VALIDATION OF MOSQUITO SALIVARY GLAND ANTIGENS AS SEROLOGICAL MARKERS OF HUMAN EXPOSURE TO *Plasmodium falciparum* INFECTED *Anopheles gambiae* MOSQUITOES IN KILIFI COUNTY, KENYA

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A Thesis Submitted to the Graduate School in Partial Fulfilment of the Requirements for the Master of Science Degree in Biochemistry of Egerton University

EGERTON UNIVERSITY

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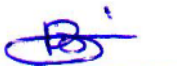
DECLARATION AND RECOMMENDATION

Declaration

I declare that this thesis is my original work and has not been presented in this University or any other for the award of a degree.

Signature: --------  .---------- Date: 13th June, 2023

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Recommendation

This thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

I dedicate this work to my supportive father Jared Oseno, loving mother Delphina Oseno, my siblings Leroy, Salvin, Collins and Melda for their undying support, my daughter Leticiah and my loving husband Douglas Mutunga.

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ABSTRACT

Malaria is a life-threatening disease responsible for more than 400,000 deaths annually worldwide, with sub-Saharan Africa being the most affected. Measurement of malaria transmission risk has been done using entomological tools which have the setback of inapplicability in low transmission settings. Due to heterogeneity in malaria exposure, serological tools which measure antibody responses to vector salivary gland antigens have been applied to measure exposure to vector bites. However, the aptitude of serological tools in distinguishing between infectious and non-infectious bites is limited. This study sought to validate biomarkers that measure exposure to infectious Anopheline mosquito bites. Nine mosquito salivary antigens, Hyp 10, Hyp 15, Hyp 37.7-2, D7r1, D7r2, D7r3, D7r4, D7l2 and SG6, were cloned into pEXP-5-CT/TOPO TA plasmid vector and *in vitro* expressed in competent BL21 (DE3) *E. coli* strain cells. Enzyme-linked immunosorbent assay (ELISA) was used to test antibody responses to the recombinant antigens using antibodies from archived plasma samples. The archived plasma samples that were used (n=684) reflect temporal variation in transmission intensity and were collected during a longitudinal study carried out in 2008 and 2014 in Junju ward, Kilifi County. All the data was analyzed using R (Version 3.5.1). After carrying out normality tests, Wilcoxon-Rank Sum Test was used to compare two groups while Kruskal-Wallis Test was used to compare multiple groups. Antibody responses to SG6, Hyp 10, Hyp 15, Hyp 37.7-2, D7r1, D7r2, D7r3, D7r4 and D7l2 reflected a temporal variation in malaria transmission intensity. Antibody responses to SG6, D7r2 and D7l2 reflected parasitaemia and malaria infection outcome. In addition, antibody responses to D7l2 were lower in individuals who used bed-net as vector control strategy. In all the analyses, statistical significance was at $P < 0.05$. The findings of this study will contribute significantly towards evaluating the effectiveness of a vector control strategy and estimating the risk of malaria transmission at both the population and individual level in areas of varying transmission intensities. Additionally, the outcomes of this study will aid studies involving naturally acquired immunity to malaria.

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LIST OF ABBREVIATIONS AND ACRONYMS

AA	:	Amino acid
cDNA	:	Complementary Deoxyribonucleic acid
DNA	:	Deoxyribonucleic acid
ELISA	:	Enzyme Linked Immuno-sorbent Assay
Hyp	:	Hypothetical
IgG	:	Immunoglobulin G
LB	:	Luria-Bertani
LC-MS/MS	:	Liquid Chromatography tandem Mass Spectrometry
mRNA	:	Messenger Ribonucleic acid
MW	:	Molecular weight
OD	:	Optical Density
PBS	:	Phosphate-buffered saline
PCR	:	Polymerase Chain Reaction
pI	:	Isoelectric point
PSM	:	Peptide-spectrum match
RNA	:	Ribonucleic acid
Rpm	:	Revolutions per minute
SDS-PAGE	:	Sodium Dodecyl Sulphate Polyacry-lamide Gel Electrophoresis
SG6	:	Salivary Gland Protein 6
TB	:	Terrific Broth

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Malaria is a life-threatening disease that is caused by a protozoan of the genus *Plasmodium* (Martinsen *et al*., 2008) and spread by the female mosquito of the *Anopheline* species (Molina-Cruz & Barillas-Mury, 2014). Malaria is most prevalent in Sub-Saharan Africa (Bhutta *et al*., 2014) and mostly affects children under the age of 5 years. In 2021, approximately 247 million cases of malaria occurred worldwide resulting in an estimated 619 000 deaths with 96% of the deaths recorded in African region (WHO, 2022).

The current strategies for malaria management and control are mainly vector control and chemotherapy; with vaccine development considered as a potential tool for malaria control. Use of drugs such as chloroquine and artemisinin has the limitation of development of drug resistance in the parasite (Niba *et al*., 2023). One of the causes of chloroquine drug resistance of *Plasmodium* in Asia, Africa and South America has been attributed to mutations in *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene (Sidhu *et al*., 2002; Wicht *et al*., 2020).

Resistance of *Plasmodium* to artemisinin has been observed in South East Asia and mutations in K13-propeller domain has been validated as one of the causes of drug resistance (Ariey *et al*., 2016). Vaccine development is therefore a potential strategy to control malaria so as to overcome the issue of multi-drug resistance in the parasite. Efforts to develop a vaccine against malaria are still underway and the RTS,S/ASO1 malaria candidate vaccine which targets the pre-erythrocytic was the first to reach the phase III trials and showed an overall efficacy of 50% in infants aged between 6 to 12 weeks at the first vaccination (Partnership, 2012). Despite the RTS,S/ASO1 vaccine using the brand name Mosquirix being approved by the World Health Organization, people who are immunized are still susceptible to repeated malarial attacks (Nadeem *et al*., 2023). Other malaria vaccine candidates that target other stages of the parasites such as mosquito sexual stages (transmission blocking), blood stages and placental sequestering are still under development (Duffy & Gorres, 2020). Attempts have been made in the use of whole sporozoites as a chemoprophylaxis but the results remain inconclusive as the study participants needed rescue treatment (van der Boor *et al*., 2023). Vector control is therefore currently critical in the control and potential elimination of the disease (Oke *et al*., 2022). Use of long-lasting insecticide impregnated bed nets and indoor residual spraying as vector control measures have contributed to 78%

reduction in clinical malaria cases in 2000-2015 (Bhatt *et al*., 2016). It is hence fundamental to evaluate the efficacy of a vector control strategy and henceforth the risk of malaria transmission. This can be achieved through measurement of exposure to vector bites.

The means used to measure exposure to vector bites include parasitological, entomological and clinical. These methods have been met with various shortcomings such as being labour-intensive, time-consuming, expensive and non-reproducible especially in areas of low-malaria transmission (Zhou *et al*., 2014). For instance, the entomological inoculation rate which is considered the 'gold-standard' to measure exposure to vector bites and the risk of malaria transmission is obtained by multiplying the prevalence of sporozoites in mosquitoes by the human biting rate (Beier *et al*., 1999). Obtaining the human biting rate typically involves exposure of human subjects to the mosquito vector which puts them at risk of infection with other diseases transmitted by the vector (Briët *et al*., 2015; Monroe *et al*., 2020; WHO, 2013).

During a blood meal on a human host, the mosquito usually injects saliva which contains various components some of which are immune-modulatory proteins so as to aid in its feeding (Arora *et al*., 2023; Fontaine *et al*., 2011; Gillespie *et al*., 2000). Whole mosquito saliva has been used as a biomarker to measure exposure to vector bites using the serological approach since the salivary proteins are immunogenic (Doucoure & Drame, 2015). Nevertheless, whole mosquito saliva has the limitation of non-specificity due to antigenic cross-reactivities with other hematophagous salivary protein epitopes (Poinsignon *et al*., 2008). It is hence necessary to develop specific biomarkers of exposure to malaria vector bites. The salivary gland proteins SG6 and cE5 have been used as biomarkers of exposure in Angola, Burkina-Faso and Senegal (Drame *et al*., 2010a; Poinsignon *et al*., 2008; Rizzo *et al*., 2014). IgG levels against SG6 and cE5 are usually measured and their quantities correlate to the level of exposure to vector bites. To date, there is insufficient information on salivary gland antigens which can be used to distinguish between infectious and non-infectious bites. This information is necessary to estimate the risk of malaria transmission.

This study therefore tested nine (Hyp 10, Hyp 15, Hyp 37.7-2, D7r1, D7r2, D7r3, D7r4, D7l2 and SG6) *A. gambiae* specific salivary gland antigens. Two of the salivary gland antigens (SG6 and D7l2) are known to be upregulated in the salivary glands of mosquitoes infected by sporozoites (Marie *et al*., 2014). The antigens were validated as biomarkers of recent exposure to infectious Anopheline bites on a longitudinal cohort established in Kilifi County. The findings of this study will contribute in the assessment and implementation of

vector control measures, measurement of the risk of malaria transmission and studies involving naturally acquired immunity to malaria.

1.2 Statement of the Problem

Measurement of exposure to malaria vector bites is used to estimate the risk of transmission and the effectiveness of vector control strategies. Estimating the risk of malaria transmission at the individual and population level in low transmission intensity areas is difficult and probably inapplicable with the current entomological tools. Serological tools have been used to measure exposure to vector bites but their capacity to distinguish between infectious and non-infectious Anopheline bites is limited. This study validated salivary gland antigens that are upregulated in the salivary glands of *A. gambiae* infected by sporozoites. This study will hence aid in distinguishing between infectious and non-infectious Anopheline bites.

