LECTIN EXPRESSION ASSOCIATED WITH *PLASMODIUM* INFECTIONS IN *ANOPHELES*

by

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DECLARATION

This is my original work and has not been presented for a degree in any other university.

Signed..... Date...26/08/03.

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DEDICATION

This thesis is dedicated to my parents, Mr. and Mrs. Amenya and my brothers, Patrick, Adams, Thomas and Florentine, for their love and support during the entire period.

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ABSTRACT

The variation of the mannose-binding protein (MBP) gene and the role of lectins during infection in mosquitoes were investigated using genomic DNA approach based on sequencing of the gene and the levels of its expression determined using semi-quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) approach.

Using shotgun cloning strategy, sequences were determined from cloned PCR products obtained from genomic DNA of *An.gambiae s.s*, *An. arabiensis* and *An. funestus* and aligned following amino acid translation. BLAST program revealed that MBP-M1, MBP-I3 and MBP-I5 clones showed high homology, with *Anopheles* protein precusor A16 (Accession Q93118). These results were further confirmed by pairwise alignment using CLUSTAL W program. The remaining clones (MBP-M2, MBP-MW1, MBP-MW4, MBP-MW5, MBP-MW12, MBP-C1 AND MBP-C15) showed no signature of C-type lectin domain but were closely related based on the positioning of these genes on the polytene chromosome. Phylogenetic analysis indicated that MBP-M1, MBP-I3 and MBP-I5 clones are closely related. Since the representatives of the MBP clones harbour comparatively short fragments, clustering of these clones on the nodes supports a more close phylogenetic relationship between and within *Anopheles sp*.

RT-PCR analysis showed that MBP is expressed differently with respect to blood-meal status of the mosquitoes. Altered expression of MBP may contribute to the risk of disease transmission by the *Plasmodium*. The studies also showed that MBP levels decreased with time post-infection. These changes may disturb normal MBP levels and create favourable condition for parasite establishment within the mosquito midgut. These observations raise the possibility that low levels of MBP may contribute to the increased malaria transmission by the mosquito and hence *Plasmodium* plays a key role in its down-regulation.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Mosquito-transmitted diseases are on the rise and have a significant impact on human morbidity and mortality on a global scale. The mosquito is a well adapted organism and a scourge of humanity. The principal mosquito-borne human illnesses of malaria, filariasis, dengue and yellow fever are almost exclusively restricted to the tropics (Holt *et al.*, 2002). Malaria is caused by members of the genus *Plasmodium* and transmitted to humans by mosquitoes of the genus *Anopheles*. The disease is thought to be responsible for 500 million cases of illness and up to 2.7 million deaths occur annually with more than 90% occurring in sub-Saharan Africa (Breman *et al.*, 2001). Despite tremendous control efforts, malaria remains a public health problem in more than 90 countries inhabited by 40% of the global population. Children that survive the early years of life develop varying levels of natural immunity, but this does not protect them from repeated infections and illness throughout life (Beier, 1998)

The intensity of malaria parasite transmission varies geographically according to vector species of *Anopheles* mosquitoes. Risk is measured in terms of exposure to infective mosquitoes, with the heaviest annual transmission intensity ranging from 200 to > 1000

bites per person (Beier, 1998). Interruption of transmission is technically difficult in many parts of the world due to the limitations of current management tactics. Historically, successful malaria control has been effected through management or control of vector mosquito populations, thereby breaking the cycle of human disease transmission (Collins *et al.*, 1994; Collins *et al.*, 1995). In recent years, the problem of malaria has been exacerbated by the development and rapid spread of resistance in *P. falciparum* to the more commonly used and affordable antimalarial drugs such as Chloroquine and Pyrimethamine-sulfadoxine (Collins *et al.*, 2000).

The emergence of insecticide resistance in African malaria vectors threatens to worsen this problem further. Even though the malaria control community is returning to strategies such as the use of bednets impregnated with pyrethroid insecticides, this method has also been shown to select for behavioural resistance in mosquitoes, induce changes in their biting cycle and this may render bednets useless in the long run (Vulule *et al.*, 1994). Although new approaches to drug therapy show tremendous potential for the control of other diseases, there are no promising strategies on the horizon for the control of malaria (Bockarie *et al.*, 1998). Vaccine development remains a viable approach but undoubtedly will require many more years of intensive research and will face continually the problems

associated with a genetically plastic pathogen (Miller *et al.*, 1999). Indeed, these problems have provided new impetus for the search of alternative sustainable management strategies for malaria.

The application of molecular biology, genomics and bioinformatics to vector biology can be considered essential for identification of new control targets and the assessment of the status of current control strategies. The ultimate goal of these efforts is the development of novel vector and disease management strategies. These include the possibility of genetically modifying mosquitoes to reduce their vector competence, improved management of drug and insecticide resistance and the pre-intervention assessment of putative control strategies. To contribute to these challenges, the International Centre of Insect physiology and Ecology (ICIPE) has been working on programmes based on an understanding of the basic ecology, behaviour and chemical ecology of mosquitoes. For example, comprehensive studies on the larval ecology, behaviour and population genetics of key Afrotropical malaria vectors and the search for more effective agents for behaviour manipulation and control of mosquitoes have been initiated. These efforts should result in integrated and sustainable tools and tactics that will contribute towards global efforts to eradicate malaria.

1.2 Literature Review

1.2.1 *Anopheles species*

Three efficient vectors in the subgenus *Cellia*: *Anopheles gambiae, Anopheles arabiensis* and *Anopheles funestus* are the most important malaria vectors in tropical Africa (Sharakhov *et al.*, 2002). These species co-occur geographically across sub-Saharan Africa and can inhabit the same villages, shelter in the same house and feed on the same individual. Yet *An. funestus* has evolved unique breeding site preferences, mating behaviour, relative seasonal abundance and degree of specialization on humans. *An. gambiae* is a complex comprising of *An. gambiae s.s.*, *An. arabiensis*, *An. quadriannulatus*, *An. merus* that are morphologically indistinguishable but exhibit distinct genetic and eco-ethological differences reflected in their ability to transmit malaria (della Torre *et al.*, 2002). Within this complex, mosquitoes may differ in their efficiency of transmitting malaria due to the environmental conditions and factors affecting their abundance, blood-feeding behaviour, survival and ability to support development of malaria parasite.

Under the natural situations, most vector-competent mosquitoes do not become infected by malaria parasites, and this is the case even when mosquitoes feed on known gametocyte carriers (Medley *et al.*, 1993). These natural, low prevalence rates make investigations of

specificity of infectivity extremely difficult within a given mosquito-malaria combination. Of the 90% or so of mosquitoes that remain uninfected, be it refractoriness or the consequence of a low infectivity to an otherwise susceptible mosquito may need clarification (Billingsley *et al.*, 1997). Mosquito infectivity is affected by factors such as, post-feeding dynamics of digestive enzyme activities (Billingsley *et al.*, 1991), the type of peritrophic matrix and the timing of its production with blood-meal ingestion (Billingsley *et al.*, 1992). The hematophagous appetite of the female mosquito is exemplified by its remarkable ability to ingest up to four times its own weight in blood. Therefore, interfering with this process would be a powerful means of management tactic (Holt *et al.*, 2002).

1.2.2 *Plasmodium species*

Plasmodium species are Apicomplexans and exhibit a heteroxenous life cycle involving a vertebrate host and an arthropod vector. Vertebrate hosts include reptiles, birds, rodents, monkeys and humans. *Plasmodium* species are quite host specific and there are no zoonoses. Four distinct species infect humans: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. The species differ in regard to their morphology, details of their life cycles and clinical manifestations exhibited by the host. *Plasmodium falciparum* and *P. vivax* are the two parasites that contribute most heavily to the malaria burden (Richie *et al.*, 2002).

Both parasites develop over 48 hours in red blood cells (RBCs) producing around 20 merozoites per mature parasite with each merozoite able to invade other RBCs. A small portion of asexual parasites convert to gametocytes that are essential for transmitting the infection to other hosts through female Anopheline mosquito but cause no disease (Miller *et al.*, 2002). *Plasmodium vivax* causes severe anaemia whereas *P. falciparum* causes many complications of cerebral malaria, hypoglycaemia, metabolic acidosis and respiratory distress (Miller *et al.*, 2002). Conventional approaches for diagnosis of *P. falciparum* infection include microscopic examination of blood smears, the use of dip sticks that assay *P. falciparum* histidine-rich protein 2 and amplification of *P. falciparum* genes using polymerase chain reaction (PCR) (Mills *et al.*, 1999).

1.2.3 Parasite-vector interactions in the life cycle of malaria

The life cycle of malaria is relatively complex requiring passage of the parasite through several organ systems and two very different hosts (Fig. 1). Malaria sporozoites may be injected into a human host when an infected female attempts to take a blood-meal, although in a substantial proportion of feeds, sporozoites will not be transmitted (Beier *et al.*, 1991a). Most sporozoites are injected into the skin from which they disappear (Ponnudurai *et al*, 1991). The sporozoites enter the circulatory system and within 30-60 minutes invade a liver cell.

Successfully transmitted sporozoites enter liver parenchymal cells where they develop and undergo a form of asexual replication. Eventually, the schizonts burst releasing thousands of merozoites into the bloodstream whereupon they enter red blood cells (Bruce-Chwatt, 1985). Once inside the erythrocyte, parasites begin to grow by ingesting haemoglobin and other nutrients. Eventually, these growing trophozoites undergo schizogony on a more modest scale than that seen in the liver. The bursting of infected red blood cells is associated with the classic malaria paroxysm of chills and fever. Released merozoites infect other red blood cells. Eventually, some of them differentiate into pre-sexual forms, the gametocytes. When a mosquito takes a blood meal from a gametocytemic human, the gametocytes differentiate into gametes inside the mosquito midgut where fertilization occurs. Soon after fertilization, the ookinete migrates through the peritrophic membrane and the midgut epithelium and attaches to the outer wall of the gut where it becomes an oocyst. Penetration of the peritrophic membrane appears to be mediated by an ookinete chitinase, as chitinase inhibitors block infection of the midgut (Shahabuddin *et al.*, 1994).

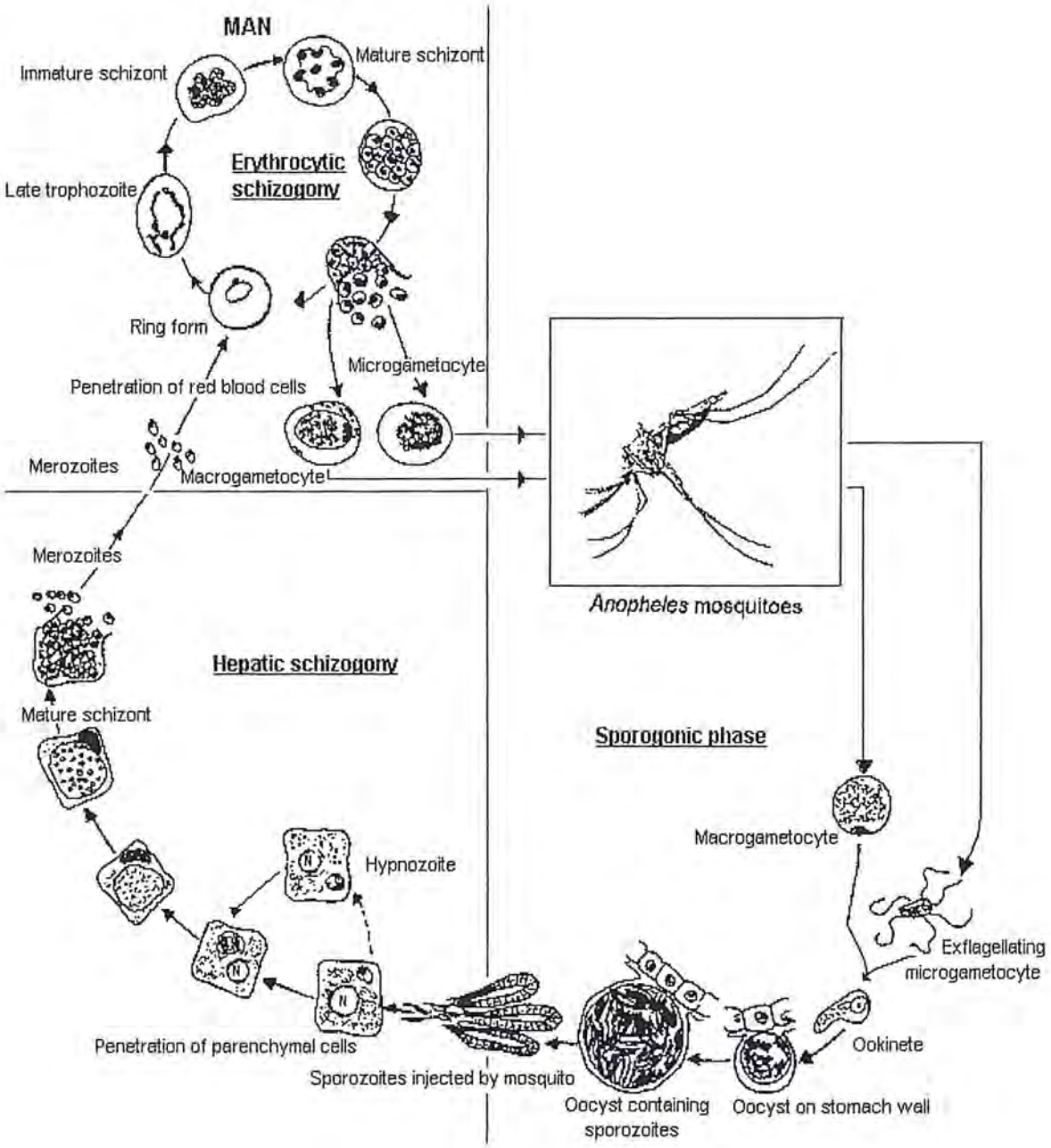

Fig.1: Life cycle of *Plasmodium sp.* (Altas of medical parasitology http://www.cdfound.to.it/-atlas.htm)

1.2.4 Mosquito midgut

Majority of mosquito species require a blood-meal for egg production and this blood mixed with saliva is pumped through the foregut and into the midgut. Apart from the physiological processes associated with digestion and initiation of egg production, the blood-meal is also a source of pathogen transmission by mosquitoes (Beernsten *et al.*, 2000). To be transmitted from one host to another, malaria parasites have to complete a complex life cycle in vector mosquitoes, starting in the midgut lumen, crossing through the midgut epithelial barrier and finally invading the salivary glands from where they can be inoculated into the next host during blood feeding (Shen *et al.*, 1999). Huge losses of parasite numbers occur during this process, partially compensated for by proliferation during the midgut-associated oocyst stage (Beier, 1998). At the extreme, the mosquito does not permit survival and transmission of the parasites. In genetically selected refractory mosquito strains, the parasites may be lysed as they transverse the midgut, or they may be encapsulated and melanized at the early oocyst stage (Collins *et al.*, 1986). Consequently, specific defense mechanisms against or in response to these foreign invaders must be considered.

