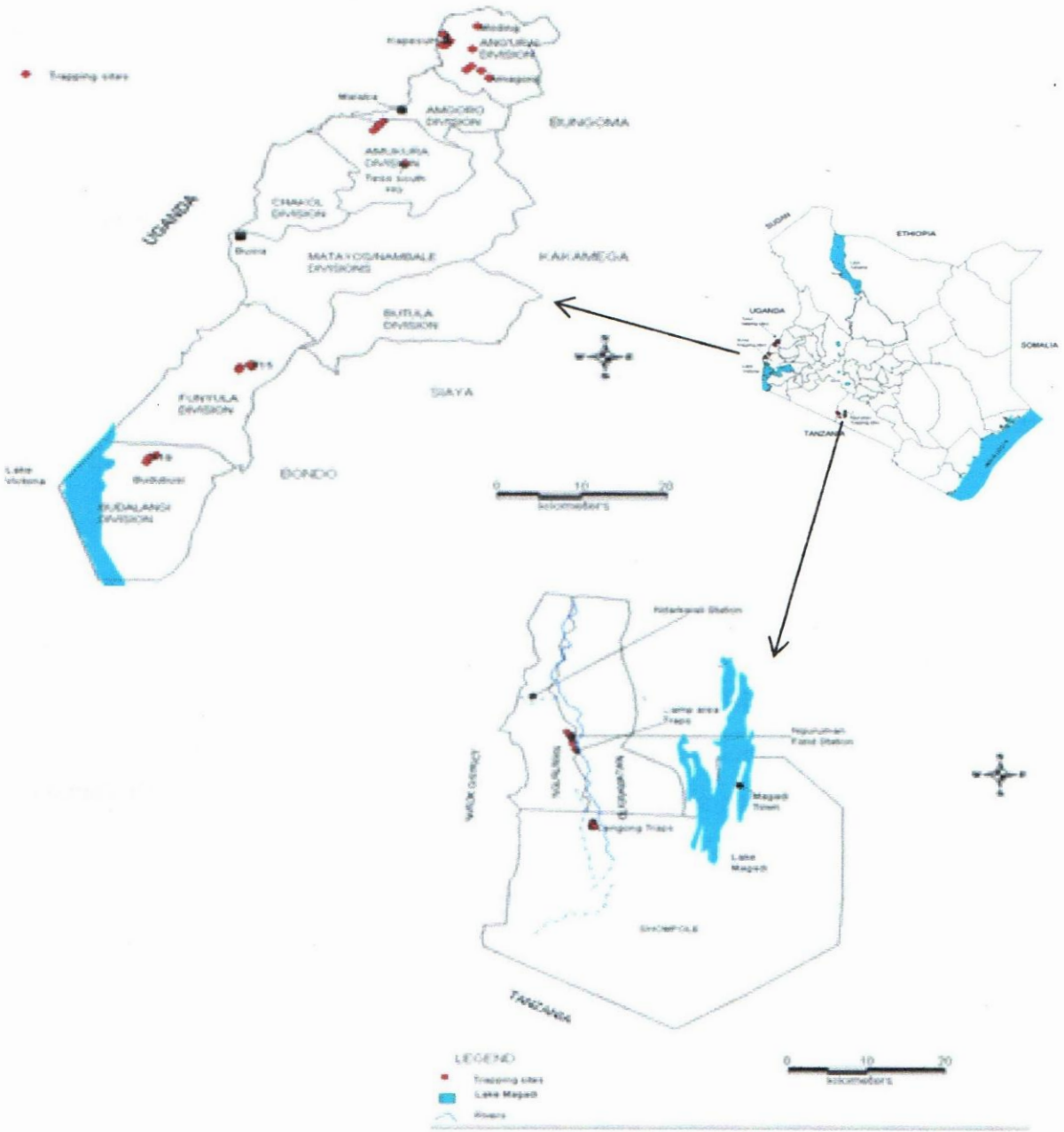


Figure 1. Sampling sites for *G. pallidipes* in Busia-Teso and Nguruman Kenya

3.1.3. Extraction of Genomic DNA from *G. pallidipes*

Genomic DNA was from the extracted from the carcases of *G. pallidipes* using Animal tissues DNeasy Blood and Tissue kit (250)(Qiagen) according to the manufacturer's instructions. According to the manufacturer's instructions. Briefly, tsetse carcasses were ground to powder in liquid nitrogen in an Eppendorf tube using a tube pestle. Buffer ATL and proteinase K were added and mixed thoroughly by vortexing. Samples were incubated at 56°C overnight to ensure complete tissue lysis. Buffer AL was added to the lysate and spun at 8000 rpm for 1 min. The clear upper phase was applied to a DNeasy mini spin column to bind the DNA and the column subsequently washed once with buffers AW1 and AW2. After the second wash, the column was transferred to a clean sterile 1.5ml Eppendorf tube and DNA eluted by adding elution buffer (100 µl). Sample (1µl) of the elute was used to quantify DNA concentration of the extract using NanoDrop spectrophotometer (Thermo-Scientific) according to the manufacturers instructions, while the rest of the DNA was stored at -20°C until when required.

3.1.4. *In silico* design of putative *G. pallidipes* specific Attacin gene primers

The only *Glossina spp Attacin* sequences available in public sequence database were for *Glossina morsitans* (cDNA and genomic with introns). Sequences were retrieved using the Sequence Retrieval System of the European Bioinformatics Institute (EBI; http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+srsq2+-noSession). The CDS option in SRS for automatically generated putative cDNA *in silico*; genomic sequences without the intervening introns. Information relating to *Glossina* Attacin genes (sequences and publications), were retrieved from the EBI database using 'Quick search' and "Complete entries" tools of EBI-SRS (http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+srsq2+-noSession), with *'Attacin'* and *'Glossina'* as search key words. 'Complete entries' on the Display Options was selected in order to retrieve.

Selected entries with complete Attacin protein coding sequences (untranslated regions [UTR] sequences were ignored) were Fasta, formatted to include the accession number of the sequence, species name and abbreviation of the gene in the header. Gaps appearing in sequences were removed using the READSEQ Sequence Conversion website (http://www-bimas.cit.nih.gov/molbio/readseq/). ClastalW2 (http://www.ebi.ac.uk/Tools/clustalw2/) software was used for multiple sequence alignment (MSA) of AttA, AttB and AttD *G. mortisans* CDS

from where regions for unique to AttA, AttB or AttD were identified, and primers designed specific for AttD. Primers generated were selected manually according to the following criteria: C+G content, >60 %; repetitive sequences, absent; repetitive bases, stretches of >3 identical bases (such as poly Ts) were avoided; sequences were selected perfectly homologous to target cDNA AttD and so that the 3' end base was preferably G or C. Four forward and four reverse primers (Table 1) were made to increase the probability of finding primers that would work on *G. pallidipes*.