1.3 Objectives

1.3.1 General Objective

To validate biomarkers that measure human exposure to *P. falciparum* infected *A. gambiae* mosquitoes in Kilifi County, Kenya.

1.3.2 Specific Objectives

i) To assess salivary gland antigens as markers that distinguish infectious from non-infectious Anopheline bites.

ii) To evaluate salivary gland antigens as markers of malaria exposures using plasma samples from areas of varying transmission intensities.

iii) To evaluate immune responses to salivary gland antigens in the presence or absence of a vector control strategy

1.4 Null Hypotheses

i) Salivary gland antigens do not distinguish infectious from non-infectious Anopheline bites.

ii) Salivary gland antigens are not markers of malaria exposure in plasma samples from areas of varying transmission intensities.

iii) Immune responses to salivary gland antigens do not vary in presence or absence of a vector control strategy

1.5 Justification

Control of malaria is crucial due to the disease life-threatening nature. Vector control using long-lasting insecticidal treated bed nets and indoor residual spraying is one of the tools for control of malaria. It is therefore necessary to evaluate the efficacy of a vector control strategy and thus to estimate the risk of malaria transmission by measurement of exposure to vector bites. This study will therefore contribute towards the measurement of human exposure to vector bites by validating *A. gambiae* specific biomarkers. Measurement of antibody levels to these biomarkers in humans will facilitate studies in evaluating the efficacy of vector control strategies and estimating the risk of malaria transmission even in low-density transmission areas at population and individual level. This study will also contribute to the development and evaluation of a malaria vaccine as protection from disease development solely due to the vaccine will be backed up by proof of exposure to infectious bites after vaccine administration. This will be instrumental in the separation of truly immune individuals from those who appear immune as they have not been exposed to infectious bites. Additionally, if antibodies produced against a salivary gland antigen or protein confer protection against clinical malaria then the antigen can serve as a potential arthropod-based vaccine candidate. This has been proven in *Leishmania braziliensis* infection whereby immunity to *Lutzomyia whitmani* saliva conferred protection against the disease (Gomes *et al*., 2016).

CHAPTER TWO

LITERATURE REVIEW

2.1 The Malaria Vector

Hematophagous arthropods are responsible for the transmission of a wide range of human diseases caused by nematodes, protozoa, bacteria and viruses which account for 17% of the global infectious diseases leading to approximately 700 000 deaths annually (Arca & Ribeiro., 2018; Gubler, 2009; WHO, 2017). Their success in disease transmission is due to multiple blood feeding per gonadotrophic cycle and insecticide resistance (Brackney *et al*., 2021; Ramalho-Ortigao & Gubler., 2020). Most notable insect vectors are the mosquitoes which transmit a variety of diseases including and not exclusive to malaria, dengue, West Nile fever, Chikungunya, Yellow fever and lymphatic filariasis (Fonseca *et al*., 2004; Ichimori *et al*., 2014; Neafsey *et al*., 2015). There are 3000 known species of mosquitoes and 100 species are responsible for transmitting human disease (Rozendaal, 1997). In the case of malaria, the female *Anopheles* mosquito is responsible for transmission of *Plasmodium*, the causative agent of malaria. Out of 400 described Anopheline, only 70 species distributed worldwide (Figure 1) have been identified as malaria vectors (Sinka *et al*., 2012). The *Aedes* mosquitoes mostly transmit Arboviral diseses such as dengue fever, Chikungunya fever, West Nile fever and Zika virus while the *Culex* mosquitoes mostly transmit filarial worms and West Nile Virus (Gao *et al*., 2020). This successive transmission is majorly aided by their microbiota.

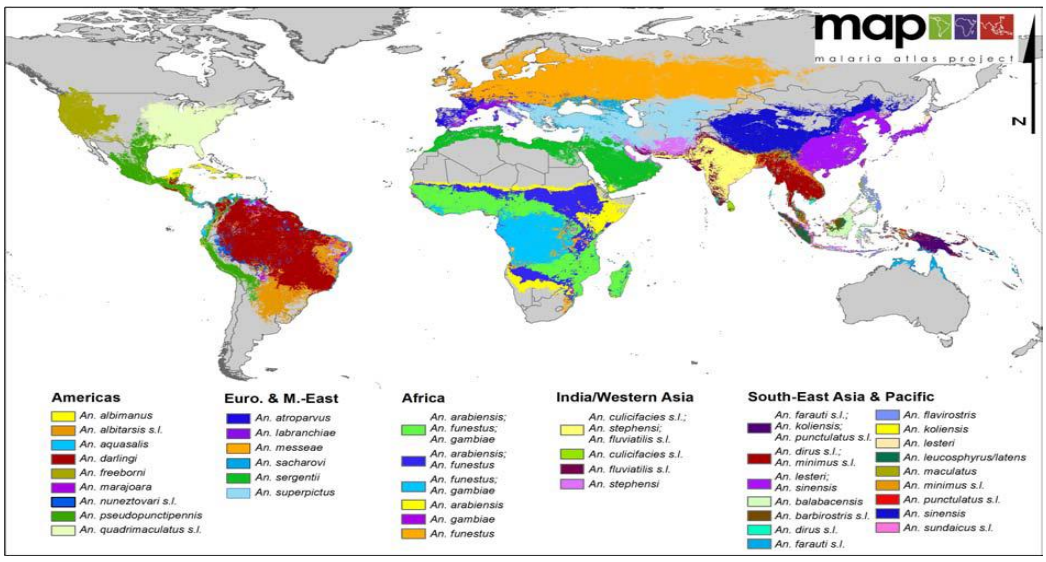

Figure 1. Global distribution of 34 dominant vectors (Sinka *et al*., 2012); s.l.: *sensu lato*, meaning 'in the broad sense' referring to species complex

A. gambiae is the most successful malaria vector mostly found in Sub-Saharan Africa and is responsible for the transmission of the deadliest malaria parasite, *Plasmodium falciparum* (Ghansah *et al*., 2014). *A. gambiae*'s success as a vector is attributed to a number of factors such as its habitation in both rural and semi-urban areas, prefers to feed on human-hosts and rests both indoors and outdoors (Sinka *et al*., 2010). The large vector burden makes vector control a crucial tool in fighting malaria.

2.2 Life Cycle of *Plasmodium*

There are six species of *Plasmodium* which are known to cause human malaria namely: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P. Ovale wallikeri* and *P. knowlesi* with *P. falciparum* causing the most severe malaria (Garrido-cardenas *et al*., 2019; Shokoples *et al*., 2009). The malaria parasite has a complex life cycle involving the vertebrate as the intermediate host and the mosquito as the definitive host (Figure 2). The life cycle in the vertebrate host is divided into exo-erythrocytic and erythrocytic stages (asexual stages) while sporogonic cycle is in the mosquito (sexual stages) (Phillips *et al*., 2017). *Plasmodium* is mostly intracellular for a larger part of its life cycle and utilizes special proteins and unique pathways during its egress events (Dvorin & Goldberg., 2022). The *Plasmodium* life cycle is tightly regulated by more than its 5,000 genes as it involves different environments, temperature and biological functions (Hollin & Le Roch., 2020).

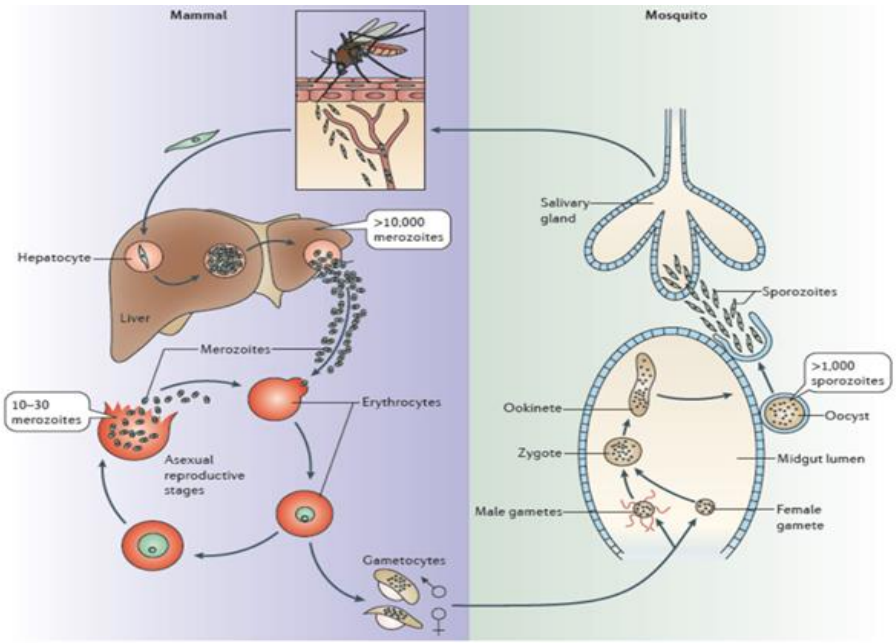

Figure 2. *Plasmodium* spp. life cycle (Ménard *et al*., 2013)

2.2.1 *Plasmodium* life cycle in the vertebrate host

The life cycle of *Plasmodium* begins when an infected blood-feeding female *Anopheles* mosquito injects sporozoites in its saliva under the skin of the human host (Amino *et al*., 2007). The sporozoites then move into the blood vessels and migrate to the liver where they cross the sinusoidal layer and enter into the hepatocytes (Tavares *et al*., 2013). Once in the hepatocytes, a sporozoite transforms into a schizont which later burst to release thousands of merozoites (Prudêncio *et al*., 2006). This constitutes the exo-erythrocytic phase. However, in the case of infection with *P.vivax* or *P.ovale*, hypnozoites remain dormant in the hepatocytes for quite a long time which later transform into merozoites under conducive conditions (White, 2011). The erythrocytic phase begin when merozoites enter into the blood vessels where they invade surrounding erythrocytes through the aid of specific receptor-ligand interactions (Wright & Rayner, 2014). The merozoites in the erythrocytes transform into trophozoites which is the feeding stage of the parasite and results in modification of the erythrocyte membrane (Sherling & van Ooij, 2016). This leads to complicated malaria such as cerebral and placental due to sequestration of the infected erythrocytes within the micro-vasculature (Wassmer & Grau, 2017).