The insect midgut is composed of a single layer of epithelial cells, which are lined at their basal side by a continuous extra-cellular layer,

the basal lamina. On the apical side, the epithelial cell membranes are folded into numerous actin-filled microvilli (Shen *et al.*, 1999). Two extra-cellular structures have been proposed to provide protection to the microvilli: the peritrophic membrane and the glycocalyx (Jacobs-Lorena *et al.*, 1996). The peritrophic membrane (pm) is an extra-cellular sac, composed of chitin, proteins and proteoglycans which completely surround the ingested food and is secreted by the gut epithelial cells. It has been proposed that the pm provides protection against pathogens, keeps protease inhibitors within the lumen and functions as a solid support as well as semi-permeable filter for digestive enzymes and blood proteins (Billingsley, 1990). The pm may influence the development of malarial parasites in mosquitoes by forming a mechanical barrier to invasion by ookinetes. However, it seems that ookinete differentiation is completed before the pm is fully formed and that ookinetes can penetrate it (Meis and Ponnudaria, 1987). Conversely, the pm could direct parasite migration from the gut lumen by chemical recognition mechanisms; in such cases, the differences between sugar components of the pm of *Aedes aegypti* and *An. stephensi* (Berner *et al.*, 1983) may be important in such a case.

Sporozoites possibly recognize characteristic sugar residues in the basal lamina of the mosquito salivary gland (Perrone *et al.*, 1986), and

the inability of sporozoites to bind lectins suggest that they do not carry exposed terminal sugars on their surface (Schulman *et al.*, 1980). The morphology (Rudin *et al.*, 1979; Schneider *et al.*, 1987) and physiology (Graf *et al.*, 1986) of the mosquito midgut are quite well understood and digestive enzymes directly affect the malarial infectivity of mosquitoes by damaging immature ookinetes in the midgut (Gass 1977; Gass and Yeates, 1979). Lectin-binding is thought to be part of a multi-step process by which parasites recognize and attach to host cells prior to invasion (Sherman 1985; Sinden 1985). For example, N-acetyl-glucosamine (GlcNAc) was found to be the most effective inhibitor for the invasion of erythrocytes by *P. falciparum* merozoites (Jungery 1995). Therefore, the carbohydrate moieties of interacting surfaces between mosquitoes and malarial parasites have been studied using the lectin-gold technique (Roth *et al.*, 1983).

1.2.5 Lectins in the gut of important disease vectors

Lectins are carbohydrate-binding proteins which are ubiquitous in protozoans, invertebrates and higher vertebrates (Liener *et al.*, 1986). The multiple roles of lectins in the life cycle of protozoan trypanosomatid flagellates have been well described in *Glossina sp.* (Ibrahim *et al.*, 1984; Maudlin and Welburn, 1987; Maudlin and Welburn, 1991). Lectins secreted in the midgut are not only

responsible for preventing the establishment of midgut infections, but also induce maturation of established midgut trypanosomes (Maudlin and Welburn, 1987; Welburn *et al.*, 1989). Inhibition of midgut lectin activity by glucosamine or GlcNAc has been fund to increase the infection of *Trypanosoma congolense* and *T. brucei* but blocked subsequent maturation of infections and anterior migration of trypanosomes (Maudlin *et al.*, 1994). Galactosamine supplement has been shown to enhance the establishment of *Leishmania major* infections in the ecto-peritrophic space of *Phlebotomus duboscqi* midgut and thus increasing the intensity of midgut infections (Volf *et al.*, 1998). In tsetse, the addition of glucosamine to the blood-meal has been found to significantly increase midgut infections by *Trypanosoma sp.* (Maudlin *et al.*, 1987; Mihok *et al.,* 1992). Also, the addition of GlcNAc has been shown to increase the number of *Brugia pahangi* microfilariae successfully migrating through the midgut wall in the mosquito *Ae. aegypti* (Ham *et al.*, 1991). Because lectin interferes with *Leishmania* development in the sandfly gut, the inhibition of lectin activity by galactosamine prevents the normal decrease of parasite loads in control flies post-infection (Volf *et al.*, 1998).

The presence and distribution of binding sites of a selection of lectins were compared in midguts of *P. gallinaceum*-infected *Ae. aegypti* and *P. berghei* infected *A. stephensi* (Rudin and Hecker, 1989). Results

showed that lectins with a high specificity to GlcNAc bound to *Ae. aegypti* whereas those lectins specific for GalNAc bound to *An. stephensi*. Therefore, it appears that a two-way lectin-carbohydrate interaction between the parasite and vector midgut may be possible and mediate malaria infectivity to the mosquito (Rudin *et al.*, 1989). There is little information pertaining to the nature of the lectin-carbohydrate interaction between the vector gut and parasites despite the fact that specific mechanisms exist that can ensure the success or failure of infection (Billingsley and Sinden, 1997; Rudin *et al.*, 1991).

1.2.6 Mannose - Binding Protein (MBP)

Mannose-binding protein also known as mannose-binding lectin (MBL) or mannan-binding lectin (MBL) is a member of the collectin family of proteins which are characterized by the presence of both a collagenous region and a carbohydrate-recognition domain (CRD). After binding to carbohydrate located on the surface of microorganisms, mammalian mannose-binding proteins (MBPs) found in serum and liver mediate immunoglobulin-independent defensive reactions against pathogens (Weis *et al.*, 1991). The proteins function by binding to specific cell-surface high-mannose oligosaccharides of various bacteria and fungi and kill these organisms by complement-mediated cell lysis (Ikeda *et al.*, 1987) or opsonization (Ezekowitz *et al.*, 1988). MBPs also act as inhibitors of human immunodeficiency

virus and influenza virus infection, presumably by binding to the high-mannose carbohydrates of the viral envelope glycoproteins and blocking attachment to the host cell (Anders *et al.*, 1990). In humans, low serum levels of MBP or the presence of variant alleles have been correlated with a common opsonic defect that causes predisposition to recurrent infections (Turner, 1996).

1.2.7 Lectins in innate immunity

A number of microbial patterns found on the surface of the pathogens have been identified that elicit immune responses in vertebrates and invertebrates (Franc and White, 2000). Insects do not possess the sophisticated immunological system of vertebrates. To defend themselves, they have developed a series of mechanisms such as recognition reactions, activation of proteolytic enzymes that lead to the coagulation of hemolymph and the production of melanin, cellular reactions and peptide synthesis of antimicrobials and inhibitors of proteases (Wilson *et al.*, 1999). These defense reactions are part of the natural immunity in insects. In insects, lectins have been detected in hemolymph, acting as opsonins and agglutinating microorganisms. They are produced during infectious processes, injuries in the tegment and development (Kawasaki *et al.*, 1996). The production of a set of lectins could therefore provide a very effective mechanism of recognition against a broad range of pathogens (Franc *et al.*, 2000).

Recent advances in the field of innate immunity in invertebrates, and in particular insects, revealed the similarities between vertebrate and invertebrate immunity (Hoffmann *et al.*, 1999). This revelation has provided clues on how pathogens may be recognized and eliminated during an infection. Preliminary studies in female *Anopheles* mosquitoes showed that blocking the MBP reduced infection of *Plasmodium* in the mosquito. Sequence analysis at the amino acid level, revealed that variations between species were negligible, but natural variations within and between species have not been examined.

1.3 Justification

Identification of the genes expressed in the digestive tract of the mosquito is of obvious importance for elucidation of the mechanisms that permit parasite development and successful passage through the midgut wall (Dimopoulos *et al.*, 1996). There is growing evidence that lectin-carbohydrate interactions mediate the infections of parasites to their insect vectors, and that their disruption could affect the subsequent establishment of the parasite within the vector (Chen *et al.*, 1999). For example, *Triatoma infestans* refractory to infection with *T. rangeli* produces an array of tissue and hemolymph agglutinins, whereas only hemolymph agglutinins are produced by the susceptible insect *Rhodnius prolixus* (Gregoria and Ratcliffe, 1991). In

trypanosome-tsetse fly interactions, lectins released into the midgut not only lyse trypanosomes and prevent midgut infections, but also provide a signal for the maturation of established trypanosomes to the procyclic forms (Maudlin and Welburn, 1987; Maudlin and Welburn, 1988).

The role of lectins and carbohydrates in mosquito-malaria interactions remain undefined. Using affinity chromatography, Chen *et al.* (1993; 1999) identified four lectins from the hemolymph of the cockroach, *Blaberus discoidalis* named BDL1, BDL2, BDL3 and GSL (β 1,3-glucan specific lectin) with respective binding affinity for mannose, GlcNAc, GalNAc and β 1,3-glucan. BDL1 was shown to have properties similar to the mammalian MBLs in terms of specificity, structure and activation of complement which forms an essential component of vertebrate non-specific immunity while GSL resembled the mammalian C-reactive protein (CRP) (Chen *et al.*, 1993;1999). As female mosquito MBL was recognized by antibodies against BDL1 (Chen and Billingsley, 1999), it may have similar structure and play an important role in mosquito defense against pathogenic microbes. Of interest therefore, was its potential interaction with the malaria parasite in the midgut and the variations of the gene within mosquito populations. This project was therefore undertaken to define the role of lectin during malaria infections of the mosquito and the variation in the lectin

gene sequence in natural mosquito populations. Consequently, the variation in the gene sequence could be related to mosquitoes that are either refractory or susceptible to parasite infection. Similarly, for a given *Anopheles* mosquito species, a correlation between MBP levels and infections could be related to either refractoriness or susceptibility in these populations

1.4 Hypothesis

1. There is heterogeneity of the mannose binding protein (MBP) gene in the mosquito populations.
2. There is a correlation between levels of MBP and the development of the malaria parasite in the mosquito midgut.

1.5 Objectives

1.5.1 Broad Objectives

The overall objective of this project was to assess the heterogeneity of the mannose-binding protein (MBP) gene in the Anopheline mosquitoes and to determine its expression levels after infectious blood-meals and oocyst development of *P. falciparum*.

1.5.2 Specific objectives

1. To amplify and sequence the MBP gene from genomic DNA of individual mosquitoes (*An. gambiae s.s.*, *An. arabiensis,*

An. funestus).

2. To analyse the sequence variations in the MBP gene between individual mosquitoes and thereby describe the heterogeneity of the gene in populations of *An. gambiae s.s.*, *An. arabiensis, An. funestus*
3. To identify by semi-quantitative RT-PCR the expression of the MBP gene in the midgut of mosquitoes infected with the oocyst stage of *P. falciparum*.
4. To identify by semi-quantitative RT-PCR the expression of the MBP gene in the midgut of mosquitoes following a gametocytaemic blood meal.

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 Experimental Insects and Parasites

2.1.1 Mosquitoes

Anopheles gambiae s.s were obtained from ICIPE colony mosquitoes (Mbita strain, Kenya and Ifakara strain, Tanzania) and were maintained at 28° C and 80% humidity. Four to five day old female mosquitoes used in the experiments were either fed on uninfected blood-meal or starved for 12 hr prior to feeding on cultured gametocytes. Female *An. arabiensis* mosquitoes were collected from houses in the Mwea Irrigation Scheme (Kirinyaga district, Kenya) while *An. funestus* were collected from North coast (Mombasa district, Kenya).

2.1.2 Culturing of malaria parasites

Plasmodium falciparum strain ENT 36 were cultured *in vitro* according to the method of Trager and Jensen (1976) as modified by Ifediba and Vanderberg (1981). Cultures (0.2% parasitaemia, 6% hematocrit of blood group O Rh^+ human erythrocytes) were set in 25-mm^3 canted neck flasks. The flasks were aerated with a gas mixture (92% N_2, 5% O_2 and 3% CO_2) and incubated at 37° C with daily media changes. The parasitaemia of the cultures was monitored by microscopic examination of Giemsa-stained thin films. At 5-6% parasitaemia, the cultures were pooled and fresh red blood cells and media added to

bring the parasitaemia to 0.5% at 6% hematocrit. Five ml of this suspension was then incubated at 37° C with daily medium changes. Cultivated *P. falciparum* gametocytes were harvested between the 14 and 16th day to coincide with exflagellation and this was used for mosquito infection.