3.1.5. PCR amplification, purification and sequencing of putative *G. pallidipes* Attacin D gene products

Attacin D gene in the DNA from *G. pallidipes* carcass was amplified using standard genomic DNA PCR procedures. Briefly, 2µl DNA products were amplified with 0.25 µl GoTaq DNA polymerase (5 u/µl) (Promega) and 1 µl dNTPs (10 mM) in 10 µl 5x Green GoTaq Flexi Buffer (Promega), 4 µl $MgCl_2$ (25 mM) in the presence of putative *G. pallidipes* Attacin D gene specific primers (forward 5` TCGGTTCAGCGTCATCCAACTCTC 3' and reverse 5` TTTTTATCAGTTTGACCATTAAAC 3`) combination at 2.5 µl (10µM) each, that provided optimal PCR product of the gene among the primers designed. Reactions were similarly prepared for Actin (Forward 5' CGCTTCTGGTCGTACTACT 3', Reverse 5' CCGGACATCACAATGTTGG3') and Tubulin (forward 5' GATGGTCAAGTGCGATCCT 3', 5' TGAGAACTCGCCTTCTTCC 3') as positive controls. The reaction volumes were adjusted to 50 µl with nuclease-free water (Promega), and overlaid with a drop of mineral oil. Reactions were carried out in Applied Bioscience thermocycler. The first cycle included 5 minutes at 95°C °C, 30 seconds at 44 °C, and 1 min at 72°C. Subsequent cycles involved one minute at 94°C, 30 seconds at 60°C, and one minute at 72 °C for 35 cycles. The final extension was conducted at for 7 min at 72 °C. Individual PCR products (10µl each) were loaded onto Ethidium Bromide 2 % agarose gels in a TAE buffer (Sambrook *et al.*, 1989). On every gel, 5µl of 100 bp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run to confirm expected molecular weights of the amplification products (Appendix 1). The gel was run for 30 minutes at 100 Volts, visualized and documented under Ultraviolet trans-illuminator.

Remaining PCR products were purified using Qiagen PCR purification kit (Qiagen, CA, USA) according to the manufacturer's instructions (Appendix 2). The purification products were assessed by electrophoresis, staining and documentation of 5 µl of eluted DNA on 2% Agarose gel. The products were submitted to BecA Hub Segolip unit for direct sequencing using BigDye™ Terminator Kit (Applied Biosystems), and the reactions analyzed on ABI 3730 or ABI3130 Genetic analyzer (Applied Biosystems). Samples with more than one allele (heterozygotes) were identified for clone based sequencing.

3.1.6. Cloning and sequencing putative *G. pallidipes* Attacin D gene products with more than one allele

PCR products with more than one allele identified through direct sequencing were cloned into pGEM-T Easy plasmid using the pGEM-T Vector System I kit (Promega, Madison, WI) according to instructions by the manufacturer. Briefly, 2 µl purified PCR product was ligated into 0.5 µl vector (50 ng/µl), in the presence of 5 µl of 2× Rapid ligation buffer , 1 µl T4 DNA ligase (3 Weiss units/µl), and 0.5 µl nuclease-free water (Promega, Madison, WI). The ligation was conducted overnight at 37°C, and stored at -20°C until required.

The recombinant vectors were electrochemically transformed in DH5α *E. coli* cells and electroporated at 1.5 volts and 25 µFD using Bio-Rad Gene Pulser® II Electroporation System (Bio-Rad, Hercules, CA). Briefly, 2 µl of ligation reaction was then added to 50 µl of electrocompetent cells and transferred to a chilled sterile electroporation cuvette (Bio-Rad Gene Pulser/E. coli Pulser Cuvettes, 0.2 cm electrode gap) and electroporated according to the manufacturer's instructions. After electroporation, the cells were transferred to a 12 ml polypropylene tube containing 1 ml 2×YT culture medium (Central Core, BecA Hub, Kenya). The Tubes were incubated at 37°C in a shaker incubator for an hour, and 100 µl of the culture plated onto a 2×YT plate containing 50 µg/ml ampicillin (Central Core, BecA Hub) with 15 µl of 10% X-gal (50 mg/ml) and 60 µl of IPTG and (0.1 M). Plates were then incubated at 37°C overnight.

A single colony containing recombinant plasmid (white) was transferred to a 12 ml tube containing 2 ml 2×YT medium containing ampicillin (50 µg/ml). The culture then incubated for 12 - 16 h at 37° C with vigorous shaking. Bacterial cells were harvest by centrifugation at 6800×g

in a micro-centrifuge for 3 min at room temperature. The pelleted bacterial cells were then re-suspended in a 250 µl buffer P2 (with lyse blue reagent) and mixed thoroughly by inversion. Buffer N3 (350 µl) was added and mixed immediately and thoroughly by inversion and centrifuged for 10 min at 17,900×g in a micro-centrifuge. The supernatant was then pipetted into a QIAprep spin column (Qiagen). Centrifuging was done for 30-60 sec and the flow-through was discarded. The QIAprep spin column washed by adding 0.75 ml PE buffer and centrifuged for 30 to 60 sec. The flow through was discarded and followed by centrifugation for an additional 1 minute to remove residual wash buffer. To elute the DNA, the QIAprep column was placed in a clean 1.5 ml microcentrifuge tube and 50µl of Buffer EB was added to the center of each QIAprep spin column, left to stand for 1 min and centrifuged for 1 min. Quantity of DNA in the plasmid was determined by assessment of DNA in 1 µl of purified plasmid through Nanodrop spectrophotometer reading. To confirm the presence of the PCR insert in the plasmid, the recombinant plasmid digested 1 µl with EcoRI endo-nuclease in 2 µl 10×EcoRI buffer, 2 µl Purified Plasmid DNA and 15 µl Deionised water at 37° C in a water bath. Ten µl of restriction products were mixed with 2 µl of 6 x gel loading buffer DNA, and electophorased on a 1.2% agarose with l. 1kb Bench-Top DNA Ladder (Inqaba) as the DNA size ladder.

The white colonies were screened to confirm presence of the recombinant plasmid via PCR. Briefly, putative positive colony (white colonies) were picked with a sterile 200 µl pipette tip and placed into 25 µl of PCR master mix in 96 well PCR plate. The master mix was composed of 5µl Green Go Taq Flexi (5×) buffer (Promega, Madison, WI), 2 µl $MgCl_2$ (25 mM); 0.5 µl dNTPs (10 mM); 0.125 µl M13 forward and reverse primers (100 pmol), 0.125 µl GoTaq polymerase (5u/ µl) (Promega, Madison, WI) and 17.125 µl of nuclease free water. Each reaction was overlaid with a drop of mineral oil. The plate was centrifuged at 2500 rpm for 1 min. PCR reactions were carried out in Applied Bioscience thermocycler as indicated above. The PCR products were likewise separated visualized and documented. The confirmed positive clones were submitted for sequencing as indicated above, but with M13 forward and reverse primers.