A trophozoite transforms into a schizont which gives rise to a new generation of merozoites. The erythrocytes then rapture and release the merozoites into the bloodstream which infect other erythrocytes (Collins *et al*., 2017). The rupture of erythrocytes to release parasites results in periodic fever which is associated with severe symptomatic malaria (Thomas *et al*., 2013). The erythrocytic cycle occurs every 24 to 72 hours depending on the type of species and results in 10 to 30-fold increase in *Plasmodium* numbers (Phillips *et al*., 2017). Some erythrocytic stage parasites do not develop into mature schizonts instead they develop into intraerythrocytic gametocytes (Greischar *et al*., 2016). Gametocyte development occurs in the bone marrow and takes 1 to 12 days depending on the *Plasmodium* species resulting in infectious male and female worms (Venugopal *et al*., 2020). There are specific differences in heterochromatin distribution which determine sex during gametocyte development (Jeninga *et al*., 2023).

2.2.2 Plasmodium life cycle in the mosquito vector

The sporogonic cycle in the mosquito starts when a female mosquito takes up intraerythrocytic gametocytes from an infected vertebrate host. The attraction of a mosquito to an infected host is facilitated by host odour and volatile products emitted by the malaria parasite (Busula *et al*., 2017). The majority of malaria-infected hosts are children aged 6 to 15

years old (Mbewe *et al*., 2023). Once in the mosquito midgut the micro-gametocytes (male) are induced to exflagellate by a fall in temperature of >5°C and elevated levels of mosquito waste product xanthurenic acid (XA) (Sinden, 2015). The micro-gametocyte undergoes three mitotic divisions to form eight haploid nuclei. Each of these nucleus then fuses with macro-gametocyte (female) nucleus to form a zygote which develops into an ookinete, a motile parasite which penetrates the mosquito midgut wall (Sinden, 2002). The ookinete encysts to form an oocyst which the ookinete nucleus divides to form thousands of sporozoites, a process which is epigenetically regulated (Ukegbu *et al*., 2015). The apetala2 (AP2) members also play a critical role during the development of ookinetes and sporozoites (Guttery *et al*., 2022). The oocyst ruptures to release sporozoites into the mosquito haemolymph where they migrate into the mosquito salivary glands. The sporozoites' invasion of mosquito salivary glands takes ten to fourteen days from the initial gametocyte-containing blood meal. The sporozoites in the salivary glands can then be injected into the next human or vertebrate host during the next blood meal (Aly *et al*., 2009).

2.3 Salivary Glands of the Malaria Vector

The salivary glands are crucial for disease transmission since the pathogens traverse them while being injected into a new host.

2.3.1 Salivary gland proteome of *A. gambiae*

The elucidation of *A. gambiae* complete genome (Holt *et al*., 2002) has facilitated the study of the transcriptome of this vector and subsequently its proteome. Of particular interest is the salivary gland proteome due to the crucial role played by the salivary glands in the transmission of *P. falciparum.* Apart from facilitating host infection, the salivary glands also play a role in midgut maturation of the parasite and may have sporozoite receptors (Dhar & Kumar., 2003; Yamamoto *et al*., 2016). The salivary gland proteome of the female mosquito has drawn more attention since both the male and female mosquitoes feed on plant sugars but it is only the female mosquito that feeds on blood (Hien *et al*., 2016). Ag5 (Antigen 5) family, apyrase/5'-nucleotidase family, cE5/anophelin, D7 family, epoxy hydrolase, hyp 4.2, hyp 6.2, hyp 8.2, hyp 13, hyp10/hyp 12, hyp15/hyp 17, hyp 37.7 family, sal amylase, sal maltase, sal peroxidase, sal ser/pro family, sal trypXII, SG1 family, SG2 family, SG5, SG6, SG7 family, SG8, SG9, 30kDa, 55.3 kDa are the main *A. gambiae* salivary protein gene and gene families documented (Arcà *et al*., 2017).

In the initial steps of cataloguing the salivary gland proteome of female *A. gambiae* mosquito, 69 unique salivary gland proteins were identified 57 of which were novel (Kalume *et al*., 2005). A large proportion of these proteins were involved in protein, carbohydrate and nucleic acid metabolism, transport and energy pathways. Twenty-five percent of the proteins could not be assigned any function while 40% of the proteins could not be assigned specific localization (Kalume *et al*., 2005).

In order to study the role of *A. gambiae* saliva and salivary gland proteins in the transmission of *Plasmodium*, expression levels of proteins in the salivary glands of *P. berghei* infected and non-infected *A. gambiae* female mosquitoes were compared (Choumet *et al*., 2007). In this comparison, the levels of gVAG (*gambiae* Venom AllerGen) increased two fold in infected salivary glands whereas the levels of gSG6, apyrase, D7 related-1 protein precursor and D7 precursor allergen AED A2 were decreased with ratios ranging from 0.67 to 0.77. A similar study also showed that the levels of gSG6 in *P. berghei* infected *A. gambiae* mosquitoes were decreased (Zocevic *et al*., 2013). Interestingly, in another study, *A. gambiae* infected with *P. falciparum* showed upregulated levels of gSG6 in infection (Marie *et al*., 2014). In addition to gSG6, the levels of gSG1, TRIO, SG5 and long form D7 were upregulated in infection (Marie *et al*., 2014). gVAG is a member of the antigen 5 family but its function is unknown; it is postulated to play a role in defense against the *Plasmodium* parasite (Arcà *et al*., 2014). gSG6 is expressed in the distal lateral lobes and is secreted with the saliva during female feeding. However, its function is unknown but it is postulated to play a role in blood feeding. This is because its silencing results in increase in probing time and reduced blood feeding aptitude (Lombardo *et al*., 2009).

PSR1 (*Plasmodium* responsive salivary 1) is novel insect gene which codes for DM9 repeat motifs and has been shown to be upregulated in the salivary glands of *Anopheles gambiae* infected with *Plasmodium falciparum*. PSR1 may play a critical role in the interaction between *Plasmodium* and the epithelia of mosquito (Chertemps *et al*., 2010). This does add more proof that mosquito salivary gland infection with mosquito sporozoites does impact expression of some of its salivary proteins.

Apyrase belongs to the 5'-nucleotidase family of proteins and inhibits ADP-induced platelet aggregation (Champagn *et al*., 1995) hence counteracts the normal homeostatic response to injury. The function of D7 precursor allergen AED A2 is still unknown; however D7 related-1 protein binds serotonin with high affinity, as well as histamine and

norepinephrine thus antagonizing vasoconstriction, platelet aggregation and pain inducing properties (Calvo *et al*., 2006).

Agaphelin, a member of the Kazal-family of inhibitors, is the first anti-hemostatic to be upregulated in the salivary glands of *P. falciparum* infected *A. gambiae* (Waisberg *et al*., 2014). The protein exhibits anti-hemostatic functions by inhibiting neutrophil elastase thereby inhibiting platelet function. However, this study failed to establish neither the upregulation nor downregulation of other anti-hemostatic proteins such as apyrase and D7 family.

2.3.2 *A. gambiae* salivary gland proteins as biomarkers of exposure

Due to the immunogenicity of some salivary proteins, they have been used as biomarkers of exposure to vector bites whereby antibody levels to specific salivary proteins are measured (Billingsley *et al*., 2006). The central principle of using salivary biomarkers involves probing salivary proteins with specific antibodies in human sera. A salivary biomarker should meet the following fundamental criteria: distinguish individuals who are exposed to the vector bites from those who are not, evaluate the density and fluctuations of vector populations, evaluate differences in exposure between different groups and individuals and be usable at the population level (Doucoure *et al*., 2015). There are two main steps in the development of a salivary biomarker: collection of human blood samples potentially containing anti-mosquito antibodies and production of salivary gland extracts which will act as the source of antigens (Doucoure & Drame, 2015).