2.2 Membrane feeding assay

Mosquitoes were fed on either cultured gametocytes or uninfected blood meal. For uninfected blood meal, mosquitoes were fed on human blood. This was carried out by putting hands into a cage for 30 min. In the case of infective meals, mosquitoes were fed through a membrane obtained by skinning Winstar rat using a sharp pair of scissors and shaving of the fur. The portion of the shaven skin was cut, tied onto a sterile Falcon tube containing the gametocytes and the tube inverted. The tube was clamped and mosquitoes which had been in a container covered with a Muslin cloth brought close to the tube and let to feed for 30 min. To assay for infectivity, midguts and abdomens were removed at 1 day and 7 days post feeding to coincide with ookinete invasion and oocyst development, respectively.

2.3 Collection and identification of mosquitoes

Collection was carried out from inside the houses by aspiration and by the pyrethrum spray collection method (Githeko *et al.*,1996).

Collections were carried out by aspiration from house walls in the morning and by pyrethrum-spray collection methods in the afternoons. Adult *An. funestus* complex mosquitoes were distinguished from other Anophelines based on morphological characteristics (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Bloodfed mosquitoes collected were kept in absolute alcohol.

All *An. gambiae* mosquitoes were identified to species using ribosomal DNA-PCR method (Scott *et al.,* 1993). Diagnostic PCR was carried out from the DNA obtained from the leg of an individual mosquito. A 25 μl PCR mix consisting of 2.5 μl 10X PCR buffer, 1,200 μM of each dNTP, 1 mM $MgCl_2$, 0.625 units of Taq polymerase, 6.25 ng of primer GA, 12.5 ng of primers UN and ME, 18.75 ng of primer AR and sufficient sterile water was prepared. This was overlaid with 2 drops of mineral oil in a 200 μl tube. PCR was carried out in a PTC-100 Programmable Thermocycler controller with a program of 30 cycles of denaturation at 94° C for 30 sec, annealing at 50° C for 30 sec and an extension at 72° C for 30 sec. After completion, the products were run on a 2.5% (w/v) agarose gel in 1X TAE buffer (40 mM Tris-acetate, 2 mM EDTA pH 8) containing Ethidium bromide. The amplified products were visualized by illumination with ultraviolet light.

2.4 Sample preparation

Midguts and abdomens were carefully removed and individually stored at -70° C in 1.5 ml Eppendorf tubes until RNA extraction. The remaining mosquito carcass was stored at -70° C in 1.5 ml Eppendorf tubes for DNA extraction. For semi-quantitative analysis, the midguts were dissected 24 hours post-infection (hpi) and 7 days post-infection (dpi) and stored at -70° C for RNA extraction.

2.5 DNA extraction

Total genomic DNA was extracted from carcasses of individual mosquitoes obtained after removal of midgut and abdomen using a modification of the proteinase K method (Collins *et al.*, 1987). In the case of field mosquitoes, DNA was extracted from the whole body. Carcasses stored at -70° C were removed, liquid nitrogen added into the tubes and the sample ground using a polypropylene pestle. To each tube, 400 µl of freshly prepared DNA extraction buffer (5 M NaCl, 0.5 M EDTA, 10 % SDS , 1 M Tris, pH 8) and 5 µl of RNase A was added, the sample homogenized and incubated at 37° C for 60 min. Ten µl of Proteinase K (20 µg/µl) was added and incubated at 50°C for 60 min. Phenol:chloroform:isoamyl alcohol (400 µl) was added to each tube, vortexed gently and centrifuged (13,000 rpm, 10 min). The supernatant fraction was transferred to a fresh tube and 2 ml of pre-chilled ethanol

(-20° C) added, mixed by inversion and incubated at -70° C for 30 min. Centrifugation was carried out (13,000 rpm, 10 min) and the supernatant discarded. Two ml of 70% ice-cold ethanol was added, vortexed briefly and centrifuged (13,000 rpm, 5 min, 4° C). The pellet (often invisible) was washed in ethanol and dried by suction. The DNA pellet was re-suspended in TE (10 mM Tris-HCl, pH 7.5 and 0.1 mM EDTA) and incubated (65° C, 30 min) until complete dissolution. DNA concentration was determined by spectrophotometry method.

2.6 Genomic DNA amplification by PCR

PCR amplifications were carried out in 200 µl tubes using a PCR kit (Fermentas, MBI, Germany). Each tube contained 200 ng of target DNA, 0.025 units of Taq DNA polymerase, 0.05 mM dNTP, 0.75 mM $MgCl_2$, 2.5 µl 10X PCR buffer, 1 µl each of the MBP forward and reverse primers (CL1/CL2 and A16A/A16B (sequences shown in the appendix) in a total volume of 25 µl. Each reaction mixture was overlaid with one drop of mineral oil to prevent evaporation. The cycling conditions were as follows: initial denaturation at 94° C for 1 min followed by 35 cycles of denaturation at 94° C for 1 min, annealing at 55° C for 30 sec and extension at 72° C for 1 min. This was followed by a final extension at 72° C for 10 min. Amplification was verified by electrophoresis on 1.5% (w/v) agarose gel in 1X TAE buffer. A 100 bp ladder (Fermentas, WBI, Germany) was used as a

molecular weight marker for confirmation of the length of the PCR products. Gels were stained with Ethidium bromide (20 μg/ml), visualized and then documented.

2.7 Cloning of PCR product

Using shotgun cloning strategy, PCR-amplified DNA were ligated into pGEM®-T Easy vector (Promega, Madison, WI, USA). A 10 μl ligation reaction consisting of 5 μl 2X rapid ligation buffer, T4 DNA ligase, 1 μl pGEM-T vector, 1 μl PCR product, 1 μl T4 DNA ligase and 2 μl deionized water was incubated overnight at 4° C. Recombinant plasmids were transformed into competent XL-1 blue cells. To 10 μl of the ligation reaction, 50 μl of cells were added and incubated on ice for 30 min. After 1 min heat shock at 42° C of the cells, 500 μl of NZY broth (10 g/l NZ amine, 5 g/l yeast extract, 5 g/l NaCl) was added and the mixture incubated at 37° C for 1 hr. The mixture was briefly centrifuged and the pellet reconstituted in 200 μl of the broth. Hundred μl of IPTG (Isopropylthio-β-D-galactosidase: 0.1M) and 40 μl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase: 50 mg/ml) were spread onto LB-agar Ampicillin (125 μg/ml) treated plates. The culture (50 μl) was evenly spread onto the plates and incubated at 37° C overnight. Abundant clones were screened using the universal primers Sp6 and T7. Positive clones were streaked onto another LB-agar Ampicillin plates and subsequently inoculated into 250 ml LB

medium for plasmid preparation. Recombinant plasmids were purified from overnight cultures using the alkaline lysis technique.

2.8 Single-Strand Confirmation Polymorphism Analysis (SSCP)

Cloned PCR products were resolved by SSCP analysis using the method originally described by Orita *et al.* (1989) with a few modifications. Briefly, each PCR product (2 µl) was mixed with 5 µl denaturing solution (95% Formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue). The samples were denatured at 95° C for 4 min to separate two strands and snap cooled on ice. The single strand DNA fragments were separated in a non-denaturing PAGE 30% cross-linking in the presence of 10% glycerol (70 v, 16 hr, 4° C). Electrophoresis was carried out in a Multiphor electrophoresis tank in 0.5X TBE buffer (2 mM EDTA, 90 mM Tris-Borate, pH 8.3). The gels were silver stained according to Wray *et al.* (1981) with minor modifications. After fixation with 5% acetic acid, 15% ethanol for 30 min, the gel was rinsed once for 2 min with distilled water and impregnated for 30 min with solution A ($AgNO_3$, (1g/L) and 100 µl 37% formaldehyde). It was then rinsed 3 times for 2 min with deionized water. Colour development was obtained with Solution B (2.5% Na_2CO_3, 100 µl 37% formaldehyde/100 ml; 10 mg/ml $Na_2S_2O_3$) and water for 5 min. The reaction was stopped with 2% w/v glycine for 10 min and finally impregnated with 5% (v/v) glycerol for 10 min.

2.9 Small scale plasmid purification (Mini-prep) by alkaline lysis technique

Recombinant plasmids from cloned products were extracted using QIAprep® spin Miniprep kit (Qiagen, Gmbh) following manufacturer's instructions. High-copy plasmid DNA from 5 ml overnight culture of *Escherichia coli* in Luria-Bertani (LB: 10 g/L Bacto tryptone, 5 g/L Bacto yeast extract, 5 g/L NaCl, pH 7)) medium containing Ampicillin (125 μg/ml) was prepared by inoculation of a single colony of the positive clone. Bacteria cells were pelleted by centrifugation (5 min) and re-suspended in 250 μl of buffer (P1) containing RNase A. The lysis buffer (P2, 250 μl) was added to the suspension and mixed by several inversions gently. The lysate was neutralized by addition of 350 μl of buffer N3 and mixed by inversion until a cloudy precipitate was obtained. The solution was centrifuged (14,000 rpm, 10 min) to obtain plasmid DNA and the supernatant fraction applied to the QIAprep column by pipetting. Bound plasmid DNA was washed by adding 750 μl of buffer PE and centrifuged briefly. The flow-through was discarded and re-cetrifuged briefly to remove residual wash buffer. The QIAprep column was put in a clean 1.5 ml Microcentrifuge tube and the DNA eluted by addition of 50 μl of triple distilled water.

2.10 Large scale plasmid preparation by alkaline lysis technique (Maxi-prep)

Plasmid DNA was preparation using the method of Birnboim and Doly (1979). Two hundred and fifty ml of LB was prepared in 1 litre flask, autoclaved and cooled (40° C). A single colony of the cells containing the plasmid was inoculated in the LB media containing Ampicillin (to final concentration 125 μg/ml) and the culture incubated overnight at 37° C with vigorous shaking at 200 rpm. Harvesting of bacterial cells was carried out by centrifugation (600 rpm, 4° C, 10 min) in a GSA rotor sorval RC-5B centrifuge) and the supernatant fraction discarded. The bacterial pellet was re-suspended in 10 ml solution 1 (50 mm glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8) and incubated (23° C, 5 min). Twenty ml of freshly prepared solution 2 (0.2 N NaOH, 1% SDS) was added, the contents mixed by inversion and incubated on ice for 10 min. After incubation, 15 ml of ice-cold solution 3 (3 M KAc, 11.5% CH_3COOH) was added to the lysed mixture and incubated on ice for 10 min.

The bacterial debris was then pelleted by centrifugation (10,000 rpm, 20 min, 4°C) using GSA rotor in a Sorval RC-5B centrifuge. The supernatant fraction was carefully transferred to a fresh 50 ml centrifuge tube and the pellet discarded. To the supernatant fraction, 0.6 volumes of isopropanol was added, mixed by inversion and

allowed to stand for 15 min at room temperature. Nucleic acids were pelleted by centrifugation (10,000 rpm, 30 min, 23° C.) and the supernatant fraction discarded. The pellet was washed in 70% ethanol, re-centrifuged and vacuum dried. The pelleted DNA was dissolved in 10 ml TE.

Purification of plasmid DNA was carried out by equilibrium centrifugation in Cesium Chloride (Cs) and Ethidium bromide (EtBr) using the CsCl-EtBr gradient method. To the 10 ml DNA solution, 10 g of CsCl and 200 µl EtBr (10 mg/ml) was added and dissolved by warming the solution at 30° C. The clear red solution was transferred to a quick-seal ultracentrifuge tube (Beckman) and the remainder of the tube filled with oil. Balancing of the tubes was carried out within 0.01 g prior to heat-sealing. Centrifugation was carried out (55,000 rpm, 22 h, 20° C) using 65Ti rotor in a Beckman ultracentrifuge. Two bands of DNA were visible (the upper band which contains less material consists of linear bacterial DNA and nicked circular plasmid DNA and the lower band consisting of closed circular plasmid DNA. The lower (super-coiled plasmid DNA) band was removed using an 18-gauge needle and syringe into a fresh tube and an equal amount of butanol saturated in H_2O added and mixed. The layers were left to settle at room temperature. The upper layer containing EtBr was collected and discarded. This was repeated several times until all the

EtBr had been extracted. Precipitation of the plasmid DNA was carried out by adding 2 ml of 70% ethanol and incubated (23° C, 30 min). Pelleting of the DNA was carried out by centrifugation (15,000 rpm, 15 min, 4° C) using a Sorval RC-5C centrifuge and the pellet dissolved in triple distilled water after vacuum drying.

2.11 Sequence Analysis

The nucleotides of the purified DNA Plasmid templates were subjected to sequencing using the ABI automated sequencer (Applied Biosystems). The DNA sequences obtained were used to search GeneBank database using BLAST program of the National Center for Biotechnology information (NCBI) and sequences from other species showing high scores and other C-type lectins were retrieved and used in multiple alignment analysis using CLUSTAL W (Altschul *et al.*, 1990). Phylogenetic tree was constructed using PHYLIP from the obtained alignment without any adjustments carried out on the gaps. The Pair-Distance analysis was determined using Pearson carried out based on the alignment.