3.1.7 Agarose gel electrophoresis

Agarose gels were prepared using 1 x TAE buffer (prepared by diluting 50 x TAE with Milli-Q water (BecA Hub Central Core). To 100 ml of 1×TAE was added 2 g of agarose in a 500

ml flask, which was then boiled in a micro-wave oven to completely dissolve the agarose. After readjusting the volume to 100 ml with water, the solution was allowed to cool to 50°C in a water bath. One µl of ethidium bromide (stock at 10mg/ml). was then added and mixed. The agarose solution was then poured into a gel casting tray (Kodak Scientific Imaging) fitted with one or two 13 tooth comb. After the gel has set the comb(s) were removed and the gel was transferred to the gel tank containing 500ml 1 x TAE buffer. DNA samples (5-10 µl) were mixed with 1-2 µl of 6x gel loading buffer and loaded into the wells. One lane on each row of the gel was loaded with 6µl of DNA size ladder (100bp and 1kb). Gels were gel was run at 100 V for 45 min to 1h. The gel was then rinsed in water and visualized and photographed on a UV light box.

Briefly, five volumes of Buffer PB were added to a volume of the PCR sample and mixed. A QIAquick spin column was placed in a 2 ml collection tube. The sample was then applied to the QIAquick spin column and centrifuged in an Eppdorf microfuge at 12000 rpm for 60 sec to bind DNA. The flow-through was discarded and the QIAquick column was placed back into the same tube. The DNA was washed by adding 0.75 ml of Buffer PE to the QIAquick column and centrifuged for 30-60s. The flow-through was discarded and the QIAquick column was replaced back in the same tube and then centrifuged for an additional 1 min. The QIAquick column was placed in a clean 1.5 m microcentrifuge tube, and the DNA was eluted with 50 µl of Buffer EB (10mM Tris-HCl, pH 8.5) or H_2O that was added carefully to the center of the QUIAquick column membrane. The column was left to stand for 1 min, and then centrifuged as before. PCR products were stored at -20°C until required.

3.2. Determination of infection rates of *G. pallidipes* populations of Nguruman and Busia-Teso in Kenya with trypanosomes

3.2.1. Extraction of RNA from *G. pallidipes* midgut and fat body tissues

RNA was extracted from the midgut tissues and fat bodies preserved in TRIzol reagent (Invitrogen, Sandiego, CA) as described by Chomczynski *et al.,* (1995). Briefly, midgut and fat bodies from non-teneral *G. pallidipes* flies from Nguruman and Busia Teso were vortexed in the Trizol reagent to homogenize the tissues, and centrifuged at 12000×g for 10 min at 6°C to remove insoluble material from the homogenate. The supernatant was transferred to a clean Eppendorf tube and incubated at room temperature for five min. Chloroform (100 µl) was added to supernatant, mixed vigorously by hand for 15 sec, incubated at room temperature for two minutes, and centrifuged at 12000×g for 15 min at 6°C. The aqueous phase, containing RNA, was

transferred to a clean Eppendorf tube and the RNA precipitated by addition of isopropanol (250 µl) and incubated at room temperature for 10 min. The mixture was centrifuged at 12000×g for 10 min at 6°C and the supernatant was discarded. The resultant RNA pellet was washed once with 75% ethanol (500 µl), gently vortexed and centrifuged at 7500xg for 5 min at 6°C. The ethanol was discarded and the pellet was air dried at room temperature for 10 min. RNA concentration and purity was determined by using a NanoDrop-1000 spectrophotometer (Thermo-Scientific) according to the manufacturer's instructions. Briefly, the spectrophotometer was blanked using water, and absorbance of respective samples (1 µl) assessed at 260 and 280 nm wave length. In this respect, 1 unit at A_{260} was considered to be equivalent to40 ng/µl of RNA while the same unit at A_{260} was equivalent to 50 ng/µl of DNA. The ratio of the absorbance at 260 and 280nm ($A_{260/280}$) was used to assess the purity of nucleic acids.

3.2.2. Nested RT-PCR of RNA from *G. pallidipes* midgut and fat body tissues

Nested RT-PCR of RNA from *G. pallidipes* midgut and fat body tissues for molecular identification of trypanosome species was conducted using Internal transcribed spacer (ITS) primers method of Cox *et al.,* (2005). cDNA derived from RNA of mid-gut and fat body dissected from non-teneral flies and subsequently amplified using OneStep RT-PCR Kit (Qiagen,) according to the manufacturer's instructions. Briefly, a master mix, was prepared composed of 15 µl nuclease-free water, 5 µl OneStep RT-PCR (5X) buffer, 1 µl dNTP (10 mM), 0.5 µl each primers (10 µM) (Outer loop primers ITS1 (forward) 5'GATTACGTCCCTGCCATTTG3' and ITS2 (reverse) 5'TTGTTCGCTATCGGTCTTCC3') and 1 ul OneStep RT-PCR enzyme mix. RNA (2 µl) of was added to 23 µl master mix to a total volume of 25 µl. Reactions were carried out in Applied Bioscience thermocycler. Reverse transcription was conducted for 30 min at 45°C. The second cycle (inactivation of the reverse transcriptase and concomitant activation of Hot-Start Taq) was conducted for 15 min at 95°C initiating the first round of PCR reaction. This reaction was further conducted for 30 seconds at 48°C, and 1 min at 72°C. Subsequent cycles involved one minute at 95°C, 30 seconds at 48°C, and one minute at 72 °C for 35 cycles. The final extension was conducted at for 7 min at 72 °C.

Second round of PCR reactions was conducted on the PCR products of the first round reactions targeting internal segment of the amplicons. Briefly, 5µl PCR product was amplified

with 0.5 µl GoTaq DNA polymerase (5 u/µl) (Promega) and 1.5 µl dNTPs (10 mM) in 10 µl 10x Green GoTaq Flexi Buffer (Promega) and 3 µl $MgCl_2$ (25 mM) in the presence of forward ITS3 (5'GGAAGCAAAAGTCGTAACAAGG3') and reverse ITS4 (5'TGTTTTCTTTTCCTCCGCT G3') primers at 2.5 µl (10µM) each. The reaction volume was adjusted to 50 µl with nuclease-free water (Promega), and overlaid with a drop of mineral oil. Reactions were carried out in Applied Bioscience thermocycler. The first cycle included 5 minutes at 95°C °C, 30 seconds at 44°C, and 1 min at 72°C. Subsequent cycles involved one minute at 94°C, 30 seconds at 44°C, and one min at 72 °C for 35 cycles. The final extension was conducted at for 7 min at 72 °C. Individual PCR products (10µl each) were loaded onto Ethidium Bromide 2 % agarose gels in a TAE buffer (Samrook *et al.,* 1989). On every gel, 5µl of 100 bp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run to confirm expected molecular weights of the amplification products. The gel was run for 30 minutes at 100 Volts, visualized and documented under Ultraviolet trans-illuminator. In this scheme, presence of bands of 1513, 1422, 1413, 14954, 1207-1224, 850, 611 or 988 base pairs would be diagnostic for presence of *T. congolense Forest, T. congolense Kilifi, T. congolense Savannah, T. congolense Tsavo, T. brucei , T. simiae, T. vivax* or *T. theileri* respectively (Cox *et al.,* 2005). All PCR reaction included similarly prepared reactions for Actin (Forward 5' CGCTTCTGGTCGTACTACT 3', Reverse 5' CCGGACATCACAATGTTGG3') and Tubulin (forward 5' GATGGTCAAGTGCGATCCT 3', 5' TGAGAACTCGCCTTCTTCC 3') as positive or internal controls.