The use of whole salivary gland extracts as the source of antigens has the limitation of difficulty in collecting salivary gland extracts and standardizing sampling since the mosquito salivary gland protein content vary according to age, sex or diet (Choumet *et al*., 2007). In addition, there is anti-saliva cross-reactivity to different antigenic epitopes of salivary gland antigens of insects of different species. For instance, evaluation of exposure to *Aedes albopictus* bites where individuals who were only exposed to *Aedes albopictus* mosquitoes were sampled, showed cross-reactivity to *Aedes aegypti* salivary gland extracts (Doucoure *et al*., 2012). It is thus necessary to produce a genus or species-specific immunogenic salivary protein and this can be achieved through a recombinant protein approach or a synthetic peptide approach (Fontaine *et al*., 2011).

The recombinant approach has been used to produce *A. gambiae* salivary protein 6 (gSG6) (Lombardo *et al*., 2009) whose orthologs have been found in *Anopheles stephensi* and *Anopheles funestus* (Calvo *et al*., 2007). gSG6 has been validated as a biomarker of

exposure to afrotropical malaria vectors (Rizzo *et al*., 2011) and has been applied in evaluating the effectiveness of vector control strategies and the risk of malaria transmission (Drame *et al*., 2010b; Idris *et al*., 2017; Stone *et al*., 2012). cE5, also a salivary biomarker, has been produced using the recombinant approach whose transcripts are found in both male and female tissues of *A. gambiae* but the protein is only expressed in the female salivary gland tissue (Ronca *et al*., 2012). cE5 aids in blood feeding by acting as a thrombin inhibitor (Pirone *et al*., 2017). IgG levels against cE5 have been used to evaluate the efficacy of insecticide treated bed nets in Angola where even weak exposure to *Anopheles* vector bites was detected (Marie *et al*., 2015). The difference between gSG6 and cE5 salivary biomarkers is that gSG6 causes a short-lived IgG4 response whereas cE5 results in a longer-lived IgG1 response (Rizzo *et al*., 2014).

The recombinant approach of producing a salivary biomarker has the limitation of producing a whole recombinant antigen with more than one epitope and complexity of the system used to produce the recombinant antigen. Consequently, designing and synthesizing a peptide by bioinformatics analysis of the sialotranscriptomic is highly recommended (Doucoure & Drame, 2015). gSG6-P1 was synthesized using the bioinformatics approach (Poinsignon *et al*., 2008). gSG6-P1 has been applied to measure low-exposure to *A. gambiae* vector bites, evaluation of vector control measures, the risk of malaria transmission and seasonal variation in malaria transmission (Drame *et al*., 2015; Kassam *et al*., 2021; Kwapong *et al*., 2023; Londono-Renteria *et al*., 2015; Montiel *et al*., 2020; Sadia-Kacou *et al*., 2019; Sagna *et al*., 2013). An interesting study showed that gSG6-P1 could be used as a marker of exposure to any *Anopheles* species (Ndo *et al*., 2022). This is because their study area, Nyabessang forest area in the south of Cameroon, was pre-dominated by *A. moucheti* and *A. paludis* yet antibody responses to *A. gambiae* gSG6-P1 could still be detected.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Population and Study Design

This study used archived plasma samples collected during a longitudinal cohort study established in 2005 in Junju, Kilifi County in Kenya (Bejon *et al*., 2007). The details of the study design are described elsewhere (Bejon *et al*., 2010). Junju has two peaks of malaria transmission seasons: May-July (during the long rains) and November-December (during the short rains). A cross-sectional survey was carried out in May of each year where a blood sample was collected for parasite detection by microscopy and plasma samples stored. Trained field workers then followed up the participants on a weekly basis for active malaria case detection. Additionally, participants experiencing malaria symptoms before the weekly visits, visited Junju dispensary where parasite detection by microscopy was conducted (Passive case detection). Participants with proven cases of malaria were treated with Artemether-Lumefantrine. For complicated cases, the participants were referred to Kilifi District Hospital. Children who were born into the participating homesteads were recruited during the longitudinal survey. This study analysed plasma samples collected during the May 2008 and May 2014 cross-sectional survey (n=684) for participants aged 1-14 years. This study measured the plasma antibody levels to *A. gambiae* salivary gland antigens (Hyp 10, Hyp 15, Hyp 37.7-2, D7r1, D7r2, D7r3, D7r4, Dl72 and SG6) so as to validate them as biomarkers of exposure to Anopheline infectious bites.

3.2 Ethical Consideration

Ethical approval was granted by the KEMRI Scientific and Ethics Review Unit No. 3139.

3.3 Mosquito Strain

Mosquitoes were maintained under controlled conditions to ensure reproducibility. The *A. gambiae* Kilifi strain was reared at 27±1°C, 75±5% relative humidity (RH) and 12h light/12h dark. The mosquitoes were fed on 10% glucose until they were five to six days old as they had reached full maturity.

3.4 Mosquito Dissection and Extraction of Salivary Gland Tissue

Mosquito dissections were carried out as described (Londono-renteria *et al*., 2010). Mosquitoes were anesthetized by cold, washed in 70% ethanol and placed in 1X PBS (phosphate-buffered saline) for salivary gland dissection. Salivary gland dissection was carried out under a light microscope. The mosquito legs were removed using hands while the

head was chopped off using forceps. The thorax was then placed on a glass slide containing 1X PBS under a light microscope. A needle probe was used to severe the attachment that connects the gland to the thorax hence obtaining intact salivary glands. After dissection, the samples were frozen at -20°C until further processing.

3.5 Preparation of cDNA from Salivary Gland

Total RNA was extracted from the obtained mosquito salivary glands. mRNA was then isolated from total RNA followed by cDNA synthesis.

3.5.1 Total RNA extraction

Total RNA was extracted from mosquito salivary gland using the TRIzol® Reagent (Thermo Fisher Scientific, San Jose, CA, USA). Briefly, the salivary gland samples were homogenized in 1ml TRIZOL Reagent per 60mg of the sample. The homogenized samples were then incubated for five minutes at room temperature so as to enhance the complete separation of nucleoprotein complexes. Centrifugation then followed to remove cell debris and the supernatant was transferred to a new tube. Phase separation was achieved by adding 0.2 ml of chloroform per 1 ml of TRIZOL Reagent. After capping the tubes securely, the samples were vortexed vigorously for 15 seconds then incubated at room temperature for 3 minutes. Afterwards, the samples were centrifuged at a speed of 12,000 x g for 15 minutes at 4 degrees Celsius. After centrifugation, the mixture separated into three phases; the lower red phenol-chloroform phase, an interphase and an upper colourless aqueous phase.

The clear layer, rich in RNA was collected and the RNA precipitated from the aqueous layer by mixing it with 0.5ml of isopropyl alcohol per 1ml of TRIZOL reagent used in the initial homogenization (Chomczynski & Mackey, 1995; Simms *et al*., 1993). The samples were then incubated at 15 degrees for 10 minutes followed by centrifugation at 12,000 x g for 10 minutes at 4 degrees Celsius. The RNA precipitate then formed a gel-like pellet on the bottom and side of the tube. The RNA pellet was then washed twice by mixing 1ml of 75% ethanol per 1ml of TRIZOL Reagent initially used during homogenization. The samples were mixed by vortexing then centrifuged at 7,500 x g for 5 minutes at 4 degrees Celsious. The total RNA samples were stored at -20°C until further processing.

3.5.2 mRNA isolation

The mRNA isolation from total RNA of mosquito salivary glands was done using Dynabeads™ mRNA Purification Kit (Thermo Fisher Scientific, San Jose, CA, USA) as recommended by the manufacturer. For RNA preparation 75µg of total RNA was adjusted to

100µl using 10mM of Tris-HCL, pH 7.5. The mixture was then heated to 65 degrees Celsious for 2 minutes so as to interrupt any secondary structures. The Dynabeads were prepared as follows; they were resuspended and 1mg was transferred to a microcentrifuge tube.

The tube was then placed on a magnet for the Dynabeads to migrate to the tube wall. The beads were then calibrated by adding 100µl of binding buffer and removing the supernatant after placing on magnet. 100 µl of binding buffer was then added to the Dynabeads. mRNA was isolated from total RNA by adding total RNA to the Dynabeads/binding buffer suspension. Mixing was then done thoroughly by vortexing for 5 minutes at room temperature. The Dynabeads were Oligo$(dT)_{25}$ hence the polyadenylated mRNA hybridized with the beads and was immobilized. Washing was then done to remove unbound mRNA and non mRNA molecules. mRNA was then eluted under low ionic strength and stored for cDNA synthesis.

3.5.3 cDNA synthesis

The cDNA was synthesized using Oligo$(dT)_{12-18}$ and SuperScript II Reverse Transcriptase (Invitrogen, San Diego, CA, USA) as per the manufacturer instructions. The first strand was synthesized using 1 µl Oligo(dT), 0.0005 µl mRNA and 1 µl deoxynucleotides triphosphates (dNTPs) then topped up with sterile distilled water to make 12 µl. The mixture was then heated at 65°C for 5 minutes to denature the secondary structure of RNA and quickly chilled on ice to allow the RNA anneal to the primer before addition of RNase as per the manufacturer's instructions. This was followed by incubation at 42°C for 2 minutes then addition of the reverse transcriptase and incubation for 50 minutes at 42°C. Heat inactivation of the reverse transcriptase was done at 94°C for 2 minutes. The PCR machine used was Veriti 96 Well Thermal Cycler (Applied Biosystems, Fullerton, CA, USA). The first strand cDNA was then used as template during consecutive amplification of specific mosquito salivary gland genes. The cDNA from mosquito salivary glands was stored at -20°C until further processing.