2.12 RNA extraction

Midgut and abdomens preserved at -70° C were used for total RNA isolation using RNAgents® Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturers instructions. The

denaturing solution was added to the tissues and subsequently disrupted using a polypropylene pestle for 15 min until no fragments of tissue of cells were visible. Sodium Acetate (2 M, pH 4.0) was added and mixed thoroughly by inverting the tube 4-5 times. Phenol-chloroform: isoamyl alcohol was then added and carefully mixed by inversion 3-5 times, shaken vigorously for 10 sec and chilled on ice for 15 min. The mixture was transferred into a sterile Microfuge tube and centrifuged (10,000 rpm, 20 min, 4° C) in a bench-top Microfuge. The top aqueous phase containing RNA was transferred into a fresh sterile Microfuge leaving the DNA and proteins in the organic phase and at the interphase. To precipitate and re-suspend, an equal volume of isopropanol was added to the aqueous phase. 1.3 µl of glycogen (20 µg/µl) was added and the sample incubated at -70° C for 30 min. Pelleting of RNA was carried out by centrifugation (10,000 rpm, 10 min, 4° C). The pellet containing RNA was re-suspended in denaturing solution and vortexed until the RNA dissolved. The RNA was once more precipitated using isopropanol as described above. The RNA was pelleted by centrifugation (10,000 rpm, 10 min, 4° C) and washed by re-centrifugation using ice-cold 75% ethanol. The RNA obtained was immediately used to prepare cDNA.

2.13 cDNA synthesis

The RNA was reverse-transcribed with the SMART™ PCR cDNA synthesis kit (CLONTECH, Laboratories, Inc., USA) according to the manufacturer's instructions. First strand oligo (dT)-primed cDNA synthesis was performed in 10 µl reaction mixture consisting of 3 µl RNA, 1 µl SMART IV™ oligonucleotide (10 µM) and 1 µl CDS III/3" PCR primer (10 µm). The tube contents were mixed and then centrifuged briefly in a Microfuge. This was followed by incubation at 72° C for 2 min and then snap cooling on ice for 2 min. The tube contents were centrifuged briefly to collect the contents at the bottom and 2 µl of 5X first-strand buffer, 1 µl Powerscript ™ RT added to make a total volume of 10 µl. The tube contents were re-centrifuged briefly, incubated at 42° C for 1 hr and cooled on ice.

To generate full-length cDNA, long distance PCR (LD-PCR) was performed. Four µl of first strand cDNA template was used in a 50 µl reaction containing de-ionized water, 10X Advantage® 2 PCR buffer, 50X dNTP mix, primers (5' PCR and CDSIII/3' PCR) and 50X Advantage 2 polymerase mix. The tube contents were briefly shaken and centrifuged to collect the contents at the bottom of the tube. The PCR cycling profile was carried out in a first denaturation at 95°C for 2 min followed by 18 cycles at 95°C for 15 sec, 65°C for 30 sec, and 68° C for 6 min. One µl of the amplified cDNA products was

separated by electrophoresis on a 1% TAE/agarose gel and then visualized by EtBr staining alongside a 1 kb-DNA size marker (Boehringer).

2.14 Reverse Transcription-PCR (RT-PCR)

MBP primers (CL1/CL2) and the nested primers (A16A/A16B) were used in separate reactions for RT-PCR. Twenty five µl reaction mixture containing 10 µM each of the primers, 1 µl of cDNA template, 0.75 mM $MgCl_2$, 0.05 mM dNTP and 2.5 µl 10X PCR buffer was prepared. PCR was carried out with a first denaturation at 94° C for 5 min followed by 32 cycles at 94° C for 45 sec, 58° C for 45 sec and 72° C for 45 sec. This was followed by a final extension of 72° C for 10 min. Amplified products were analyzed by electrophoresis on 1% agarose gel (including 0.25 µg/ml EtBr). 100 bp DNA marker (MBI, Fermentas, Germany) was run alongside to confirm the fragment size and the bands visualized under UV light.

2.15 Assessment of midgut infections

Prior to quantitative analysis, the PCR method was used to check for infection in the cDNAs prepared from infected midguts and abdomen using *Plasmodium* parasite-specific primers, MSP1-A and MSP1-B (Sigma-Genosys Ltd, UK). The lyophilized primers were re-constituted to a stock concentration of 100 µM. For PCR reactions, 10 µM was

prepared from the stock and used. The PCR conditions and programme were similar to those used in genomic DNA amplification except for the number of cycles which were reduced to 25. The PCR products were analyzed on a 1.5% agarose gel stained with EtBr.

2.16 Determination of Expression levels using Semi-quantitative RT-PCR

Quantification assays were carried out using Multiplex titration RT-PCR method as described by Nebenführ and Lomax (1998) with slight modifications. For each primer set, preliminary PCR amplifications were carried out under standard conditions to determine the number of cycles necessary to obtain visualizable PCR products while avoiding saturation. cDNA prepared from non-infected and infected midguts were used as templates. The program used was as similar to that described for genomic DNA amplification. A 1:2 7-fold dilution was carried out on the original concentration of cDNA with a starting concentration of 50 ng. Briefly, multiplex titration RT-PCR was performed with 2 μl of the RT products in a total volume of 25 μl in the presence of 0.05 mM dNTP, 0.025 units of *Taq* polymerase, 0.75 mM $MgCl_2$, 10 μM of primers CL1/CL2 or A16A/A16B including rp7S primers as the internal control for normalization. PCR products (10 μl) were separated by electrophoresis on 1.5% agarose gel (including 0.25 μg/ml EtBr) in TAE buffer. The gels were visualized and bands

counted on a UV transilluminator and dilution step of the last visible band noted. The gels were photographed using a gel documentation system (Biosystematica, UK).

2.17 Determination of MBP Expression by RT-PCR

The expression levels of MBP was evaluated following *P. falciparum* infection by RT-PCR analysis from midguts dissected 24 hr and 7 days following an infective blood meal. cDNA prepared from the midguts and abdomens 24 hr after non-infective feeding was also screened using rp7S as a positive control in the PCR. The PCR conditions and program was similar to that described for genomic DNA amplification. The PCR products were separated on 1.5% agarose gel and 100 bp DNA marker (MBI, Fermentas, Germany) run alongside to verify the sizes of the fragments.

CHAPTER 3

3.0 RESULTS

3.1 The variation of the MBP gene sequence between and within *Anopheles sp*.

3.1.1 PCR amplification and cloning of genomic DNA encoding MBP gene

In an attempt to amplify genomic DNA using MBP primers, two fragments of size 600 bp and 400 bp were obtained (Fig. 2). This revealed that there is more than one lectin gene in the mosquito. The amplicons obtained were found to be 200 bp bigger compared to expected products size to the cDNA, indicating that genomic loci for these genes harbour major introns. The PCR products were ligated in pGEM-T vector and cloned into *E.coli* XL1-Blue cells. Positive colonies, determined by their white colour as opposed to the negative ones which were blue in colour were selected. Appendix B shows the three *Anopheles sp* and the total number of clones that were analyzed by SSCP.

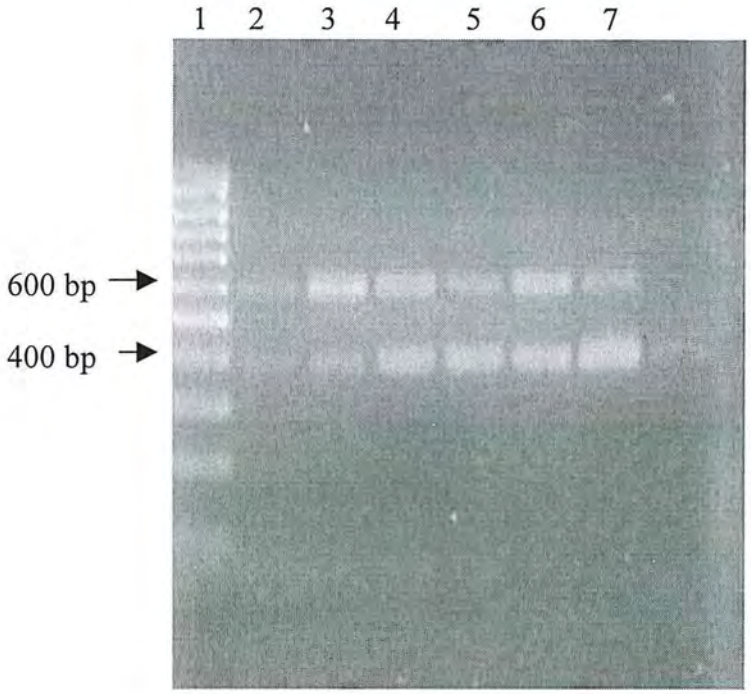

Fig. 2: Typical result of a PCR amplification of MBP fragments.

PCR products were analyzed in a 1% agarose gel and stained using Ethidium bromide.

Lane 1: 100 bp ladder (Fermentas, MBI, Germany)

Lane 2 - 4: Ifakara colony (Tanzania)

Lane 5-7: Mbita colony (Kenya)

3.1.2 Screening for mutations using SSCP

A native polyacrylamide gel was used to separate DNA clones based on single-stranded conformation polymorphisms prior to sequencing. Single stranded DNAs fold to form stable and meta-stable secondary structures that influence their mobility in a native acrylamide gel. Analysis of the cloned PCR products obtained from *An. gambiae s.s.* produced two bands representing a single DNA fragment for each of the clones (Fig. 3). Three conformational patterns were detected with frequencies of 25% for each of the conformational patterns. In contrast, SSCP analysis of the various clones obtained from different *An. arabiensis* revealed more than two bands for each clone with certain bands appearing as discrete (Fig. 4). There was a high level of polymorphism allowing for the detection of ten different conformational patterns. The frequencies in this case were almost 10% for all the patterns exhibited. In both cases, some bands were of very high intensity while the others low intensity.

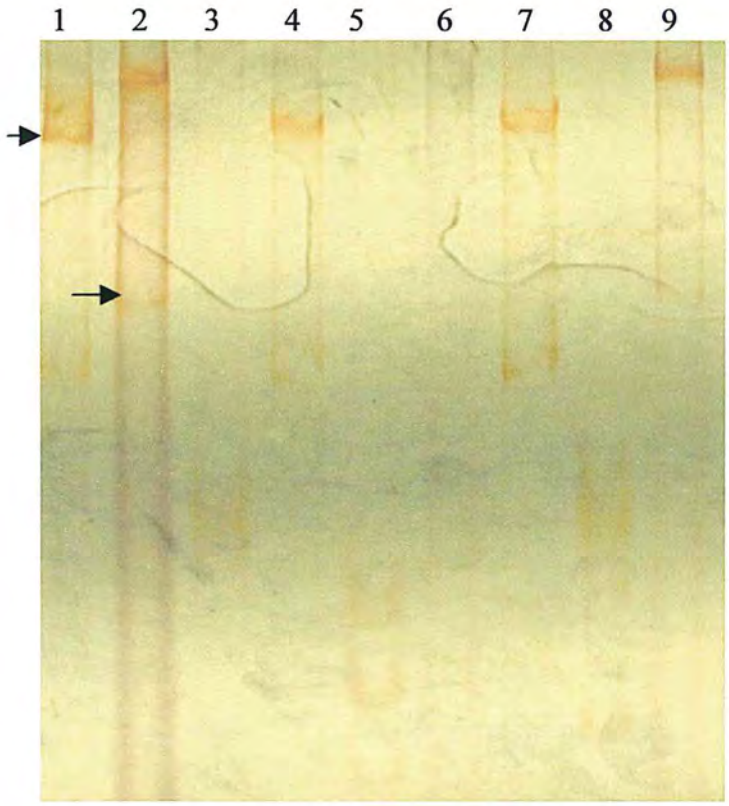

Fig. 3: SSCP analysis of MBP clones from *An. Gambiae s.s*.

Arrows indicate the clones that were purified and sequenced.

Conformation patterns are denoted by numbers.