3.3. Differences between *Attacin* D in *G. pallidipes* and other homologues using bioinformatic approaches.

DNA sequences of Attacin D from the *G. pallidipes* from Nguruman and Busia-Teso Nguruman were obtained in this study and their functional homologues in Anopheles (taxid: 7164), *Glossina* (taxid: 7393), *Aedes* (taxid: 7158) and *Rhodnius* (taxid: 13248) genera determined by BLAST (Altschul *et al.,* 1997) analysis and presence of domains and motifs identified through InterProScan (Mulder *et al.,* 2003) analysis at the European Bioinformatics Institute (http://www.ebi.ac.uk/InterProScan).

CHAPTER FOUR

RESULTS

4.1. Attacin D immune gene polymorphisms in *G. pallidipes* populations of Nguruman and Busia-Teso in Kenya

4.1.1. Tsetse sampling in Nguruman and Busia-Teso and selection of *G. pallidipes* Attacin D specific primers

Summary of tsetse collected from Nguruman and Busia-Teso study sites is presented in Table 1. A total of 770 *G. pallidipes*, 49 *G. longipennis*, and 14 *G. f. fuscipes* were collected. *Glossina pallidipes* was predominant species at both sites, although many more individuals were collected at Nguruman compared to Busia-Teso. *Glossina longipennis* was only present in Nguruman, and *G. fuscipes* in Busia-Teso. In addition, 11 'biting flies' were collected from Nguruman, and 8 from Busia-Teso. The species of these 'biting flies' were not determined. Putative Attacin D gene primers, designed *in silico* from *G. m. morsitans* genome, successfully amplified its homologous in *G. pallidipes* (Fig 1), establishing their suitability for downstream application in Attacin gene polymorphism studies. Sequences were successfully (clean and unambiguous) generated from a total of 98; 76 through direct sequencing of PCR product and 22 through clone (plasmid) based sequencing. Among the samples, 58 were from Nguruman and while 36 were from Busia-Teso sampling sites in addition to 4 insectary fly from TRC.

Table 1. Tsetse species and number collected from each study site

Study area	*G. pallidipes (non teneral)*	*G. pallidipes (teneral)*	*G. longipenis*	*G. fuscupes*	*Total*
Nguruman	586	151	49	0	786
Busia-Teso	17	16	0	14	47
Total	603	167	49	14	833

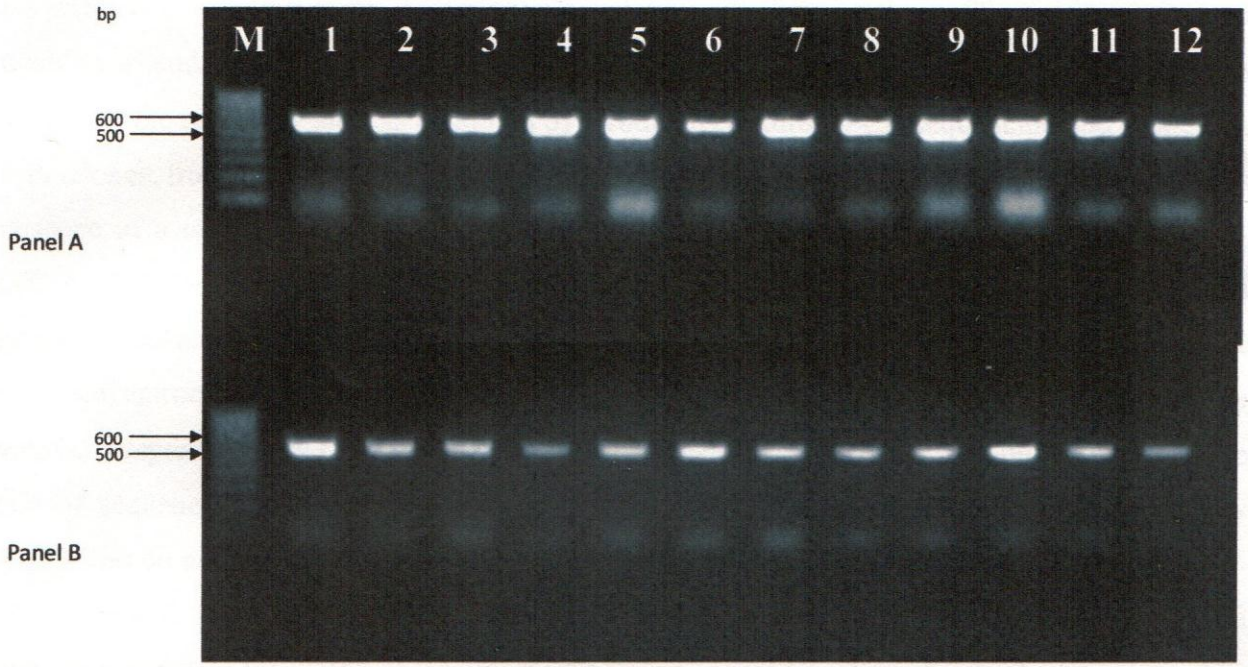


Figure 2.Representative PCR amplification of genomic DNA extracts from *G. pallidipes* carcasses using *G. m. morsitans* Attacin D specific primer homologous.

Panel A represents *G. pallidipes* sampled from Nguruman, Panel B represents *G. pallidipes* sampled from Busia-Teso, lane M represents 100bp molecular weight maker while lanes 1-12 represents individual samples. The amplicons were separated on the 2% agarose.

4.1.2. Comparative analysis of Attacin D DNA from *G. pallidipes* from Nguruman and Busia-Teso and G. *m. morsitans*

Summary of multiple sequence alighments of the Attacin D from *G. pallidipes* against that of other *Glossina* species are presented in Fig 3. The most polymorphism types observed in this sequence alignment are Mutations, deletions, substitutions but with predominance of mutation in coding sites. There are 473 sites, excluding sites with gaps. Two types of AttD is present in the two study sites as exhibited by productds of direct PCR sequencing and sequences from clones from individual alleles from each individual's flies. The analysis also reveals presence of a conserved conserved region (366-AGT CGT ACG CAT TTG GAC AAT GGT TTC AAT TTG ATC GTG TTG GCG GCG G-415). Comparison of the *G. pallidipes* sequences against the *attacin* D sequence of *Glossina morsiatns morsitans* and *G. pallidipes* reveal presence of several introns in all individuals flies sequences in position 170 to 180, also present in *G.m morsitans* represented by CTTTCGTATAAT. Nevertheless, in Busia-Teso, another characteristic type of sequences is observed with introns and having a polymorphic region. The insactary (TRC) flies do not have any particular difference to Gp73 used as a control for direct sequences.