3.6 Protein Expression

Mosquito salivary gland proteins were expressed using bacterial protein expression system.

3.6.1 Selection of mosquito salivary gland antigens for expression

The following eighteen (18) mosquito salivary gland antigens were selected for expression: 30kDa, Agaphelin, D7l2, D7r1, D7r2, D7r3, D7r4, Hyp 10, Hyp 12, Hyp 15, Hyp 17, Hyp 37.7, Hyp 37.7-2, Hyp 4.2, Hyp 6.2, Hyp 8.2, Hyp 13 and SG6. All the mosquito

salivary gland antigens to be expressed had a signal peptide which implied that they were secreted proteins. The other unique characteristics were some of them were found in Anopheline mosquitoes only which included cE5, Hyp 10, Hyp 12, Hyp 15, Hyp 17, Hyp 4.2, Hyp 6.2, Hyp 8.2, and SG6. All the selected antigens were specific to female mosquitoes except Hyp 4.2, Hyp 10 and Hyp 12. Agaphelin, D7l2 and SG6 are known to be upregulated in *P. falciparum* infection while the upregulation or downregulation of the remaining antigens during infection is unknown.

3.6.2 Cloning

The cDNA synthesized from mosquito salivary glands was PCR amplified in Veriti 96 Well Thermal Cycler (Applied Biosystems, Fullerton, CA, USA). Gene specific primers to the specific salivary gland antigens were used as shown in Table-1. All the PCR reagents were thawed on ice and briefly vortexed before use. A 50 µl PCR reaction was prepared using 25 µl of Platinum^{TM} Hot-Start PCR Master Mix (2X) (Invitrogen, Carlsbad, CA, USA), 1 µl of the cDNA, 1 µl forward primer for each specific gene, 1 µl reverse primer for each specific salivary gene and nuclease free water (used for top up after addition of all PCR components). The components were then gently vortexed. The PCR conditions were: initial denaturation at 94°C for 3 minutes, 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final elongation of 72°C for 30 seconds.

Following PCR reaction, the PCR products were separated on 1.5 % agarose gel (Lee *et al*., 2012). 5µl of SYBR Safe DNA stain was added to the melted agarose so as to ensure the fluorescence of the DNA fragments. A loading dye was added to each sample so as to increase weight of the sample and aid visibility while loading. 100 base pairs DNA ladder (Invitrogen, Carlsbad, CA, USA) was added for approximating the size of the double stranded DNA. After loading, the gel was run for 35 minutes at 50 volts. Visualization was done under UV using BIO-RAD Molecular Imager (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were excised from the gel and purified using PureLink™ Quick Gel Extraction Kit (Invitrogen, San Diego, CA, USA) following the manufacturer's instructions.

The PCR products were cloned into pEXP-5-CT/TOPO TA using pEXP-5-CT/TOPO™ TA Expression Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. 6 µl of TOPO cloning reaction was prepared for electroporation using 1 µl of the gel excised PCR products, 1 µl of pEXP-5-CT/TOPO TA vector, 1 µl of dilute salt solution and 4 µl of distilled water. The reaction was then mixed gently and

incubated for 5 minutes at room temperature then placed on ice. pEXP-5-CT/TOPO TA is a T7 based plasmid vector which has a C-terminal 6X his tag for protein purification purposes.

Table 1. Sequences of gene specific primers used for PCR amplification of the selected mosquito salivary gland genes.

Mosquito salivary gland gene	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
30kDa	ATGGCTAGCCCGGCTGACGACACGTGGAT	GGATCCCTCTGCATCGCGCTTGTCAA
Agaphelin	ATGGCTAGCGACATCAACTCGAAATGGCG	GGATCCGAACTCTCCATTGCAATTGCCA
D7l2	ATGGCTAGCGACATCAACTCGAAATGGCG	GGATCCGAAATGGACAGTTGTTTAGATG
D7r1	ATGGCTAGCAACACGGTTAAGAAGTGTGAGA	GGATCCGTTGCAAATCTTGTCATCGA
D7r2	ATGGCTAGCCGAAAGGAGTCAACGGTGGA	GGATCCGCACAAACCATCATCGATTTCCT
D7r3	ATGGCTAGCAGACAAGAGGAAACGGTTGAAGA	GGATCCGTTACACAGCCCATCATCAA
D7r4	ATGGCTAGCGAGACTGTGCAAGATTGTGAGA	GGATCCGCAGTTTAATGCCTTATCATAATCCT
Hyp 15	GATCCACTGCCGGGCAGA	CATGTTTGTTAATACACCGCCA
Hyp 17	GATCCACTGCCGGGCCAAA	AAGAGCGGAAAATATGCCACC
Hyp 37.7	ATGGCTAGCACTAAGCGAATCCCGACAGC	GGATCCAGCGCGTGATCGTTTCGAA
Hyp 37.7-2	ATGGCTAGCGCCGCTCGCCGACCCACAAC	GGATCCACCGTTTACTGCTCGTTCTACCG
Hyp 4.2	GTACCCATAACATTGAGCAGTGA	TCCTTCATCAGGAGACATGCTGT
Hyp 6.2	GCTCCACAAGTGACTGAGGC	CTTTTTCACTCGCAAAAAATCA
Hyp 8.2	GAAGAAGCTAGTACCGCAGCAGA	GCCTGAAAACGAGAAGGGCA
Hyp10	GAAGACCCCCGTACCGAGC	GCGAATATCCTTTGTACAGTGGT

Hyp12	AACGATCCAGTCGATGCA	TTGTATATTCTTAGTACAGTAACTGT
Hyp 13	AACGAAATCATACAAAATGTTGT	TTGCGATCCGGAGTCACT
SG6	GAAAAGGTGTGGGTCGA	CTGCTCCAGGAAGGCCT

3.6.3 Transformation, selection and analysis of colonies, isolation of plasmid DNA

The recombinant pEXP-5-CT/TOPO TA was used to transform TOP10 competent *E.coli* cells (Thermo Fisher Scientific, San Jose, CA, USA) (Froger & Hall, 2007). Freshly thawed TOP10 competent *E.coli* cells were mixed with recombinant plasmid and incubated in ice for 30 minutes. The cells were then heat shocked at 42°C for 30 seconds then immediately placed back in ice followed by addition of pre-warmed SOC medium (Invitrogen, Carlsbad, CA, USA). The transformed cells were then incubated in Innova 42 Incubator Shaker series for at 37 degrees 1 hour at 200 rpm for cell recovery. Afterwards, the cells were cultured in LB (Luria-Bertani) agar (Invitrogen, Carlsbad, CA, USA) containing ampicillin and incubated overnight at 37°C.

A few of the resulting colonies were randomly picked and each colony inoculated and cultured separately in LB broth containing ampicillin overnight. These colonies were also PCR screened using T7 forward primers and reverse primers for each individual gene. A 50 µl PCR reaction was prepared using 25 µl of Platinum^{TM} Hot-Start PCR Master Mix (2X) (Invitrogen, Carlsbad, CA, USA), a micropipette tip of the colony, 1 µl T7 forward primer, 1 µl reverse primer for each specific salivary gene and nuclease free water (used for top up after addition of all PCR components). The components were then gently vortexed. The PCR conditions were: initial denaturation at 94°C for 3 minutes, 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final elongation of 72°C for 30 seconds. Following PCR reaction, the PCR products were separated on 1.5 % agarose gel. 5µl of SYBR Safe DNA stain was added to the melted agarose so as to ensure the fluorescence of the DNA fragments.

A loading dye was added to each sample so as to increase weight of the sample and aid visibility while loading. 100 base pairs DNA ladder (Invitrogen, Carlsbad, CA, USA) was added for approximating the size of the double stranded DNA. After loading, the gel was run for 35 minutes at 50 volts. Visualization was done under UV using BIO-RAD Molecular Imager (Bio-Rad Laboratories, Hercules, CA, USA).

The bacterial cells from the positively PCR identified colonies were harvested by centrifugation at 6,800 x g for 3 minutes at room temperature. The supernatant containing the medium was then drained. Plasmid DNA were isolated using QIAprep® Miniprep (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The QIAprep® Miniprep is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The plasmid DNA was then stored at -20°C until further processing.

3.6.4 Transformation and culture for protein expression

Competent BL21 (DE3) *E.coli* strain cells (Thermo Fisher Scientific, San Jose, CA, USA) were transformed with recombinant pEXP5-CT-TOPO/TA. The transformed BL21 (DE3) cells were cultured in TB auto-induction media (Invitrogen, Carlsbad, CA, USA) and incubated overnight at 37°C at 200 rpm. The culture was then centrifuged at 4°C at 12000 rpm using Centrifuge 5810 R (Eppendorf AG, Hamburg, Germany) to obtain bacterial pellet.

3.6.5 Protein purification, quantification and validation

BugBuster® Protein Extraction Reagent (Sigma-Aldrich, St. Louis, USA) was used to lyse the cells in the bacterial pellet and release the cellular contents in solution as per the manufacturer's instructions. Benzonase nuclease (Sigma-Aldrich, St. Louis, USA) was added in the cellular lysate to degrade the nucleic acids. The sample was then gently shaken for 30 minutes at 4°C then centrifuged at 12000 rpm at 4°C using Centrifuge 5810 R (Eppendorf AG, Hamburg, Germany).