Lanes 1,4,7; Lanes 2, 9 and Lanes 3, 8 have similar conformation patterns

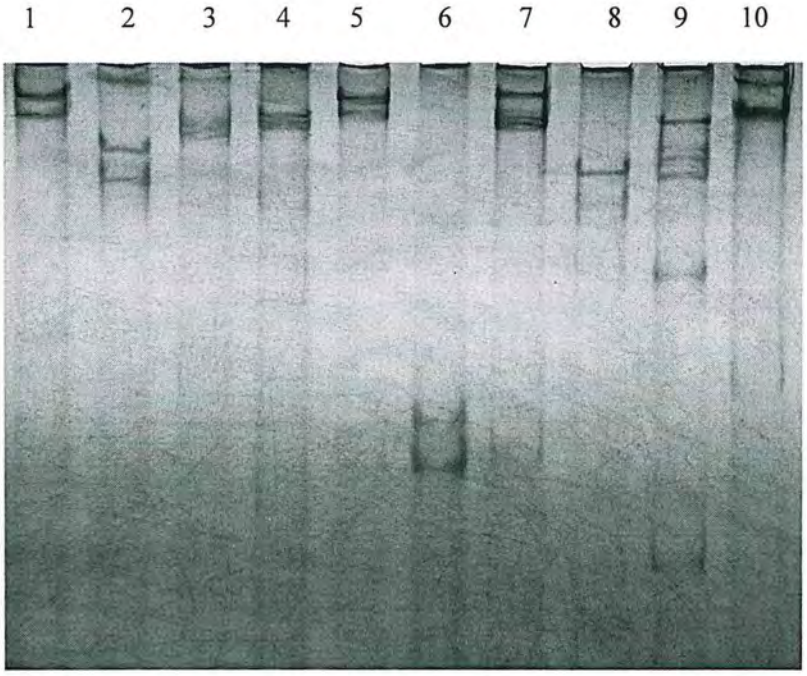

Fig. 4: SSCP analysis of the cloned MBP fragments of *An. arabiensis*

Numbers show the various conformation patterns

3.1.3 Genomic sequence analysis

The resultant sequences were queried against the GeneBank database and three MBP clones (MBP^{M1}, $MBP^{I3}$and MBP^{I5}) sequences had significant similarity to C-type lectin 2. These gene transcripts had relatively high similarity to ANOGA A16 (above 90 % identity at the nucleotide level). The remaining sequences of clones (MBP^{M1}, MBP^{M1}, MBP^{M1}, MBP^{M1}, MBP^{M1}, $MBP^{M1}$and MBP^{M1}) were predicted to encode the protein Kinase c using INTERPRO SCAN (www.expasy.org). BLAST results from AnoBase revealed similarity of all the nucleotide sequences with *Anopheles gambiae*. The cytological location of the sequences obtained from the clones of the *Anopheles* genes encoding MBP showed the positions of all these genes on the polytene chromosomes. Five genes (MBP-M1, MBP-M2, MBP-I3, MBP-I5 and MBP-MW1) were located on chromosome 2R, 4 genes (MBP-MW5, MBPMW12, MBP-C1 and MBP-C15) on chromosome 3L and 1 gene (MBP-MW4) on chromosome 3R. An amino acid alignment of the 10 clones encoding MBP and other Anopheles sequences retrieved from GeneBank is shown in Fig. 5. The conserved amino acid residues proposed to be crucial in insects are indicated by an asterisk. In addition, motifs characteristic of C-type lectins are shown in bold.

```
MBP-M1             ------------------------------------------------------------
MBP-I5             ------------------------------------------------------------
AgCP2787           -----GTFSKSFKMLLANTAAAVLLLIVCIGASVGLPTVDEENVVQAEQLPILPTADSSK 55
A16_ANOGA          -------------MLLANTAAAVLLLIVCIGASVGLPTVDEENVVQAEQLPILPTADSSK 47
MBP-I3             ---------------------PWLTIICP------------------------------- 8
AgCP2709           RIVICRSARIVIDLPPSSGRMEVLYKNTLLSFVVAVVLVLSIVAATTNPEPAGTKTEVKS 60
AAAB01008807_419   ------------------------------------------------------------
MBP-MW4            ------------------------------------------------------------
MBP-C15            ------------------------------------------------------------
MBP-C1             ------------------------------------------------------------
AAAB01008984_393   ----------------------------MPISPLVPMSPGRPGAPTLPGRPIAPGKPGSP 32
MBP-M2             ------------------------------------------------------------
MBP-MW1            ------------------------------------------------------------
MBP-MW12           ------------------------------------------------------------
MBP-MW5            ------------------------------------------------------------

                                                           **** *      **
MBP-M1             ---------------LLPADFAVENKKFTIGTLGVGSFF-RAWRNCIDEGKGLATIE-SE 43
MBP-I5             ---------------LLPADFAVKNKKFTIGTLGVGSFF-RAWRNCIDEGKGLATIE-SE 43
AgCP2787           PTDDTVKAIAPQPRYLLPADFAVKNKKFTIGTLGVGSFF-RAWRNCIDEGKGLATIE-SE 113
A16_ANOGA          PTDDTVKAIAPQPRYLLPADFAVKNKKFTIGTLGVGSFF-RAWRNCIDEGKGLATIE-SE 105
MBP-I3             SADDTVKAIAPQPRYLLPADFAVKNKKFTIGTLGVGSFF-RAWRNCIDEGKGLATIE-SE 66
AgCP2709           YSPEDVKDVNNFPVNGLPP-LTYSSKKYTLHTEVVN-FF-EAWNRCRDMGKQFASIENSQ 117
AAAB01008807_419   -----------HGRVKVRTDDVAKAATFCCSLSHYNSSY-AGIPTCFEYSNSFFIAT--- 45
MBP-MW4            ---------------MLLANTAVKRAVCVARLNPLHPSA-KRASECMRAPNSPTQGTAHR 44
MBP-C15            ------MACRALRVRLDHEVMTVRRVSRVNRAVSVNGAT-RAKRAISELLDRLVLWA-VR 52
MBP-C1             ---------------MLLANTAVLPRGVAFPLSGLS------ASPCIHR-KKVGPFP--- 35
AAAB01008984_393   LSPLMAAAVAGRPGEPGTPRSPLRPISPTGPISPWSPFSPSSPRSPMRPGSPLKPFSPSY 92
MBP-M2             --------------MLPTTRYVYLLNNDSKNFCSFSLSM---VANPLPSSMQLRHAR--- 40
MBP-MW1            ----LTPARARKTGNFIIRSNHADCSPSNPNITTLGWTLAGASGKHTEQPRRHTTDN--- 53
MBP-MW12           -------------MTASSRRVWLNGRLKWEAKCPFG-------HLASDDNDDIIITT--- 37
MBP-MW5            -----ACSLQTLPPNDDSRAYFYFGVVSIGRYAEVALLLLPSPWTPICCEFVALIYS--- 52

                                      ****
MBP-M1             KEEKFLESSLKASSTGSNYWIGAANIGASNTN--KLTWITTEITSEF------------- 88
MBP-I5             KEQKFLESLLKASSTGSNYWIGATNIGASNTN--KLTWITTEC----------------- 84
AgCP2787           KEQKYLESLLKASSTGSNYWIGATNIGASNTN--KLTWITTDLPVQTKPPFLNVVAKSTC 171
A16_ANOGA          KEQKYLESLLKASSTGSNYWIGATNIGASNTN--KLTWITTDLPVQTKPPFLNVVAKSTC 163
MBP-I3             KEQKFLESLLSK------------------------------------------------ 78
AgCP2709           DFAAYRDAVQPYANVNYTFWLAGTNVGARSNDYRKFYWITNDRPVSYVSGFQ-------- 169
AAAB01008807_419   --FLYFSNLFHH------------------------------------------------ 55
MBP-MW4            PVRVLLRSNRTPQLIASVPTVRNANTPGQ------------------------------- 73
MBP-C15            VIVVYLVRLVFPQLLPP-LRVTRENLASRALLADLVRWEYRDYRVTWVRRERWAFRDYLD 111
MBP-C1             ----YQPVRATFAFV--------------------------------------------- 46
AAAB01008984_393   PRGPAGPSAPGSPSMHGAPGTPGNPVVPLSPFTPGNPGTPMSPFTPFKPG---------- 142
MBP-M2             -KNDPTPSVPMVNFLFFTAKSAGSK----------------------------------- 64
MBP-MW1            --RTTLPSAPTYGPPLFGALCTLCTLDYWQCVSPHSAAVFARSM---------------- 95
MBP-MW12           ---IALVWLCATGVN--------------------------------------------- 49
MBP-MW5            --RSTQTHKCDGGDDDVIIIITAQVAKGAFCFPF-------------------------- 84

MBP-M1             -----------------------------
MBP-I5             -----------------------------
AgCP2787           IALTPTGSWTLRNCLNPLNIFPYICEEYF 200
A16_ANOGA          IALTPTGSWTLRNCLNPLNIFPYICEEYF 192
MBP-I3             -----------------------------
AgCP2709           -----------------------------
AAAB01008807_419   -----------------------------
MBP-MW4            -----------------------------
MBP-C15            CRARLD----------------------- 117
MBP-C1             -----------------------------
AAAB01008984_393   -----------------------------
MBP-M2             -----------------------------
MBP-MW1            -----------------------------
MBP-MW12           -----------------------------
MBP-MW5            -----------------------------
```

3.1.4 Phylogenetic analysis of Anopheles MBP gene

The retrieved sequences from (skonops.imbb.forth.gr/AnoBase/-8k) and their putative amino acid sequences were aligned against the sequences from MBP clones with the CLUSTAL W program. Table 1 shows the percent similarity between the deduced amino acid sequences of the *Anopheles* and the MBP clones calculated using Pearson method. Evolutionary distances were calculated using Point Accepted Mutation (PAM) matrix and the tree constructed using Phylogenetic Inference Package (PHYLIP). Fig. 6 shows a phylogenetic tree illustrating the relationships between these sequences based on a CLUSTAL W amino acid alignment.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		6	5	4	7	12	5	2	3	10	10	7	6	6	5
2			2	10	2	12	5	2	13	17	12	12	9	9	2
3				14	7	6	5	7	3	8	4	5	7	7	4
4					4	6	6	6	8	4	4	6	10	10	8
5						3	64	94	3	6	6	10	87	87	22
6							7	3	4	13	20	5	9	9	7
7								67	3	13	5	10	83	83	15
8									3	6	7	10	96	96	25
9										6	3	5	4	4	12
10											23	4	10	10	10
11												7	6	6	4
12													10	10	9
13														100	22
14															22

Table 1: Pairwise percentage similarities between derived amino acid sequences of *Anopheles sp*. and *Anopheles* MBP clones

Matrix analysis was carried out using point Accepted Mutation (PAM)

1: MBP-MW1; **2**: MBP-MW4; **3**: MBP-MW5; **4**: MBP-MW12; **5**: MBP-M1; **6**: MBP-M2; **7**: MBP-I3; **8**: MBP-I5; **9**: MBP-C15; **10**: MBP-C1; **11**: AAAB01008984 (Accession number for 3R chromosome); **12**: AAAB01008807 (Accession number for 2L chromosome); **13**: AgCP2787 (Accession EAA1326); **14**: A16_ANOGA (Accession Q93118); **15**: AgCP2709 (Accession EAA1326)

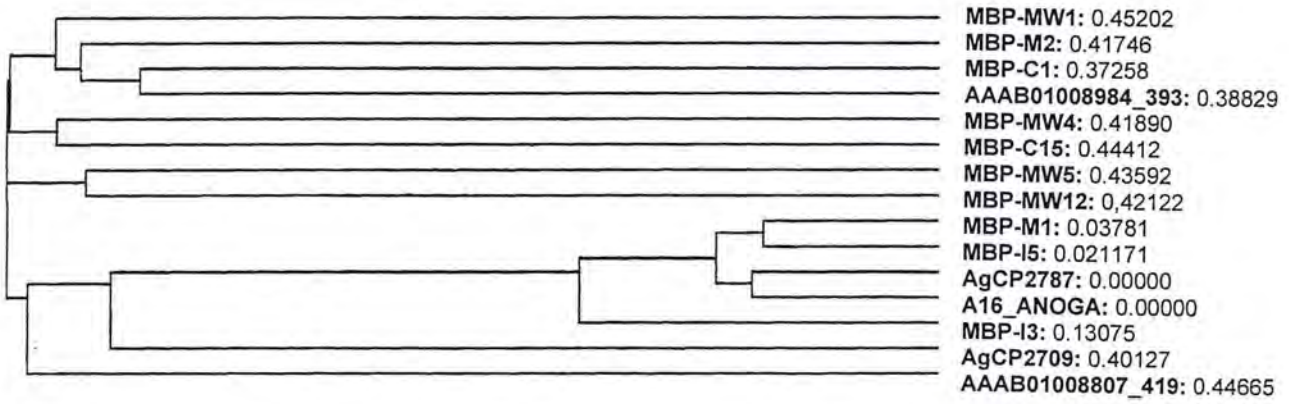

Fig. 6: Phylogenetic tree showing the relationship of MBP sequences obtained from clones of *An. gambie s.s*, *An. arabiensis* and *An. funestus* to other C-type lectins.

The relationships among the various groups were analyzed be Pearson method based on the alignment of the deduced amino acid sequences performed with CLUSTAL W software. The numbers on the branches show the PAM values

3.2 The role of MBP during malaria infections

3.2.1 cDNA synthesis

RNA extraction from individual mosquito midgut and abdomen was performed using RNAgents ® Total RNA Isolation System (Promega, Madison, WI, USA). The cDNA synthesized from the total RNA was reflected as a smear on a 1% agarose gel. (Fig 7). The cDNA obtained was used as template for semi-quantitative RT-PCR expression level analysis.