Overall DNA polymorphism of Nguruman and Busia-Teso *G. pallidipes* sequences including insactary fly (TRC) shows strong DNA polymorphism downstream from position 199 to position 298 reaching Theta value of 0.037 with corresponding Pi value of 0.012± 0.0033 (Fig 3) , with 19 polymorphic sites and weak polymorphism upstream toward 473 polymorphic sites. The G+C content at coding positions was 0.445 for the 473 sites, Total number of mutations (Eta) was 65 with 12 haplotypes (Nucleotide Diversity), 0.278 haplotype (gene) diversity, Hd: and 0.00817 Nucleotide diversity (per site) Pi. The were 62 polymorphic sites, (S) and 0.00355 Haplotype diversity variance. The standard deviation of Haplotype diversity: was 0.060 with a sampling variance (Pi) of 0.0000108 with SD.0.0033. The sequences had conservation (C) of 0.869. The overall / average nucleotide difference (k) was 3.866.

In Nguruman, the there were 4 polymorphic/segregating sites (S), selected from region 1 to 473 with 473 sites. There were four mutations and five haplotypes with a diversity of 0.134; the variance of haplotype diversity was 0.00370 with a standard deviation of 0.061. The nucleotide diversity was 0.00029 with a Theta (per site) from Eta of 0.00183. The average number of nucleotide differences was 0.138.

In Busia–Teso, there were 43 Parsimony informative two variants sites and three Singleton variable sites two variants site positions 59; 206 and 386. There were seven haplotypes, with a diversity of 0.4762 ±0.1. The nucleotide diversity was 0.01842 with a haplotype of diversity variance of 0.01003. The population exhibited a standard haplotype deviation diversity of 0.100. The nucleotide diversity was 0.01842 with theta (per site) from Eta of 0.02345. The average number of nucleotide differences was 8.713.

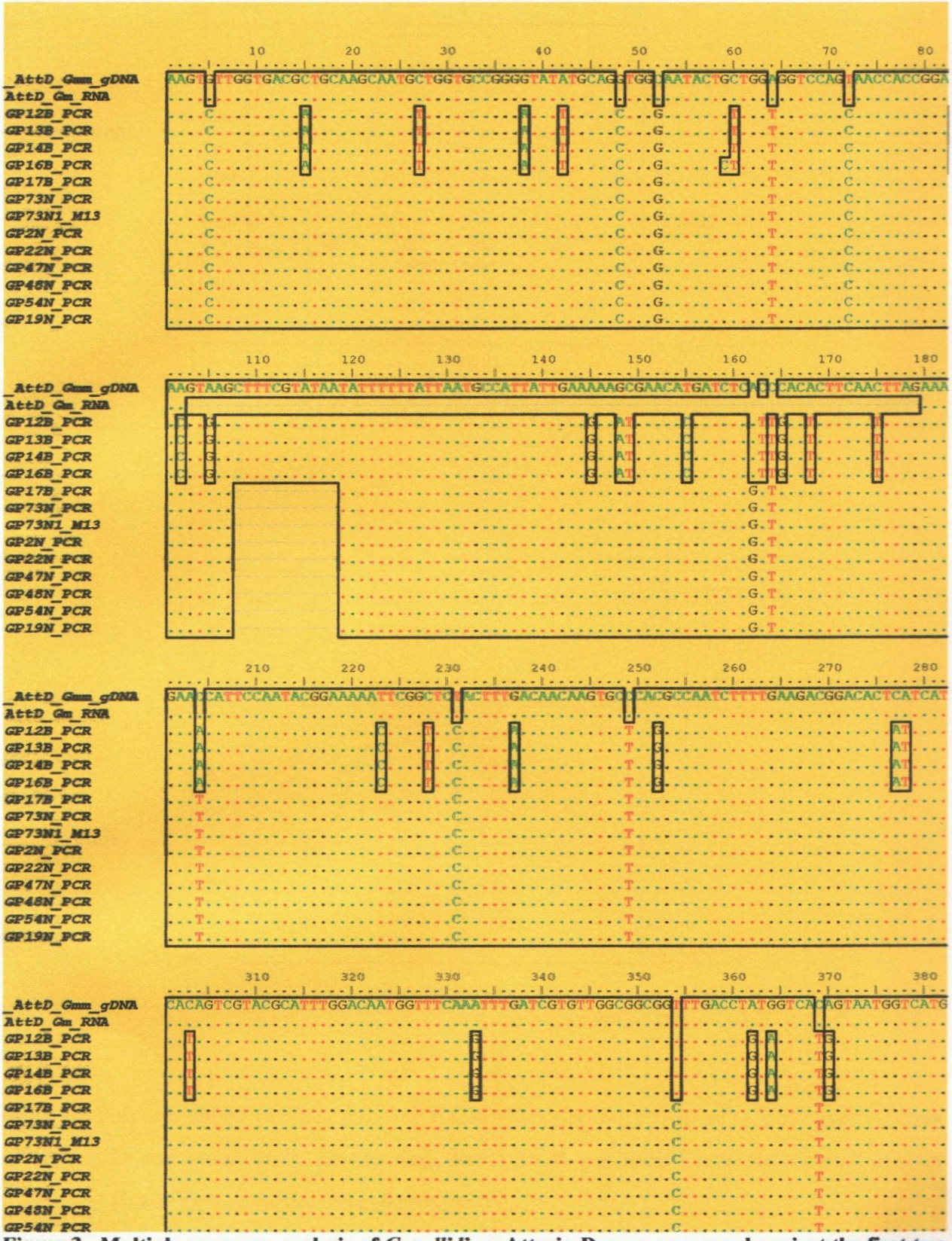
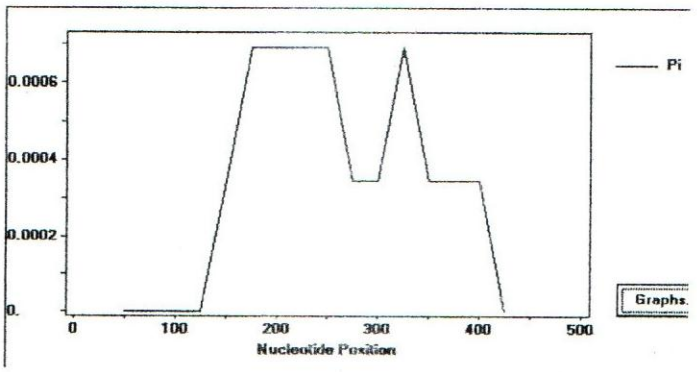
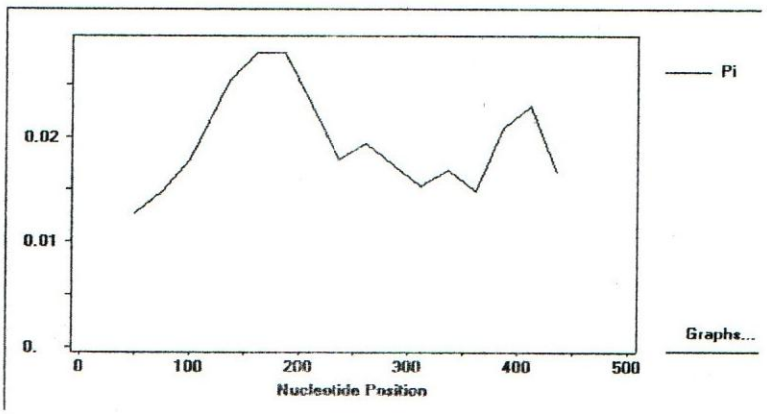