To ascertain whether the protein was in the pellet (inclusion bodies) or supernatant (soluble form), the lysate was resolved in 16% Tricine SDS-PAGE (sodium dodecyl sulfate polyacry-lamide gel electrophoresis**)**. Protein extraction under denaturing conditions was done for the proteins found in the pellet so as to solubilize the inclusion bodies and the histagged proteins. This improved binding to the matrix and reduced nonspecific binding as the tag was fully exposed. The denaturing reagent used was 8M urea.

The Ni-NTA purification system (Invitrogen, Carlsbad, CA, USA) was used to purify the proteins as recommended by the manufacturer. The Ni-NTA purification system is based on IMAC (immobilized metal ion affinity chromatography) whereby the Nickel (II) ions immobilised in the resin interact with the polyhistidine tagged proteins (Kielkopf & Urbatsch, 2020) .

The proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, San Jose, CA, USA) following the manufacturer's instructions. The BCA protein

assay combines reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium with the colorimetric detection of the cuprous cation by bicinchoninic acid (BCA). The Optical Density of BCA/copper complex was read at 562nm using microplate reader Synergy 4 (BioTek, Winooski, Vermont, USA).

3.6.5.1 SDS-PAGE (Sodium Dodecyl Sulfate Polyacry-lamide Gel Electrophoresis)

To confirm the purity of the proteins, the recombinant proteins were resolved in 16 % SDS-PAGE gel for 120 minutes at 110 volts using Mini PROTEAN® Tetra Electrophoresis System (Biorad, Hercules, CA, USA). To estimate the molecular weights of the proteins, the gels were stained with Coomassie brilliant blue R-250 (Thermo Scientific, Rockford, IL, USA) and comparison was done with Full-Range Rainbow Molecular Weight Marker (GE Healthcare Life Sciences, Malborough, MA, USA) which was run alongside the proteins in the gels.

3.6.5.2 Mass Spectrometry (MS) Analysis

To confirm the identity of the proteins, Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) was carried out on the purified proteins. Twenty (20) µg/µl of each purified recombinant protein was used for MS. Each sample volume was topped up to 100µl using 100mM triethyl ammonium bicarbonate (TEAB). The samples were then reduced using 40mM Dithiothreitol (DTT) at 70°C for 1 hour followed by alkylation using 80mM Iodoacetamide (IAA) at room temperature in the dark for 1 hour. The samples were then precipitated using 400µl of cold acetone (-20°C) for 1 hour at -20°C followed by centrifugation at 15,000g for 10 minutes at room temperature. The pellet was air-dried for 5 minutes at room temperature and resuspended in twenty microlitres of 8M urea in 100mM TEAB. Ten microliters of one µg/µl of proteomics grade trypsin/Lys-C mix (Promega, Madison, USA) was added to each sample and digestion performed overnight at 37°C in Innova 42 Incubator Shaker series at 150 rpm.

The pH of the peptides was adjusted to acidic state using twenty microlitres of 2.5% Trifluoroacetic acid (TFA) then concentrated using P10 C18 pipette tips Zip-Tips (Millipore, Billerica, MA, USA.) according to the manufacturer's instructions. The eluted peptides were dried in Speedvac concentrator (Thermo Fisher Scientific, San Jose, CA, USA) and re-suspended in fifteen microlitres of 99% H_2O, 1% acetonitrile and 0.1% formic acid.

Five microlitres of obtained peptides were loaded on to a 75µm x 2cm C18 trap column (Thermo Fisher Scientific, San Jose, CA, USA) using Dionex Ultimate 3000 nano-

flow ultra-high pressure liquid chromatography system (Thermo Fisher Scientific, San Jose, CA, USA). A reverse-phase 50cm long column (Thermo Fisher Scientific, San Jose, CA, USA) maintained at 40°C over a 60-min elution gradient (2 to 40% of mobile phase B; 80% acetonitrile with 0.1% formic acid) at a flow rate of 0.3µl/min was used for chromatographic separation of the peptides. Measurement of the peptides was done using LC instrumentation consisting of a Dionex Ultimate 3000 nano-flow ultra-high pressure liquid chromatography system (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) via a nano-electrospray ion source (Thermo Fisher Scientific, San Jose, CA, USA). The settings of ms^1 were: Resolution, 7000; Automatic gain control (AGC) target, 3e6; maximum injection time, 100ms; scan range, 380-1600m/z; ms^2 settings were: Resolution, 17500; AGC target, 5e4; maximum injection time, 100ms; isolation window, 1.6m/z. ms^2 for the top ten most intense ions involved fragmentation with higher energy collision using normalized energy of twenty-eight followed by exclusion for the next twenty seconds.

The raw files obtained from mass spectrometry were searched on a Proteome Discoverer software version 1.3.0.339 (Thermo Fisher Scientific, San Jose, CA, USA) using the Mascot server (Matrix Science) against a concatenated database of *A. gambiae* and *P. berghei* protein FASTA sequences. Fixed modification was set as cysteine carbamidomethylation while deamidation of asparagine or glutamine and methionine was set as variable modification. The false discovery rate (FDR) for both proteins and peptides was set at 0.01 and a maximum of two missed cleavages were allowed in the database search. For positive identification of a protein, a minimum of two unique peptides was considered.

3.7 ELISA (Enzyme Linked Immunosorbent Assay)

Indirect 3-day ELISA was carried and all the samples were run in duplicate in different plates (Hornbeck *et al*., 2001). ELISA Immulon® 4HBX Flat Bottom Microtiter® Plates (Thermo Fisher Scientific, Rochester, NY, USA) were used. The concentration of the recombinant salivary gland antigen used for ELISA varied for each antigen after optimization. The salivary gland antigen dissolved in sodium bicarbonate coating buffer pH 9.6 was immobilized in a polystyrene plate at 4°C overnight. The following day, washing was done four times with 1X PBS Tween (0.05%) using ELx405 Select washer (BioTek, Synergy HT). The plates were blocked using 1% skimmed dry milk powder (Marvel, UK) dissolved 0.05% PBS tween for 5 hours then washed four times. Plasma/serum was diluted in blocking

buffer (0.05% PBS tween dissolved in 1% skimmed dry milk powder) then added and incubated overnight at 4°C.

The following day, the plates were washed four times using 1X PBS Tween (0.05%) in ELx405 Select washer (BioTek, Synergy HT). HRP (Horseradish peroxidase)-conjugated polyclonal rabbit anti-human IgG (Agilent/Dako) was then added, incubated for 3 hours then washed four times under similar washing conditions. HRP substrate OPD (phenylenediamine dihydrochloride) (Sigma-Aldrich, St. Louis, USA) was added according to manufacturer's instructions. After optimization, incubation was done for 40 minutes in the dark. The reaction was stopped using 25µl of 2M sulphuric acid in each well. The OD (Optical density) at 492 nm was recorded using microplate reader-BioTek, Synergy 4 (Promega, Madison, USA).

To account for plate-plate variation, a well containing pooled hyper-immune plasma from malaria immune African adults was included in each plate to serve as positive control. Plasma from malaria naïve European adults served as negative control. A well containing no plasma sample; blocking buffer was used; served as background for each plate. For all the analyses, duplicates whose CV $\geq$ 35% were excluded.

3.8 Data Analysis

All data was analysed using R version 3.5.1 (Vienna, Austria). All the analyses except for temporal variation in malaria transmission intensity were carried out on the Junju 2014 cohort. After confirming that the data was not normally distributed, the Wilcoxon Rank Sum Test was used for comparing two groups while the Kruskal Wallis Test was used for comparing more than two groups. The correlations of the antibody responses to the recombinant mosquito salivary gland antigens were determined using the Spearman method. For all analyses, a $P < 0.05$ was considered as statistically significant.

CHAPTER FOUR

RESULTS

4.1 PCR Amplification of Mosquito Salivary Gland Genes, Cloning and Selection of Positive Colonies

Eighteen (18) salivary gland genes were successfully amplified from mosquito salivary gland cDNA using their specific primers (Figure 3). The successfully amplified salivary gland genes were: SG6, Hyp 17, Hyp 12, Hyp 6.2, Agaphelin, Hyp 10, Hyp 4.2, Hyp 15, Hyp 8.2, Hyp 13, Hyp 37.7-2, Hyp 37.7, D7r4, 30kDa, D7r1, D7l2, D7r3 and D7r2.

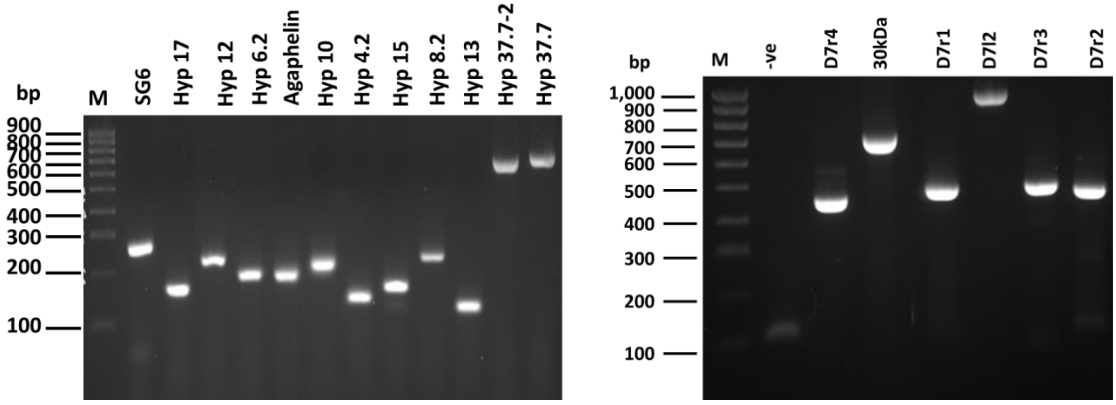

Figure 3. 1.5 % agarose gel image showing amplified PCR products of 18 mosquito salivary gland genes.