3.2.2 Reverse Transcription-PCR

Reverse transcription (RT) PCR was carried out using cDNA prepared from *An. gambiae* colony mosquitoes from Mbita (Kenya) and Ifakara (Tanzania) to ascertain the fragment sizes as described in Materials and Methods. PCR data confirmed that cDNA fragment of expected sizes (575 bp and 389 bp) were amplified for the Mbita colony with both CL1/CL2 and A16A/A16B primers, respectively, but not with the Tanzania colony (Fig. 8). Bright bands of 800 bp and 600 bp were obtained with Tanzania mosquito when amplified with CL1/CL2 and A16A/A16B primers, respectively. As shown in Fig. 8, faint bands were also obtained from each of the amplified samples

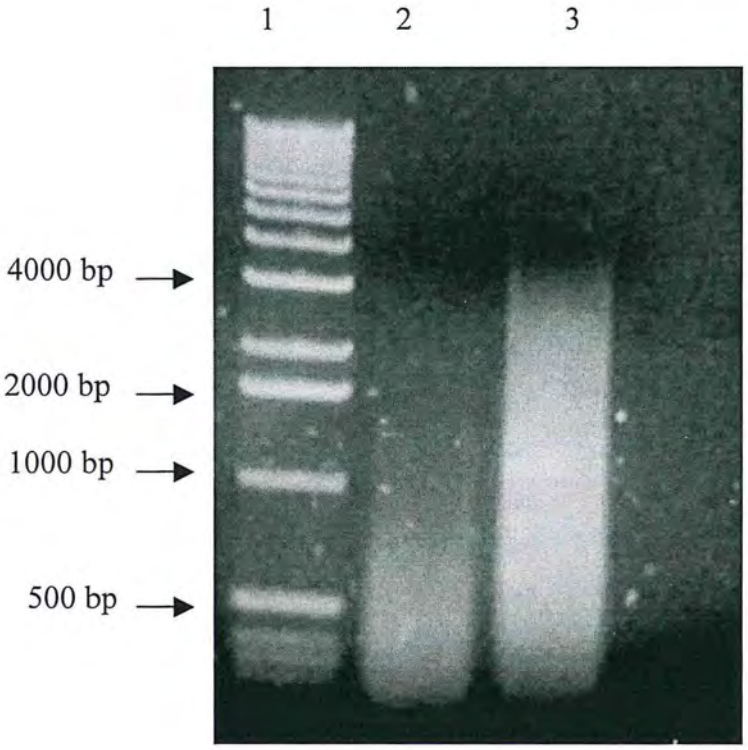

Fig. 7: Analysis of cDNA on a 1% EtBr stained agarose gel.

Lane 1: 1kb DNA marker (Boehringer)

Lane 2: 2 μl cDNA (Mbita strain)

Lane 3: 2 μl cDNA (Ifakara strain)

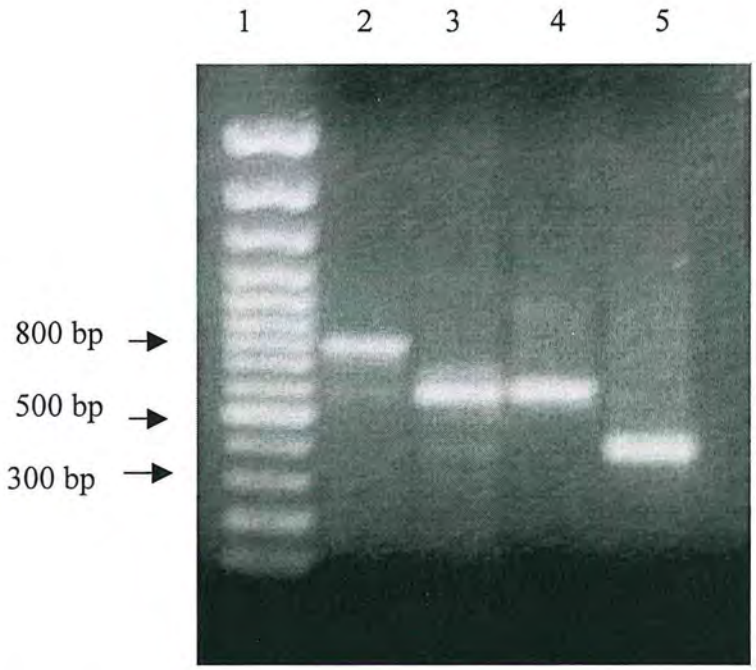

Fig: 8: EtBr stained agarose gel electrophoresis of RT-PCR products.

Lane 1: 100 bp DNA ladder (MBI, Fermentas, Germany);

Lane 2: CL1/CL2 Ifakara strain (Tanzania);

Lane 3: A16A/A16B Ifakara strain;

Lane 4: CL1/CL2 Mbita strain (Kenya);

Lane 5: A16A/A16B Mbita strain.

3.2.3 Determination of expression levels using semi-quantitative RT-PCR

The ability of Multiplex titration PCR (MTRP) to determine the relative concentrations of the template for MBP gene was assessed using CL1/CL2 and A16A/A16B primers and a control gene primer (rp7S). For the estimation of the template abundance, the primer pairs used were combined to yield reliable and approximately the same level of amplification. The number of lanes represented the dilution step at which the template was limiting thereby providing an indication of its abundance in the original mixture. cDNA prepared from midguts after a non-infective feed and from midguts of a non-infected female were screened. MBP expression could be induced by parasite ingestion or by the blood-meal. As shown in Fig. 9 and 10, MBP expression pattern was less pronounced in an infected sample compared to uninfected sample. There was a linear relationship between the concentration of the PCR templates used and the dilution step at which the product could no longer be detected. This was particularly evident in Fig. 9 where in the seventh dilution step, no template could be detected. This was also in contrast to Fig. 10, which had an abundant template at the same dilution step.

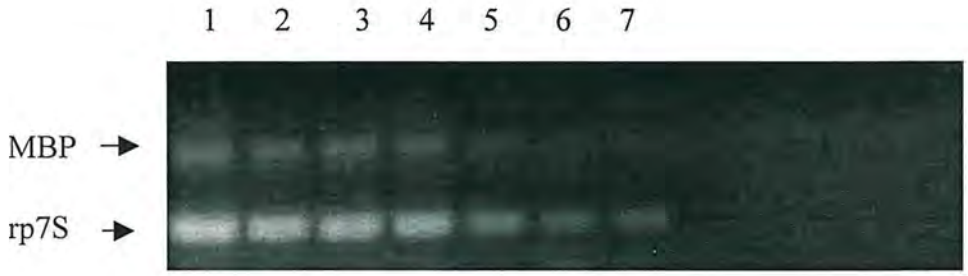

Fig. 9: Quantification of gene expression levels by Multiplex titration RT-PCR of uninfected midgut cDNA

Lanes on the 1.5% agarose gel stained using EtBr correspond to the dilution steps taken from the original template with a starting concentration of 50 ng. The number of lanes with detectable bands was counted. A 1:2.7-fold dilution series was followed.

Fig. 10: Quantification of gene expression levels by Multiplex titration RT-PCR from infected midgut cDNA.

Lanes on the 1.5% agarose gel EtBr stained correspond to the dilution steps taken from the original template with a starting concentration of 50 ng. The number of lanes with detectable bands was counted. A 1:2.7-fold dilution series was followed.

3.2.4 Expression of MBP

The expression of MBP was evaluated in midgut tissue at 24 hr and 7 days after an infective blood-meal. At each time-point, total RNA was prepared from a single mosquito midgut and abdomen and cDNA prepared as described in Section 2.17. Parasite-specific primers (MSP1) were used to confirm infection in the cDNA prior to expression analysis (data not shown). For each time-point, cDNA was used as a template in the analysis using MBP gene-specific primers. The results showed that MBP was expressed at both time points but at significantly different levels. At 24 hours post-infection, which coincided with ookinete invasion, MBP abundance was found to be much higher as compared to 7 days post-infection which coincided with oocyst infection (Fig. 11). MBP expression level was high at 24 hr in the mosquito that received an infective blood-meal. Significant differences in time after blood-meal ingestion was evident; gene expression decreased with time after infective blood-meal ingestion.

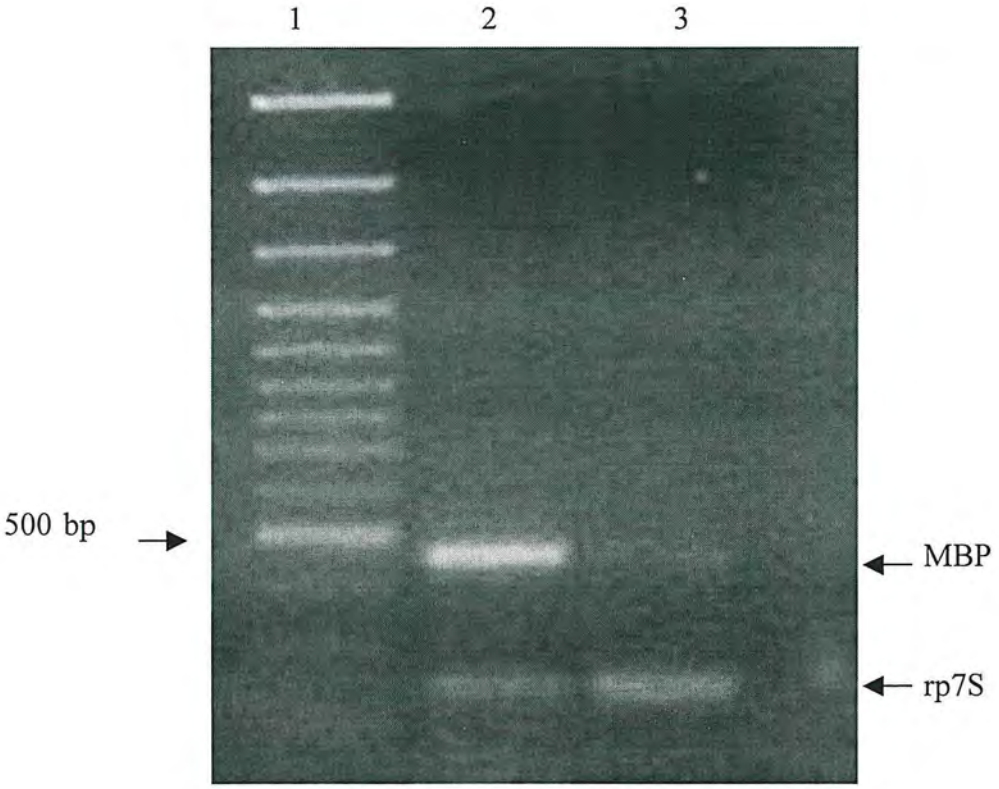

Fig. 11: Expression of MBP

DNA bands for target (MBP) and internal control (rp7S) are indicated. Template concentrations were maintained at 50 ng.

Lane 1: 100 bp DNA marker (MBI, Fermentas, Germany);

Lane 2: 24 hours post-infection

Lane 3: 7 days post-infection

CHAPTER 4

4.0 DISCUSSION AND CONCLUSION

4.1 General Discussion

Interactions between the immune system of the mosquito vector and the Plasmodium parasite can hinder or abort its development. The mosquito is known to mount robust immune reactions which accounts in part for the major parasite losses that occur within the vector (Dimopoulos *et al.,* 2001). Malaria transmission is possible because some parasites are able to survive vector's defences. This is because the key interaction is between the ookinete parasite stage and the midgut epithelial cells that it invades (Hans *et al.,* 2000). In invertebrates, the role of lectins as mediators of non-self recognition in the innate immune response have been well documented (Epstein *et al.,* 1996).

Insects have a rapid and effective system for defense against microbial infection, which shares many characteristics with the innate immune system of vertebrates (Hoffman *et al.*, 1999). Proteins that specifically bind to microbial components play a key role in non-self recognition and clearance of invading microbes (Yu *et al.,* 2000). These proteins are known as pattern recognition receptors since they bind to certain molecular patterns present in the array of carbohydrate components on the surface of microorganisms for example, lectins (Franc *et al.,* 2000). There is lack of antibodies or clonal selection of lymphocytes in insects

hence molecules such as lectins can recognize infectious pathogens and stimulate protective responses which is an essential component of their immune system (Janeway, 1992). Individual insect species contain several lectins, including C-type lectins of different specificities, for detecting a variety of pathogens (Yu *et al.,* 2000). For example, Wilson *et al.* (1999) concluded that multiple endogenous serum lectins in the cockroach, *B. discoidalis*, play important roles in insect innate immunity.

4.2 Analysis of variation and heterogeneity of MBP gene in *Anopheles*

4.2.1 MBP gene variation using SSCP

It was hypothesized in this study that there is genetic variation of the MBP gene in mosquitoes and that this could be linked to refractoriness or susceptibility of mosquitoes to Plasmodium. Even though sequence variations at the protein level were found to be negligible, it was expected that more polymorphism would be detected at the DNA level. The present study focused on genomic DNA analysis within and between each mosquito species. With this in mind and the fact that several clones of mosquitoes had to be sequence, SSCP was carried out to avoid sequencing similar clones since this would have resulted in duplication. SSCP data showed a high level of polymorphism within *An. arabiensis* clones compared to *An. gambiae s.s.* clones. This could be due to the fact that *An. gambiae s.s.* used in this study were colony mosquitoes kept under

controlled conditions unlike the *An. arabiensis*, which were field-collected.

Due to limited time available for sample collection and the fact that different mosquito species can only be found at certain times of the year, field-collected *An. gambiae s.s.* were not analyzed. PCR analysis of the mosquitoes from Mwea (Kirinyaga district) revealed that they were all *An. arabiensis*, while those collected from Coast province were all *An. funestus*. These field-collected samples were used for sequence variation studies. The clones from both *An. gambiae s.s.* and *An. arabiensis* mosquito species were different in that *An. arabiensis* showed quite a different migration pattern of both DNA strands. Since it is clear that the strands represent single DNA strands, the differences in this feature in *An. gambiae s.s.* and *An. arabiensis* provides evidence that there could be variation in the mosquitoes. To verify this observation and to exclude degradation artefacts (as this might have occurred during DNA extraction), SSCP was carried out at room temperature (23° C) and at 4° C with other conditions kept constant. The results showed that the best resolution was obtained at 4° C.

Theoretically, in an SSCP gel, there can be a maximum of four single strands for heterozygous samples. In this case, more than four bands were observed in *An. arabiensis*. Orita *et al.* (1989)

showed that occasionally, such results would be expected although the sequence is the same.