Figure 3 . Multiple sequence analysis of *G. pallidipes* Attacin D gene, compared against the first two *G. m. morsitans* genomic AttD and RNA coding sequence

Nguruman



Busia-Teso



Overall

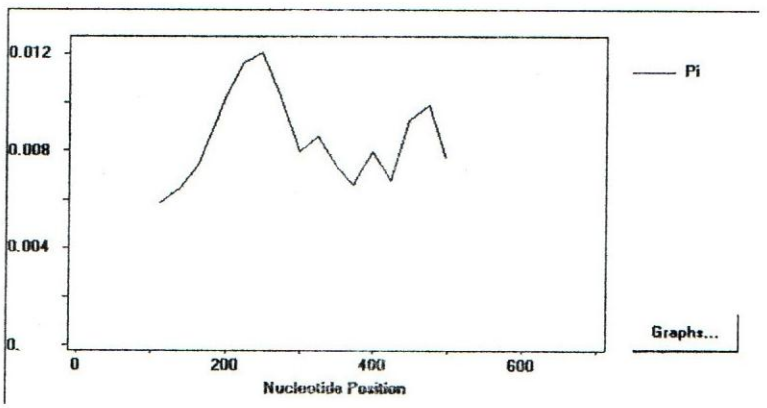


Figure 4. Polymorphism profiles (DNAsp nucleotides diversity (Pi)) of *G. pallidipes* Attacin D sequences from clones and direct PCR products from Nguruman and in Busia-Teso, and the overall perspective.

4.2. Infection rates in *G. pallidipes* populations of Nguruman and Busia-Teso in Kenya with trypanosomes in relation to polymorphisms of *Attacin* D immune genes in the respective flies

4.2.1 Microscopy detection of trypanosome infection in midgut of fatbody tissues from *G. pallidipes* from Nguruman and Busia-Teso, Kenya

There were 586 and 17 non-teneral *G. pallidipes* sampled from Nguruman and Busia-Teso respectively. This represented 76% (n= 770) and 51.51% (n = 33) of the *G. pallidpes* sampled in Nguruman and Busia-Teso respectively. From the 205 and all of the none tenerals from Nguruman and Busia-Teso respectively dissected to remove the midgut and fat body, only two of the flies, one each from Nguruman and Busia–Teso were infected trypanosomes, as revealed by microspcopic examination. In both cases, the infections were detected in the midgut and proboscis, suggesting mixed infection with *T. congolense* and *T. vivax*.

4.2.2. PCR detection and/or confirmation of trypanosome infection in midgut and fatbody of *G.palidipes* from Nguruman and Busia–Teso, Kenya

Application of trypasosome specific ITS based nested PCR on cDNA from midgut and fat body tissues of *G. pallidipes* from Nguruman and Busia–Teso revealed presence of putative Trypanosome DNA in the tissues of flies sampled from both study sites (Fig 4.). Fragments obtained were about however 605bp against the 611bp expected for *T.vivax* infections. Among the 31 and 19 flies selected for this analysis from Nguruman and Busia–Teso respectively, two and four flies respective flies had putative trypanosome DNA in their midgut and fatbody tissues.

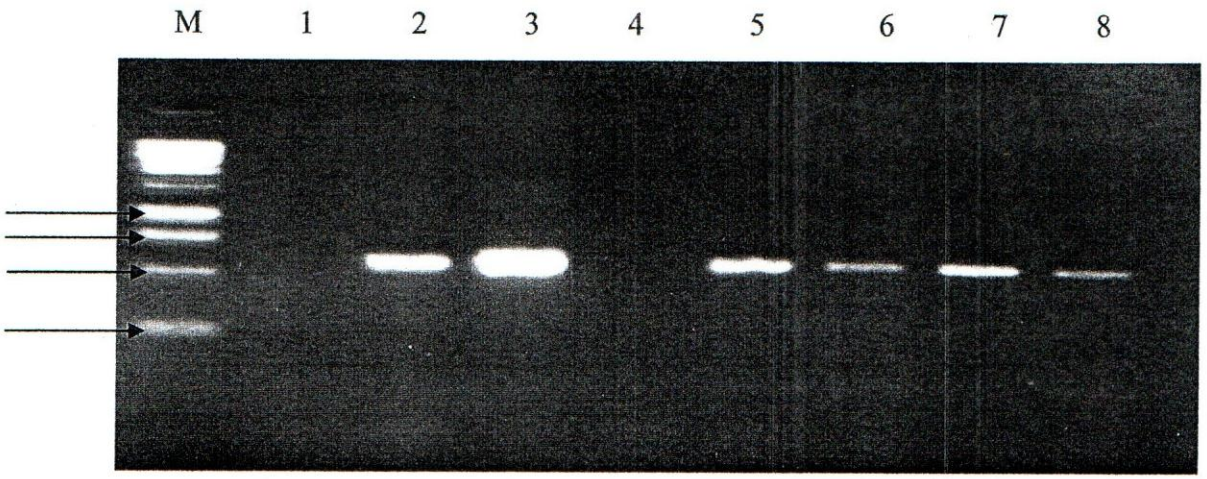


Figure 5. Detection of trypanosomes in midgut and fat body of *G. pallidipes* from Nguruman and Busia-Teso using ITS primers

lane M represents 100bp molecular weight maker. Lanes 1-4 and 5-8 represents cDNA PCR amplicons from midgut or fat body from *G. pallidipes*in Nguruman and Busia-Teso respectively. The amplicons were separated on the 2% agarose.

4.3. Putative differences between *G. pallidipes* populations of Nguruman and Busia-Teso in Kenya, in genomes of *Anopheles*, Glossina, *Aedes* and *Rhodnius* genera, based on *Attacin* D homolog comparative analyses using bioinformatic approaches

The putative *G. pallidipes* Attacin D homologous were identified only in *G. m. morsitans* and in *Aedes aegypti* among insect vectors of pathogens in *Glossina, Aedes* and *Rhodnius* genera. The *Ae. aegypti* homologue is only putative and has not been further characterized. Interproscan analyses of the putative *G. pallidipes* Attacin D sequences confirm presence of a C terminal Attacin domain, and two other unidentified and unitegrated domains. The analysis did not reveal any differences in domain architecture among the putative *G. pallidipes* attacin D sequences.