Bands correspond to the expected sizes of the genes as referenced by the DNA ladder. Following the cloning of the genes, a diagnostic PCR was used to screen for successful clones using T7 primer and their respective specific forward primers. Clones of seventeen (17) out of the eighteen (18) mosquito salivary gland genes were positively identified as successfully cloned (Figure 4). The salivary gland genes of the positively identified clones were: SG6, Hyp 17, Hyp 12, Hyp 6.2, Agaphelin, Hyp 10, Hyp 4.2, Hyp 15, Hyp 8.2, Hyp 13, Hyp 37.7-2, Hyp 37.7, D7r4, D7r1, D7l2, D7r3 and D7r2 One clone from each gene was picked and used to grow bacterial cultures where plasmid DNA was extracted and used in the expression of their respective proteins.

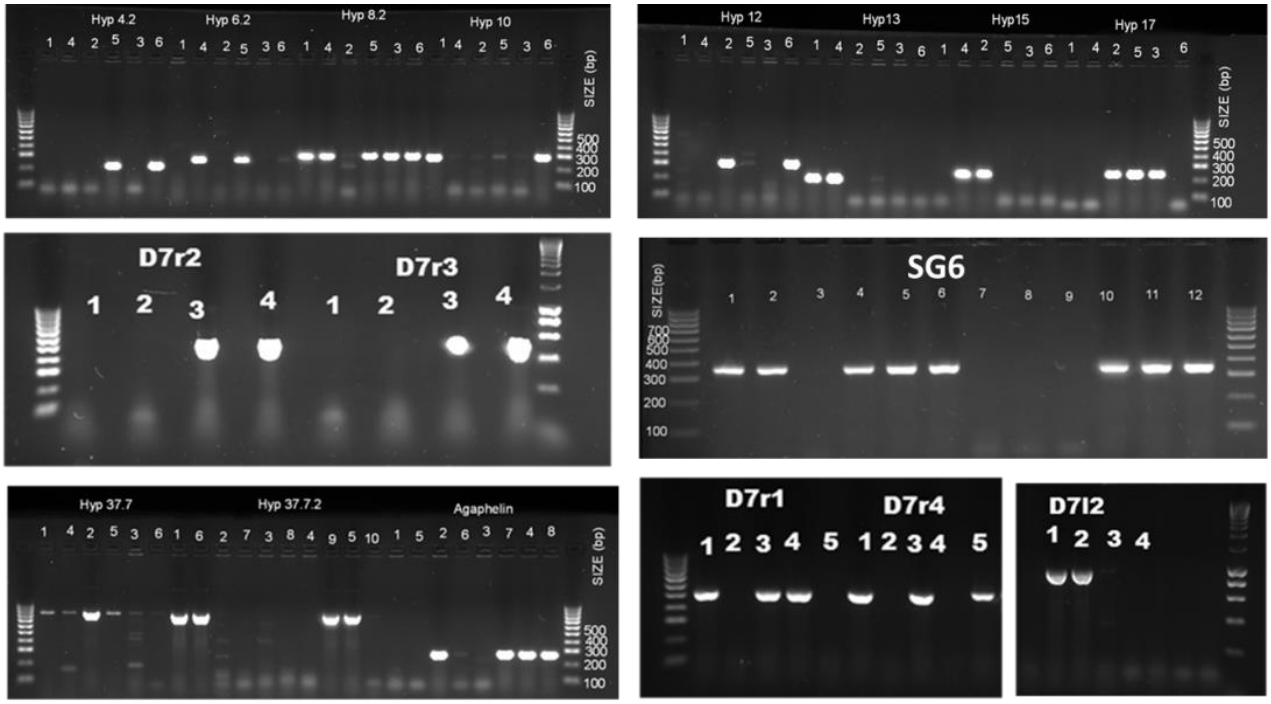

Figure 4. 1.5 % Agarose gel image for T7 PCR screening for positive colonies.

The bands amplified correspond to the expected sizes of the respective genes as referenced by the DNA ladder.

4.2 Production of Recombinant Mosquito Salivary Antigens, Purification and Validation

The recombinant plasmids were used to transform BL21 (DE3) *E.coli* cells where the recombinant salivary antigens were expressed. The recombinant salivary antigens were resolved in 16% Tris-Tricine SDS-PAGE. B-mercaptoethanol was used as the reducing agent and coomassie blue was used for staining to confirm their expression and purity. Nine antigens out of the eighteen (18) salivary gland antigens were successfully expressed and purified (Figure 5). The successfully expressed salivary gland antigens were; Hyp 10, D7r4, SG6, D7r3, D7l2, D7r1, Hyp 37.7-2, D7r2 and Hyp 15. Hyp 10, D7r4, SG6, D7r3 and D7r2 have two prominent bands. The lower bands represent the actual sizes of the proteins while the upper bands may be as a result of dimerization or oligomerization that was wasn't completely reduced with β-mercaptoethnol.

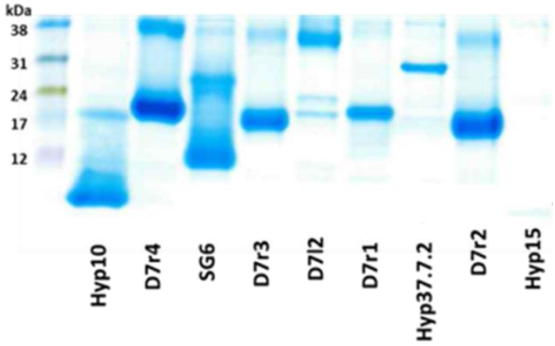

Figure 5. Purified recombinant mosquito salivary gland antigens resolved in 16% SDS-PAGE.

The approximate sizes of the recombinant antigens are: Hyp 10 is 10kDa, D7r4 is 19kDa, SG6 is 13kDa, D7r3 is 18kDa, D7l2 is 36kDa, Hyp37.7-2 is 28kDa, D7r2 is 18kDa and Hyp15 is 8kDa.

4.3 Identification and Confirmation of the Recombinant Salivary Glands Protein

The case of subsequent ELISA experiments, the recombinant mosquito salivary gland antigens were subjected to identification and confirmation analysis using Liquid-Chromatography tandem mass spectrometry (LC-MS/MS). All the 9 antigens except Hyp 15 had their target antigen gene name matching the antigens identified by LC-MS/MS. The lower concentration of Hyp 15 might have led to the differing results hence a larger quantity of Hyp 15 would have been required for mass spectrometry. Since all the antigens had a Mascot score greater than 100, then they were considered fit for subsequent ELISA experiments. The results are as shown in table-2 below.

Table 2. Identification of the recombinant mosquito salivary antigens by mass spectrometry

Target antigen	Target antigen gene name	Theoretical MW (kDa)	Antigen(s) identified by Mass Spec	Number of Ms/Ms unique peptide sequences	Sequence coverage	Mascot score
D7L2	AGAP008279	36.138	AGAP008279-PA Long form D7 salivary protein	19	68.67	4918.46
D7r1	AGAP008284	18.738	AGAP008284-	1	26.51	243.70

			PA hypothetical protein			
D7r2	AGAP008282	18.483	AGAP008282-PA hypothetical protein	8	61.54	9522.16
D7r3	AGAP008283	18.653	AGAP008283-PA hypothetical protein	6	56.47	4542.68
D7r4	AGAP008281	19.309	AGAP008281-PA hypothetical protein	10	63.25	4463.76
Hyp 15	AGAP000152	8.023	AGAP008307-PA hypothetical protein	1	18.68	235.15
Hyp 37.7-2	AGAP001989	28.713	AGAP001989-PA hypothetical protein	10	62.02	3707.50
Hyp10	AGAP008307	10.016	AGAP008307-PA hypothetical protein	5	69.23	7933.92
SG6	AGAP000150	13.091	AGAP000150-PA GSG6 salivary protein	5	44.83	1673.83

4.4 Determination of ELISA Coating Concentration for Each Recombinant Mosquito Salivary Gland Antigen

In order to determine the coating concentration for each recombinant salivary antigen, the recombinant mosquito salivary gland antigens were serially diluted and equal concentrations of serum were added in each well. A pool of serum from malaria immune African adults (PHIS-Pooled Hyper-Immune Serum) were used as positive control while a serum from a malaria naïve European adult (EU 02) was used as negative control (Appendix 3). Malaria immune refers to individuals living in a malaria endemic region while malaria naïve refers to individuals living in a non-malaria endemic region. Each data point represents

a mean of double wells. The point at which antibody response in the malaria immune serum saturated was taken as the coating concentration.

4.5 Seropositivity and Antibody Responses According to Age and Sex of Recombinant Mosquito Salivary Antigens

To determine whether individuals mount immune responses to the mosquito salivary gland antigens an ELISA was conducted. Seropositivity was determined as mean of OD readings of malaria naïve individuals ± 3SDs. The cut off for seropositivity and percentage for seropositive individuals for each recombinant antigen are as shown in Table-3. More than 60% of the individuals had antibody responses to the recombinant mosquito salivary gland antigens.