4.2.2 Variation of coding sequences of MBP clones and significant homology to known genes

The sequences of 10 clones from genomic DNA of *An. gambiae s.s.* colony mosquitoes, *An. arabiensis* and *An. funestus* were analysed. INTERPRO SCAN results revealed that the sequences of MBP-M1, MBP-I3 and MBP-I5 exhibited the C-type lectin domain signature and profile. This was characterized by positional conservation of one of the conserved six cystein residues which allows for the formation of disulphide bonds and are typical of invertebrate serine proteases. Disulphide bonds help to maintain a digestive protease-resistance structure (Shen *et al.,* 1999). The comparative analysis of amino acid sequences of the proteins from these 3 clones using the BLAST programme showed that there is significant similarity with A16 *An. gambiae* (Accession Q93118) which is expressed in the guts of adult female mosquitoes (Dimopoulos *et al.,* 1996). Previous work by Dimopoulos *et al.* (1996) showed that A16 had localized similarity to the carbohydrate recognition domains of numerous C-type lectins, most significantly to human L-selectin and the human macrophage mannose receptor and to a lesser extent to a lectin of the fly, *Sarcophaga peregrina*. L-selectin belongs to the family of selectin cell adhesion molecules which mediate leukocyte-endothelial cell

interactions (Siegelman *et al.,* 1989), while the macrophage mannose receptor is involved in the discrimination of self from non-self and is thought to play an important role in the innate immune response to pathogenic microorganisms (Taylor *et al.,* 1990). In the present study, in addition to the conserved motifs, these 3 sequences were found to exhibit similarities, indicating close phylogenetic relatedness of their host insect. These results contrast with the remaining 7 (MBP-M2, MBP-MW1, MBP-MW4, MBP-MW5, MBP-MW12, MBP-C1 and MBP-C15) clones which did not show any significant similarity to C-type lectins. Apart from MBP-M2 which was a sequence obtaind from *An. gambiae s.s.*, 4 clones were from *An. arabiensis* (MBP-MW1, MBP-MW4, MBP-MW5 and MBP-MW1) and 2 from *An. funestus* (MBP-C1 and MBP-C15). Nevertheless, these genes showed the greatest identity with the polytene chromosome of *An. gambiae*.

Sequence alignment revealed a remarkable size difference between all the different clones. These differences could have been due to the short sequences obtained as a result of the method of cloning used. The protein Kinase C signature, which was evident in these sequences also, suggests that the fragments were rather short hence making the comparison difficult. The open reading frames were interrupted by introns indicating that their genomic loci harbour major introns. The large number of clones obtained suggested that MBP is an abundant molecule in *Anopheles sp*. This

raises the possibility that it could be involved in mosquito-parasite interaction.

4.2.3 Phylogenetic relationship and heterogeneity of MBP

Using the uncorrected alignment, it was not possible to conclude whether the DNA sequences obtained harboured sufficient information to unequivocally resolve phylogenetic relationships between and within the species. Quite noteworthy, all the clones harboured phylogenetic information. Highly conserved and derived regions were found in MBP-M1, MBP-I3 and MBP-I5, which were *An gambiae s.s.* but from different strains. These 3 show a phenotypically similar pattern with other *An. gambiae* (A16_ANOGA and AgCP2787) but contrasts to the results obtained with *An. arabiensis* and *An. funestus* clones are clustered together, suggests that these species could be similar. A full-length genomic sequence is necessary to unravel the phylogenetic relationship between and within *Anopheles* mosquitoes.

4.3 Role of MBP during malaria infection in the mosquito

This experiment was undertaken to determine whether MBP has a role during infection. Previous studies have shown cross-reactivity between the cockroach lectin, BDL1 (a lectin with structural and functional similarities to vertebrate lectin) and the mosquito lectin (Chen and Billingsley, 1999). Subsequent work on *An. stephensi* showed that blocking this particular lectin reduced the infection of

mosquitoes by *Plasmodium*. Initial association of low levels of MBP was based on expression of MBP from cDNA prepared from infected midguts and abdomen 24 hours and 7 days post infection to coincide with ookinete invasion and oocyst development in the midgut respectively. In this study, the abundance of MBP decreased significantly at 7 days post infection suggesting that the presence of the *Plasmodium* in the midgut may suppress the levels of the protein in circulation. The expression patterns observed showed that no over-expression of MBP was detected at 24 hour after an infective blood-meal suggesting that the mRNA is rapidly degraded.

Adult female *Anopheles* mosquitoes require blood for ontogeny and it is during this period that MBP is induced in midgut (Chen *et al.*, 1999). This observation is in agreement with the results of this study. Interestingly, MBP levels were found to be much higher in cDNA prepared from uninfected than in infected miduts and the decrease in levels was greatest in the latter case compared to the former. This difference in expression levels following uninfected compared to infected blood-meals suggests that MBP could be involved in the infection process. In humans, MBP forms an essential component of the innate immune system, since MBL-deficient individuals are prone to recurrent infections during infancy (Super *et al.*, 1992). From the current studies, it is possible that the mosquito's immune system is down-regulated by *Plasmodium*.

The underlying reasons for this observation are presently unclear but it is possible that this down-regulation favours survival of the parasite in the midgut. The differences observed in RT-PCR of the cDNA prepared from the Mbita and Ifakara mosquito strains could possibly be due to geographical differences although further confirmatory work is required in this area.

4.4 CONCLUSION AND RECOMMENDATIONS

The fact that low numbers of parasites are found in natural mosquito populations suggests the existence of a fine-tuned equilibrium between the propagative capacity of the parasite and the mosquito's ability to control Plasmodium infection. It is likely that a heavy infection would affect the mosquito's fitness, leading to intense natural selection for refractory vector populations, whereas a very low infection rate would not allow transmission of the parasites (Dimopoulos *et al.,* 2001). The strength of the hypotheses in this study is difficult to weigh since the mosquitoes used in the study were not confirmed as either refractory or susceptible stains. It would be therefore worthwhile examining both strains. It may also be premature to conclude that there is indeed variation between and within individual mosquitoes based on the data obtained so far. Nonetheless, it is worth noting that the molecular morphology data obtained so far seem to provide preliminary results which could form basis for further investigation hence the two hypotheses need to be tested more rigorously with

additional species from different geographical areas. A number of genes have also been implicated in vector competence of mosquitoes, for example the mucin gene (Morlais *et al.,* 2001: Shen *et al.,* 1999). Molecular analysis of these genes on natural vector populations may facilitate manipulation of vectors in the hope of lowering their vectorial capacities. Application of genomics could generate new information on the mechanisms that naturally regulate the successful development of the parasites in vector species and lead to new tools for interrupting parasite transmission.

REFERENCES

Altschul, S.F., Gishi, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology* **215**, 403-410

Anders, E.M., Hartley, C.A. and Jackson, D.C. (1990). Bovine and mouse serum β inhibitors of influenza A viruses are mannose-binding lectins. *Proceedings of the National Academy of Sciences of USA* **87**, 4485-4489

Beernsten, B.T., James, A.A. and Christensen, B.M. (2000). Genetics of mosquito vector competence. *Microbial and Molecular Biology Review*. p. 115-137

Beier, J.C. (1998). Malaria parasite development in mosquitoes. *Annual Review of Entomology* **43**, 519-543

Beier, J.C.,Davis, J.R.,Vaughan, J.A.,Noden, B.H. and Beier, M.S.(1991a). Quantification of *Plasmodium falciparum* sporozoites transmitted *in vitro* by experimentally infected *Anopheles gambiae* and *Anopheles stephensi. American Journal of Tropical Medicine and Hygiene* **44**, 564-570.

Berner, R., Rudin, W. and Hecker, H. (1983) Peritrophic membranes and protease activity in the midgut of the malaria mosquito, *Anopheles stephensi* (Liston) (Insecta, Diptera) under normal and experimental conditions *Journal of Ultrastructural Research* **83**, 195-204

Billingsley, P.F. (1990). The midgut ultrastructure of hematophagous insects. *Annual Review of Entomology* **35**, 219-248

Billingsley, P.F. and Hecker, H. (1991) Blood digestion in the mosquito *Anopheles stephensi* Liston (Diptera: Culicidae): activity and distribution of trypsin, aminopeptidase and α-glucosidase in the midgut. *Journal of Medical Entomology* **28**, 865-871

Billingsley, P.F. and Rudin, W. (1992) The role of the mosquito peritrophic membrane in bloodmeal digestion and infectivity of *Plasmodium species*. *Journal of Parasitology* **78**, 430-440

Billingsley, P.F. and Sinden, R.E. (1997). Determinants of malaria-mosquito specificity. *Parasitology Today* **13**, 297-301.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline lysis procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513-1522

Bockarie, M.J., Alexander, N.D., Hyun, P., Dimber, Z., Bockarie, F. and Ibam, E. (1998). Randomised community-based trial of annual single-dose diethylcarbamzine with or without ivermectin against *Wuchereria bancrofti* infection in human beings and mosquitoes. *Lancet* **351**, 162-168

Breman, J.G., Egan, A. and Keusch, G.T. (2001). The intolerable burden of malaria: a new look at the members. *American Journal of Tropical Medicine and Hygiene* **64** (suppl.1), iv-vii.

Bruce-Chwatt. Essential Malariology. *Oxford University Press, United Kingdom*

Chen, C. and Billingsley, P.F. (1999). Detection and characterisation of a mannan-binding lectin from the mosquito, *Anopheles stephensi* (Liston). *European Journal of Biochemistry* **263**, 360-366

Chen, C., Ratcliffe, N.A. and Rowley, A.F. (1993). Detection, isolation and characterization of multiple lectins from the hemolymph of the cockroach, *Blaberus discoidalis. Biochemical Journal* **294**,181

Chen, C., Rowley, A.F., Newton, R.P. and Ratcliffe N.A. (1999). Identification, purification and properties of a beta-1,3-glucan-specific lectin from the serum of the cockroach, *Blaberus discoidalis* which is implicated in immune defense reaction. *Comparative Biochemistry and Physiology B. Biochemistry and Molecular Biology*. **122**, 309-19

Collins, F.H., Sakai, R.K., Vernick, K.D., Paskewitz, S., Seeley, D.C., Miller, L.H., Collins, W.E., Campbell, C.C. and Gwadz, R.W. (1986). Genetics of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. *Science* **234**, 607-610

Collins, F.H., Mendez, M.A., Rasmussen, M.O., Mehaffey, P.C., Besansky, N.J. and Finnerty, V. (1987). A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *American Journal Tropical Medicine and Hygiene* **37**, 37-41

Collins, F.H. and Besansky, N.J. (1994). Vector biology and the control of malaria in Africa. *Science* **264**, 1874-1875

Collins, F.H. and Paskewitz, S.M. (1995). Malaria: current and future prospects for control. *Annual Review of Entomology* **40**, 195-219

Collins, F.H., Kamau, L., Ranson, H.A. and Vulule, J.M. (2000). Molecular entomology and prospects for malaria control. *Bulletin of the World Health Organization* **78**(12), 1412-1419

della Torre, A., Constantini, C., Besansky, N.J., Caccone, A., Petrarca, V., Powell, J.R. and Coluzzi, M. (2002). Speciation within *Anopheles gambiae*-the glass is half full. *Science* **298**, 115-117

Dimopoulos, G., Richman, A., della Torre A. and Kafatos, F.C.(1996). Identification and characterization of differentially expressed cDNAs of the vector mosquito, *Anopheles gambiae*. *Proceedings of National Academy of Science of USA*. **93**, 13066-13071

Dimopoulos, G., Müller, H, Levashina, E.A. and Kafatos, F. (2001). Innate immune defense against malaria infection in the mosquito. *Current Opinion in Immunology* **13**, 79-88

Epstein, J., Eichbaum, Q., Sheriff, S. and Ezekowitz, R.A.B. (1996). The collectins in innate immunity. *Current Opinion in Immunology* **8**, 29-35

Ezekowitz, R.A., Day, L.E. and Herman, G.A. (1988). A human mannose-binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins. *Journal of Experimental Medicine* **167**, 1034-1046

Franc, N.C. and White, K. (2000). Innate recognition in insect immunity and development; new approaches in *Drosophila*. Special;

issue: Innate recognition systems in host defense. *Microbes and Infection*, **30**(2), 243-250

Gass, R.F. (1977). Influences of blood digestion on the development of *Plasmodium gallinaceum* in the midgut of *Anopheles Aegypti*. *Acta Tropica* **34**, 127-140

Gass, R.F. and Yeates, R.A. (1979) *In vitro* damage of cultured ookinetes in the midgut of *Anopheles atroparvus* mosquitoes and *in vitro*. *Parasitology* **91**, 219-225

Gillies M.T. and Meillon B De. (1968). The Anophelinae of Africa South of the Sahara. Johannesburg: *South African Institute for Medical Research* no. 54

Gillies, M.T. and Coetzee M. (1987). A supplement to the Anophelinae of Africa South of the Sahara, Johannesburg: *South African Institute for Medical Research*. no. 55

Githeko, A.K. Service, M.W., Mbogo, G.M. and Atieli, F.K. (1996). Resting behaviour, ecology and genetics of malaria vectors in large-scale agricultural areas of Western Kenya. *Parassitologia* **38**, 481-489