Table 2: Results of homology searches for putative *G. pallidipes* Attacin D functional homologous in *Glossina, Aedes,* Anopheles and *Rhodnius* genera

Peptide	Vector	Accession	Max Score	Total Score	Query Coverage	E-Value	Max Identit
Antimicrobial peptide Attacin D	*G. m. morsitans*	CAP78962.1	209	209	64%	1e-54	96%
Antimicrobial peptide Attacin B	*G. m. morsitans*	CAP78961.1	159	159	64%	2e-39	71%
Antimicrobial peptide Attacin A11/12/21	*G. m. morsitans*	CAP78958.1	159	159	64%	2e-39	71%
Antimicrobial peptide Attacin A1	*G. m. morsitans*	AAT35580.1	159	159	64%	2e-39	71%
Antimicrobial peptide Attacin AttA	*G. m. morsitans*	Q8WTD3.1	155	155	64%	2e-38	69%
Antibacterial peptide, putative	*Aedes aegypti*	XP_001656768.1	69.7	69.7	64%	1e-12	36%
None	*Anopheles genera*	-	-	-	-	-	-
None	*Rhodnius genera*	-	-	-	-	-	-

CHAPTER FIVE

5.1 DISCUSSION

Insects produce a limited variety of antibacterial peptides to combat a wide diversity of pathogens. These peptides are often conserved across evolutionarily distant taxa, but little is known about the level and structure of polymorphism within species. The general results analysis of Attacin D multiple sequences shows two different types of sequences among and within the two *G. pallidipes* populations that also differ both from the *G. m. morsitans* sequence, used as a reference for *Attacin D* in nucleotide level. The gDNA PCR product direct sequencing may amplify all possible existant alleles and the PCR product and therefore was the necessity to clone the allels in pGEM-T vector to select one single allele of *Attacin D.* The differences observed between a direct PCR sequence and a clone of the same sequence might be just an error added by Taq polymerase activity.

Many different genes known to be associated with various diseases have been identified, and for which sequence polymorphisms inducing protein changes have been documented (Van den Bossche *et al.,* 2006). The *Attacin* gDNA sequences analysis shows polymorphisms that could arise either from allelic variations or from the presence of additional Attacin genomic loci (Wang *et al.,* 2008). All sequences were compared against the *G. m. morsitans,* the only sequence available in NCBI database for *Glossina's Attacin D.* These sequences has been use to adjust the primer design in *G. pallidipes* species for this study. A designed primer has been successful to amplify AttD gene in *G. Pallidipes* with of 529 base pairs sequences size total in DNA level. The Attacin D has two loci which are genetically different and 62 variables sites, the selected region of 641 sites with exact sites size of 473. This sequence analysis shows that mutation are the salient feature with total of 65 mutations and 12 different haplotypes and Haplotype (gene) diversity, Hd: 0.278. AttD in *G. pallidipes* is a diploid genome (one genomic position) with sequence variation and located in an autosomal chromosome, interspecific divergence between loci 1 is 210 over 18 in locus2 (χ^2=6.086 and P value 0.0136). The Busia-Teso *G.pallidipes* population is highly polymorphic. It is also observed that the polymorphism in the two populations is high upstream and goes lower downstream the DNA sequences.

In comparison to the infection positive flies' sequences (Gp73, 22 for Nguruman and 1, 2, 3 and4 for Busia-Teso) does not show any significant differences from most of negative flies

sequences in the sequence alignment in both PCR and clones. The major feature is that Lake Victoria-south hills flies shows flagrant polymorphism characterized by substitution of C, G, T in contrast to other normal sequences. The population genetic studies have shown that the two populations are highly differentiated from each other (Ouma *et al.*, 2005), from that fact, the presumption can be made that the infection in wild tsetse does not affect the genomic statute of an infected fly in AttacinD response the infection.

Infection rate by PCR is difficult to conclude (Sample size inequality), disproportion 17 on one side and 31 on the other for PCR. The small sample size of Busia-Teso can be explain by the fact that the ongoing PATTEC project in the region is more efficient, with tsetse target placed in approximately two to three targets per 10 meters square. The catch ratio is three non-teneral *G.pallidipes* per day and the infection rate for macroscopic diagnosis was 0.48% in Nguruman (1/205) and 5.88% in Busia-Teso (1/17) from midgut and fate body examination, This microscopic results have shown that sample were positive in both midgut and proboscis which is the parasitological characteristic for *T. vivax* by microscopy.

There is no relationship between the infection level and the location, but the Busia–Teso greater value may be due to the fact that the fly population in the region has reduce and wild and domestic animals(reservoir) responsible for parasites transmission cycle are exposed to carry infection even if the tsetse population has reduced. Meaning that little tsetse population can infect great number of animal, for instance one infected fly can infect more than two animals. In contrast the low Nguruman *G.pallidipes* infection rate may be explained in the fact that during tsetse collection period the Nguruman (semi-arid region), domestics and wild animals (trypanosomes reservoirs) has moved to the neighboring region of Tanzania because the region has experienced extended dry season of twelve month and there is no green feeding grass still tsetse population is larger .

An examination of the relationships between trypanosome infection rates and natural hosts of three tsetse species (Moloo *et al.*, 1971) showed that *vivax* –type infections originated from bovid while *congolense*-type infections came from bovids and bushpig. Ashcroft (1959), discussing the feeding habits of tsetse in relation to wild animals as reservoirs of trypanosomes, suggested that the number of tsetse carrying trypanosomes and the relative proportion of the different trypanosome species may be closely related to the host animals on which they feed.

The availability of the host can influence infection rates in tsetse. At the Kenyan coast, *G. pallidipes* had higher rates of trypanosome infections in areas with high densities of domestic animals than in areas with wild animals only (Tarimo *et al.*, 1984). This was attributed to higher parasitaemias in domestic animals compared to wild animals. Allsopp (1972) investigated the role of game animals in the maintenance of trypanosomiasis in the Lambwe valley in Kenya and found an overall trypanosome infection rate of only 16% in the wild animals. However, 90% of bushbucks, the preferred host of *G. pallidipes,* were infected thereby illustrating the importance of the host on the probability that a tsetse fly picks up a trypanosome infection. The interaction animals-food might play a major role in conditioning tsetse –infection factor in these two different locations. It has been shown that tsetse flies from Nguruman have lower survival rates and are less susceptible to trypanosome infection than those from Busia-Teso. However, there were no significant differences in infection rates of the proboscis, midgut and salivary gland between the two subpopulations (Okoth et *al.,* 2006).

The Nested PCR for parasite identification has shown evidence accuracy with ITS rDNA primers (Cox, *et al.*, 2005) with infection frequency of 23.53% and 6.45% in Busia-Teso and Nguruman areas respectively. The DNA fragment size served for parasite identification shows bands size of litle greater than 605 bp positive for one genotype corresponding to *Trypanosoma vivax* as confirmed in the bioinformatic NCBI database. The microscopy remains the most appropriate methods for the clinical diagnosis but lacks sensitivity to be consider as the gold standard, but the molecular methods provids an alternative gold standard for the detection of trypanosomiasis (Thumbi *et al.*,2008). This means the disproportional level of infection in the two populations is due to the bias of sample size. The MSA of PCR and clones sequences show DNA polymorphism. Most Nguruman PCR product of direct sequencing sequences are identical to Busia-Teso and have no nucleotides variations including positive flies sequences. Results of homology searches indicate that the Attacin D appears to be an anti-microbial peptide specific to tsetse flies, with a distant relative in *Ae. aegypti.* This presents the peptide a tsetse specific molecule that can be applied in control of trypasomiasis transmission using through manipulation of the tsetse vector.

5.2. CONCLUSION

There is putative and viable homolog of *G. m. Morsitans* Attacin D gene in *G. pallidipes* populations from Nguruman and Busia-Teso. There are to major types of Attacin D between the two populations, with one present in both Nguruman and Busia-Teso and the other epecific to Busia-Teso. Both microscopy and PCR revealed trypasosmome infection in the *G. pallidipes* sampled in Nguruman and Busia-Teso, with PCR being more sensitive. Putative *G. pallidipes* Attacin D homologous were present only in *G. m. morsitans* and in *Aedes aegypti* among insect vectors of pathogens in *Glossina, Aedes* and *Rhodnius* genera.

5.3. RECOMMENDATIONS

1. Further studies should be conducted to establish role of the polymorphism observed on the competence of *G. pallidipes* to transmit trypansosomes.

2. Comparative *in silico* and *in vitro* studies should be conducted on Attacin D from other hematophagous vectors of human and veterinary diseases should be conducted.

3. The findings might facilitate identification of putative relationship among them in relation to their effect on immunity of the respective vector.

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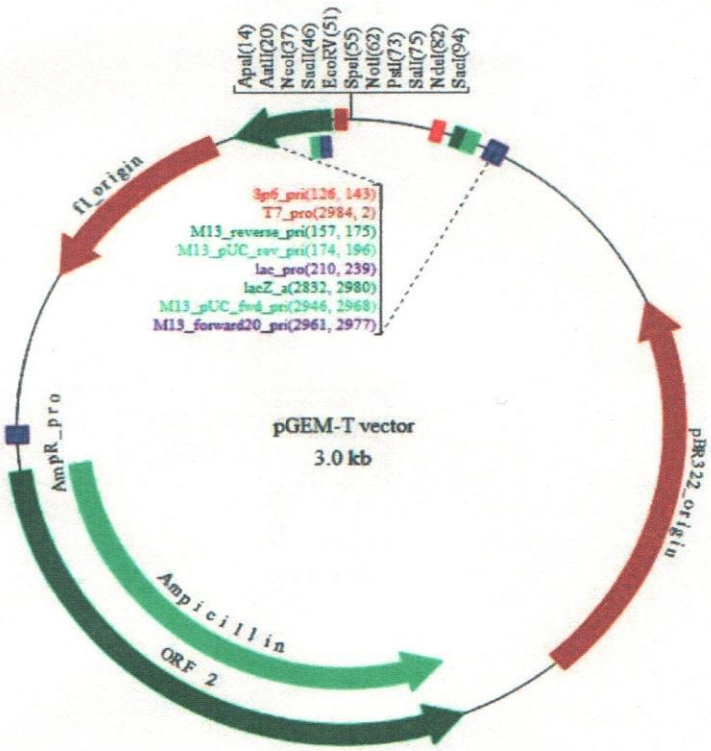
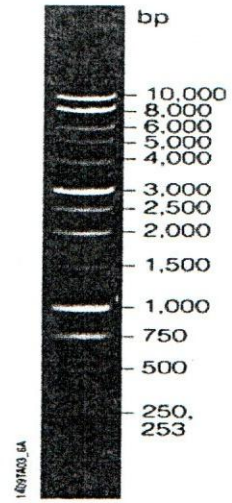
APPEDICES

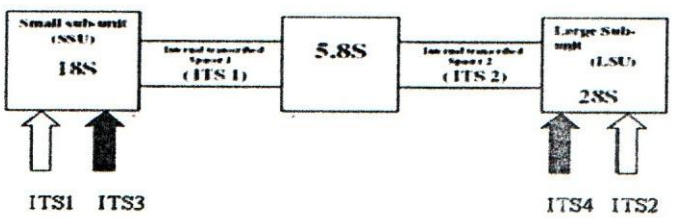
Appedix 1: pGEM-T vector





1 Kb ladder 12ul bench top

Appedix 3: Priming sites for ITS primers



Appendix 4. DNA-Polymorphism analysis features using DNAsp software

Window	Midpoint	Pi	Theta	S
1-162	112	0.00585	0.02318	12
88-198	137	0.00643	0.02318	12
113-223	162	0.00740	0.02411	13
138-248	198	0.01009	0.02704	14
163-273	223	0.01164	0.03284	17
199-298	248	0.01204	0.03670	19
224-323	273	0.01029	0.03284	17
249-448	298	0.00798	0.02704	14
274-373	323	0.00858	0.03090	16
299-398	348	0.00740	0.02511	13
324-423	373	0.00661	0.02125	11
349-448	398	0.00798	0.02704	14
374-473	423	0.00679	0.01931	10
399-498	448	0.00930	0.02511	13
424-523	473	0.00989	0.02704	14
449-641	497	0.00769	0.01774	9

S: Number of segregation per sites; Pi: average number of Nucleotide differences/diversity per site

Appendix 5. Anaysis of Attacin D polymorphism in the *G. pallidipes* from Busia–Teso

Window	Midpoint	Pi	Theta	S
1-100	50	0.01243	0.01677	7
26-136	75	0.01441	0.01916	8
51-161	100	0.01745	0.02395	10
76-186	136	0.02483	0.03114	13
101-211	161	0.02736	0.03593	15
137-236	186	0.02736	0.03593	15
162-261	211	0.02234	0.02875	12
187-286	236	0.01745	0.02395	10
212-311	261	0.01889	0.02395	10
237-336	286	0.01691	0.02156	9
262-361	311	0.01492	0.01916	8
287-386	336	0.01640	0.02156	9
312-411	361	0.01441	0.01916	8
337-436	386	0.02036	0.02635	11
362-461	411	0.02234	0.02875	12
387-484	435	0.01820	0.02200	9

Appendix 6. Anaysis of Attacin D polymorphism in the *G. pallidipes* from Nguruman

Window	Midpoint	Pi	Theta	S
1-100	50	0.00000	0.00000	0
26-125	75	0.00000	0.00000	0
51-150	100	0.00000	0.00000	0
76-275	125	0.00000	0.00000	0
101-200	150	0.00034	0.00216	1
126-225	175	0.00069	0.00432	2
151-250	200	0.00069	0.00432	2
176-275	225	0.00069	0.00432	2
201-300	250	0.00069	0.00432	2
226-325	275	0.00034	0.00216	1
251-350	300	0.00034	0.00216	1
276-375	325	0.00069	0.00432	2
301-400	350	0.00034	0.00216	1
326-425	375	0.00034	0.00216	1
351-450	400	0.00034	0.00216	1
376-473	424	0.00000	0.00000	0

Appedix 7. Comparative analysis of Hudson, Kreitman and Aguadé's test (HKA test) (1987)

	Locus 1	Locus 2
Intra-specific polymorphism		
Segregating sites(Observed)	9	8
Segregating sites(Expected)	13.48	3.52
Total number of sites	414	414
Sample size	81	81
Interspecific Divergence	210	18
No. Difference (Obs.)	205.52	22.48
No. Difference (Exp.)	4052	324
Chromosomal location	Autosome	Autosome
X-square value:	6.086	P:0.0136