Graf, R., Raikhel, A.S., Brown, M.R., Lea, A.O. and Briegel, H. (1986). Mosquito trypsin: immunocytochemical localization in the midgut of blood fed mosquito *Aedes aegypti*. *Cell Tissue Research* **245**, 19-27

Gregorio, E.A. and Ratcliffe, N.A. (1991). The distribution of agglutinins and lytic activity against *Trypanosoma rangeli* and erythrocytes in *Rhodnius prolixus* and *Triatoma infestans* tissue extracts. *Mem.Inst.Oswaldo Cruz, Rio Janeiro* **86**, 181-186

Ham, P.J., Phiri, J.S. and Nolan, G.P. (1991). Effect of N-acetyl-D-glucosamine on the migration of *Brugia pahangi* microfilariae into the haemocoel of *Aedes aegypti*. *Medical and Veterinary Entomology* **5**, 485-493

Han, Y.S., Thompson, J., Kafatos, F.C. and Barillas-Mury, C. (2000). Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinetes invasion of mosquitoes. *European Molecular Biology Organization Journal* **19**(22), 6030-6040

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., and Ezekowitz, R.A. (1999). Phylogenetic perspectives in innate immunity. *Science* **84**, 1313-1318

Holt, R.A. *et al.*, (2002). The genome sequence of the malaria mosquito *Anopheles gambiae*. *Science*. **298**, 129-149

Ibrahim, E.A.R., Ingram, G.A. and Molyneux, D.H. (1984). Haemagglutinins and parasite agglutinins in haemolymhp and gut of *Glossina*. *Tropenmedizin Parasitology*. **35**, 151-156

Ifediba, T. and Vanderberg, J.P. (1981). Complete *in vitro* maturation of *Plasmodium falciparum* gametocytes. *Nature* **294**, 364-366

Ikeda, K., Sannoh, T., Kawasaki, T. and Yamashina, I. (1987). Serum lectin with known structure activates complement through the classical pathway. *Journal of Biological Chemistry* **262**, 7451-7454

Jacobs-Lorena, M. and Oo, M-M. (1996). The peritrophic matrix of insects, In The Biology of disease vectors. (Beaty, B.J. and Marquardt, W. *eds*), pp 318-332, University press of Colorado.

Janeway, C.A.Jr. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunology Today* **13**(1), 11-16

Jungery, M. (1985). Studies on the biochemical basis of the interaction of the merozoites of *Plasmodium falciparum* and the human red cell. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**, 591-597

Kawasaki, K., Kubo,T. and Natori, S. (1996). Presence of the *Periplaneta* lectin-related protein family in the American cockroach *Periplaneta americana. Insect Biochemical and Molecular Biology* **264**, 355-364

Liener, I.E., Sharon, N. and Goldstein, I.J. (1986). The Lectins. Academic press, Inc., (London) Ltd., UK.

Maudlin, I. and Welburn, S.C. (1987). Lectin-mediated establishment of midgut infections of *Trypanosoma congolense* and *Trypanosoma brucei* in *Glossina morsitans*. *Tropical Medicine 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 Parasitology* **39**, 56-58.

Maudlin, I. and Welburn, S.C. (1991). Rickettsia-like organisms, puparial temperature and susceptibility to trypanosome infection in *Glossina morsitans*. Parasitology **102**, 201-206

Maudlin, I. and Welburn, S.C. (1994). Maturation of trypanosome infection in tsetse. *Experimental Parasitology* **79**, 202-205

Medley, G.F., Sinden, R.E., Fleck, S., Billingsley, P.F., Tirawanchai, N. and Rodriguez, M.H. (1993) Heterogeneity in patterns of malarial oocyst infections in the mosquito vector. *Parasitology* **106**, 441-449

Meis, J.F.G.M. and Ponnudarai, T. (1987). Ultrastructural studies on the interaction of *Plasmodium falciparum* ookinetes with the midgut epithelium of the *Anopheles stephensi* mosquitoes. *Parasitology Research* **73**, 500-506

Mihok, S., Otieno, L.H., Darji, N. and Munyinyi, D. (1992). Influence of D(+)-glucosamine on infection rates and parasite loads in tsetse flies (*Glossina spp*) infected with *Trypanosoma brucei*. *Acta Tropica* **51**, 217-228

Miller, D.M., III, Desai, N.S., Hardin, D.C., Piston, D.W., Patterson, G.H., Fleenor, J., Xu,S. and Fire, A. (1999). Two-color GFP expression system for *C.elegans*. *Biotechniques* **26**, 914-918,920-921

Miller, L.H., Baruch, D.I., Marsh, K. and Doumbo, O.K. (2002). The pathogenic basis of malaria. *Nature* **415**, 673-679

Mills, C.D., Burges, D.C., Taylor, H.J. and Kain, K.C. (1999). Evaluation of a rapid and inexpensive dipstick assay for the diagnosis

of *Plasmodium falciparum*. *Bulletin of World Health Organization* **77**, 553-559

Morlais, I. and Severson, D.W. (2001). Identification of a polymorphic mucin-like gene expressed in the midgut of the mosquito, *Aedes aegypti*, using an integrated bulked segregant and differential display analysis. *Genetics* **158**, 1125-1136

Nebenführ, A. and Lomax, T.L. (1998). Multiplex Titration RT-PCR: Rapid determination of gene expression patterns for a large number of genes. *Plant Molecular Biology Reporter* **16**, 323-339

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. (1989). Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of sciences of USA* **86**, 2766-2770

Perrone, J.B., De Maio,J., Spielman, A. (1986). Regions of mosquito salivary glands distinguished by surface lectin-binding characteristics. *Insect Biochemistry* **16**, 313-318

Ponnudurai, T., Lensen, A.H.W., Gemert, G.J.A.Van, Bolmer, M.G.and Meuwissen, J.H.E. (1991). Feeding behaviour and sporozoite ejection by infected *Anopheles stephensi*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **85**, 175-180.

Richie, T.L. and Saul, A. (2002). Progress and challenges for malaria vaccine. *Nature* **415**, 694-701

Roth, J., Brown, D. and Orci, L. (1983). Regional distribution of N-acetyl-D-galactosamine residues in the glycocalyx of glomerular podocytes. *Journal of Cell Biol*ogy **96**, 1189-1196

Rudin, W. and Hecker, H. (1979). Functional morphology of the midgut of *Aedes aegypti* L.(Insecta, Diptera) during blood digestion. *Cell Tissue Research* **200**, 193-203

Rudin, W. and Hecker, H. (1989). Lectin-binding sites in the midgut of the mosquitos *Anopheles stephensi* Liston and *Aedes aegypti* L. (Diptera, Culicidae). *Parasitology Research* **75**, 268-279.

Rudin, W., Billingsley, P.F. and Saladin, S. (1991) The fate of *Plasmodium gallinaeum* in *Anopheles stephensi* Liston and possible barriers to transmission. *Annual Society Belg.Med.Trop.***71**, 167-177

Schneider, M., Rudin,W. and Hecker, H. (1987). Absorption and transport of radioactive tracers in the midgut of the malaria mosquito, *Anopheles stephensi*. *Journal of Ultrastructural Research* **97**, 50-63

Schulman, S., Oppenheim, J.D. and Vanderberg, J.P. (1980) *Plasmodium berghei* and *Plasmodium knowlesi*: Serum binding to sporozoites. *Experimental Parasitology* **49**, 420-429

Scott, J.A., Brogdon, W.G. and Collins, F.H. (1993). Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reactions. *American Journal of Tropical Medicine and Hygiene*.**49**(4), 520-529

Shahabuddin, M. and Kaslow, D.C. (1994). *Plasmodium*: parasite chitinase and its role in malaria transmission. *Experimental Parasitology* **79**(1), 85-88

Sharakhov, I.V.,Serazin, A.C., Grushko, O.G., Dana,A., Lobo,N., Hillenmeyer, M.E., Westerman, R., Romero-Severson,J., Constantini,C., Sagnon,N., Collins, F.H. and Besansky, N.J. (2002). Inversions and gene order shuffling in *Anopheles gambiae* and *An. funestus*. *Science* **298**(5591), 182-185

Shen, Z., Dimopoulos, G., Kafatos, F.C. and Jacobs-Lorena, M. (1999). A cell surface mucin specifically expressed in the midgut of the malaria mosquito *Anopheles gambiae*. *Proceedings of the National Academy of Sciences of USA*.**96**, 5610-5615

Sherman, I.W. (1985) Membrane structure and function of malaria parasites and the infected erythrocyte. *Parasitology* **91**, 609-645

Siegelman, H.M. and Weissman, I.L. (1989). Human homologue of mouse lymph node homing receptor: Evolutionary conservation at tandem cell interaction domains. *Proceedings of the National Academy of sciences of USA* **86**, 5562-5566

Sinden, R.S. (1985). A cell biologist's view of host cell recognition and invasion by malarial parasites. *Transactions of the Royal Society of Tropical Medicine and Hygiene***79**, 598-605

Super, M., Thiel, S., Lu, J., Lewinsky, R.J. and Turner, M.W. (1989). Association of low levels of mannan-binding protein with a common defect of opsonization. *Lancet* **2**, 1236

Super, M., Gillies, S.d., Foley, S., sastry, K., Schweinle, J. -E., Silverman, V.J. and Ezekowitz, R.A.B. (1992). Distinct and overlapping functions of allelic forms of human mannose binding proteins. *Nature Genetics* **2**, 50

Taylor, M.E., Conary, J.L., Lennartz, M.R., Stahl, P.D. and Drickamer, K. (1990). Primary structure of the mannose receptor contains multiple motifs resembling carbohydrate-recognition domains. *Journal of Biological Chemistry* **265**, 12156-12162

Trager, W. and Jensen, J.B. (1976). Human malaria parasites in continuous culture. *Science* **193**, 503-509

Turner, M.W. (1996). Mannose-binding lectin: the pluripotent molecule of the innate immune system. *Immunology Today* **17**, 532-539

Volf, P., Kiewegova, A. and Svobodová, M. (1998). Sandfly midgut lectin: effect of galactosamine on *Leishmania major* infections. *Medical and Veterinary Entomology* **12**, 151-154

Vulule, J.M., Beah, R.F., Atiell, F.K., Roberts, J.M., Mount, D.L. and Mwangi, R.W. (1994). Reduced susceptibility of *Anopheles gambiae* to permethrin associated with the use of permethrin-impregnated bednets and curtain in Kenya. *Medical and Veterinary entomology* **8**, 71-75

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

Weis, W.I., Richard, K., Fourme, R., Drickamer, K. and Hendrickson, W.A. (1991). Structure of the calcium-dependent lectin domain from a rat mannose-binding protein determined by MAD phasing. *Science* **254**, 1608-1615

Wilson, R., Chen, C. and Ratcliffe, N. (1999). Innate immunity in insect? The role of multiple endogenous serum lectin in the recognition of foreign invaders in the cockroach *Blaberus discoidalis. Journal of Immunology* **162**, 1590-1596

Wray, W., Boulikers, T., Wray, V.P. and Hancock, R. (1981). Silver staining of proteins in polyacrylamide gels. *Analytical Biochemistry* **118**, 197-203

Yu Xiao-Qiang and Kanost, M. R. (2000). Immulectin-2, a Lipopolysaccharide specific lectin from an Insect, *Manduca Sexta*, Is induced in Response to Gram-negative Bacteria. *Journal of Biological Chemistry* **275**(48), 37373-37381

APPENDICES

Appendix A : Primer sequences

Oligo name	Sequence (5'-3')
MSP1-A	GTGTAAACACATGAAAGTTATCAAGAACTTGTC
MSP1-B	CGTAGGATCTTGTGATTTAGGTGTTACATCAAC
rp7S-A	GGCGATCATCATCTACGTTGC
rp7S-B	GTAGCTGCTGCAAACTTCGG
A16A	TGCATTGGCGCCTCAGTA
A16B	TCCGTCGTTATCCACGTT
UN	GTGTGCCCCTTCCTCGATGT
GA	CTGGTTTGGTCGGCACGTTT
ME	TGACCAACCCACTCCCTTGA
AR	AAGTGTCCTTCTCCATCCTA
CL1	GCATGCTCCTTGCAAACACTGCCG
CL2	AGATCTAAAATATTCTTCACAAATGTATGG

1. The underlined sequences are introduced restriction sites (Sph I and Bgl II for CL1 and CL2 primers respectively) and the rest sequences are the true primer sequences.

2. CL1 and CL2 pair code for the full lectin protein sequence, the expected product size for RT-PCR is 575 bp.

3. A16A and A16B pair are nested primers, the expected product size is 389 bp

Appendix B: Approximate sizes (in base pairs) of the clones sequenced from *Anopheles sp.*

Anopheles sp.	**Number of individuals**	**Designation**	**Approximate sizes in bp**
An. gambiae (Mbita strain)	1	MBP-M1	450
		MBP-M2	750
An. gambiae (Ifakara strain)	1	MBP-I3	450
		MBP-I5	750
An. arabiensis (Mwea)	1	MBP^{MW}	750
		MBP^{MW}	800
	1	MBP^{MW}	500
		MBP^{MW}	700
		MBP^{MW}	300
		MBP^{MW}	750
		MBP^{MW}	600
		MBP^{MW}	800
	1	MBP-MW5	500
		MBP-MW12	600
An. funestus (Coast)	1	MBP-C1	500
	1	MBP-C15	600

Key:
MBP-C = Mannose Binding protein from Coast
MBP-I = Mannose Binding protein from Ifakara
MBP-M = Mannose Binding protein from Mbita