A moderate increase in antibody responses to SG6, D7r2 and D7l2 correlated with age increase (Figure-6). A higher and significant increase of antibody responses to D7r2 and D7l2 correlated with increase in age. On the other hand, a significant decrease in antibody responses to Hyp 15 and D7r1 in correlation with increase in age was observed. Sex had no significant impact on the antibody responses to the recombinant salivary antigens except for SG6 where females had significantly higher antibody responses than males (Figure-7).

Table 3. Seropositivity of antibody responses to recombinant mosquito salivary gland antigens

Antigen name	Cut-off OD for Seropositivity	% of seropositive individuals
Hyp 10	0.0780	93.8983
D7l2	0.1682	92.7900
D7r1	0.1176	99.6795
D7r2	0.0201	99.6785
D7r3	0.0346	99.3548
D7r4	0.1155	97.9310
Hyp 15	0.0198	99.6885
SG6	0.0868	62.7193
Hyp 37.7-2	0.0271	99.6774

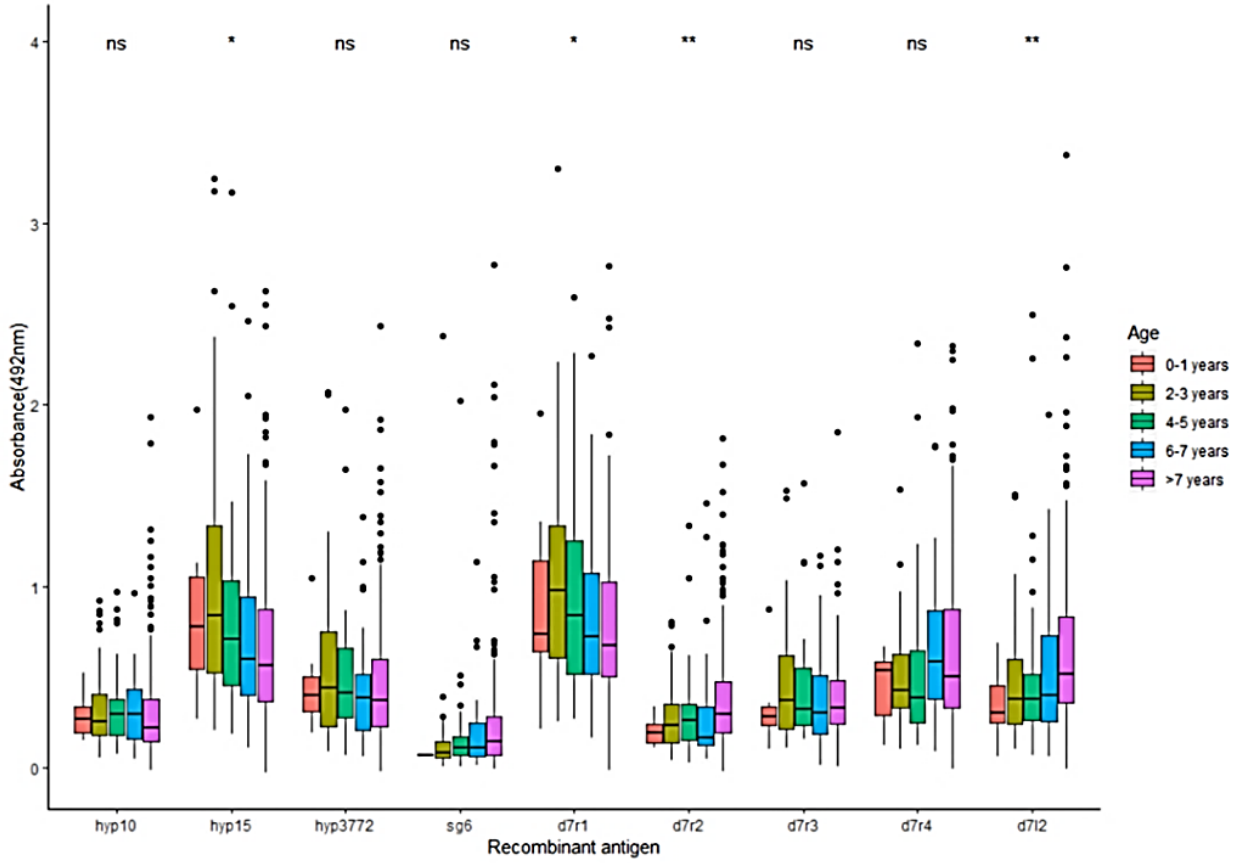

Figure 6. ELISA data showing IgG responses to recombinant mosquito salivary antigens in relation to age.

Increase with age caused a significant increase in responses to D7r2 and D7l2. Increase with age caused a significant decrease in responses to Hyp 15 and D7r1. (ns, not significant; *, $p < 0.05$; **, $p < 0.01$).

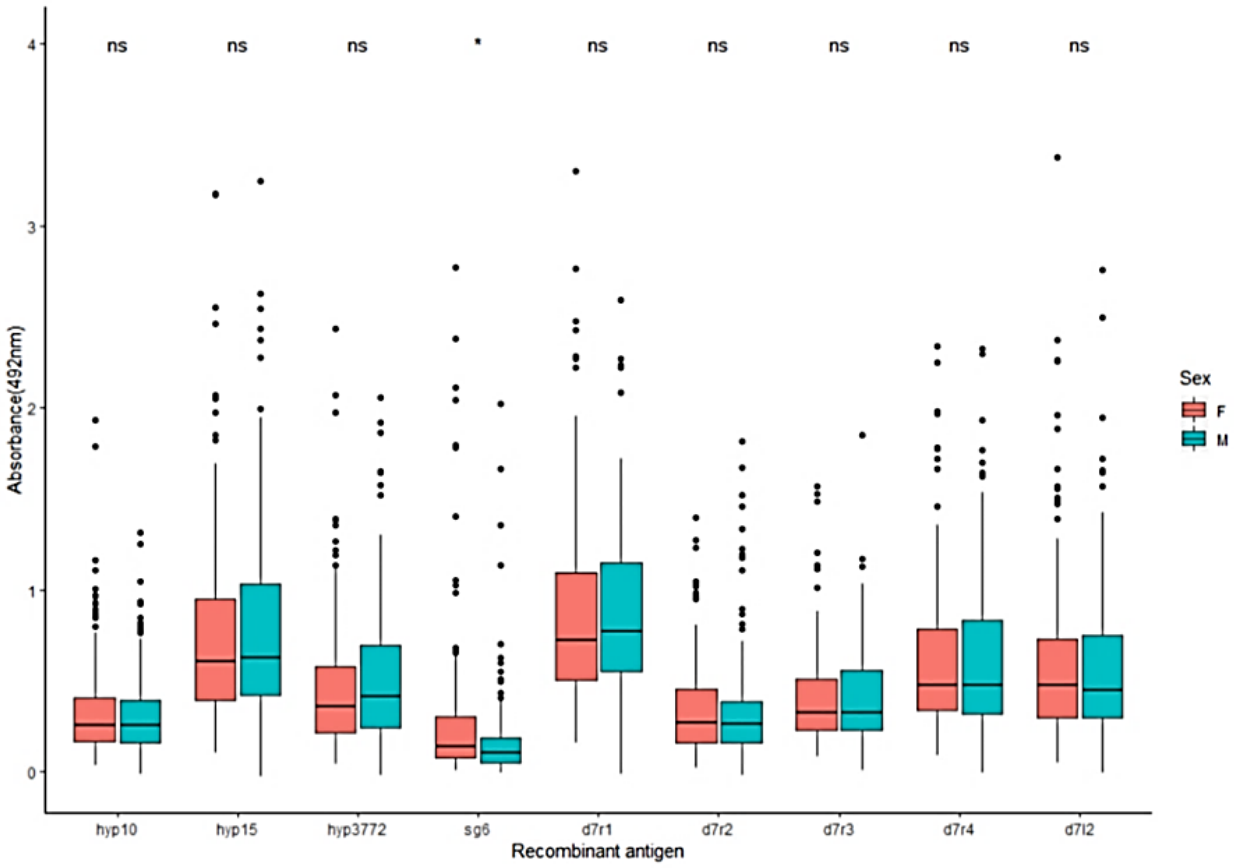

Figure 7. IgG responses to recombinant mosquito salivary gland antigens in relation to; sex.

IgG responses to recombinant SG6 was higher in females than in males. (ns, not significant; *, $p < 0.05$).

4.6 Mosquito Salivary Antigens as Markers of Malaria Exposure

Immune responses to the recombinant mosquito salivary antigens were compared in plasma samples reflecting variable transmission intensities.

4.6.1 Antibody responses according to the levels of malaria exposure

In order to determine whether antibody responses to mosquito salivary gland antigens reflect malaria endemicity, ELISA was used. Antibody responses to the recombinant mosquito salivary gland antigens were compared in plasma samples reflecting varying degrees of malaria exposure Antibody responses in pooled plasma sample from malaria immune African adults, malaria naïve European adults and African children from a malaria endemic region (Junju, Kilifi County) were compared. IgG responses to the recombinant salivary antigens were significantly higher in the malaria exposed individuals as compared to the malaria naïve individuals (Figure 8).

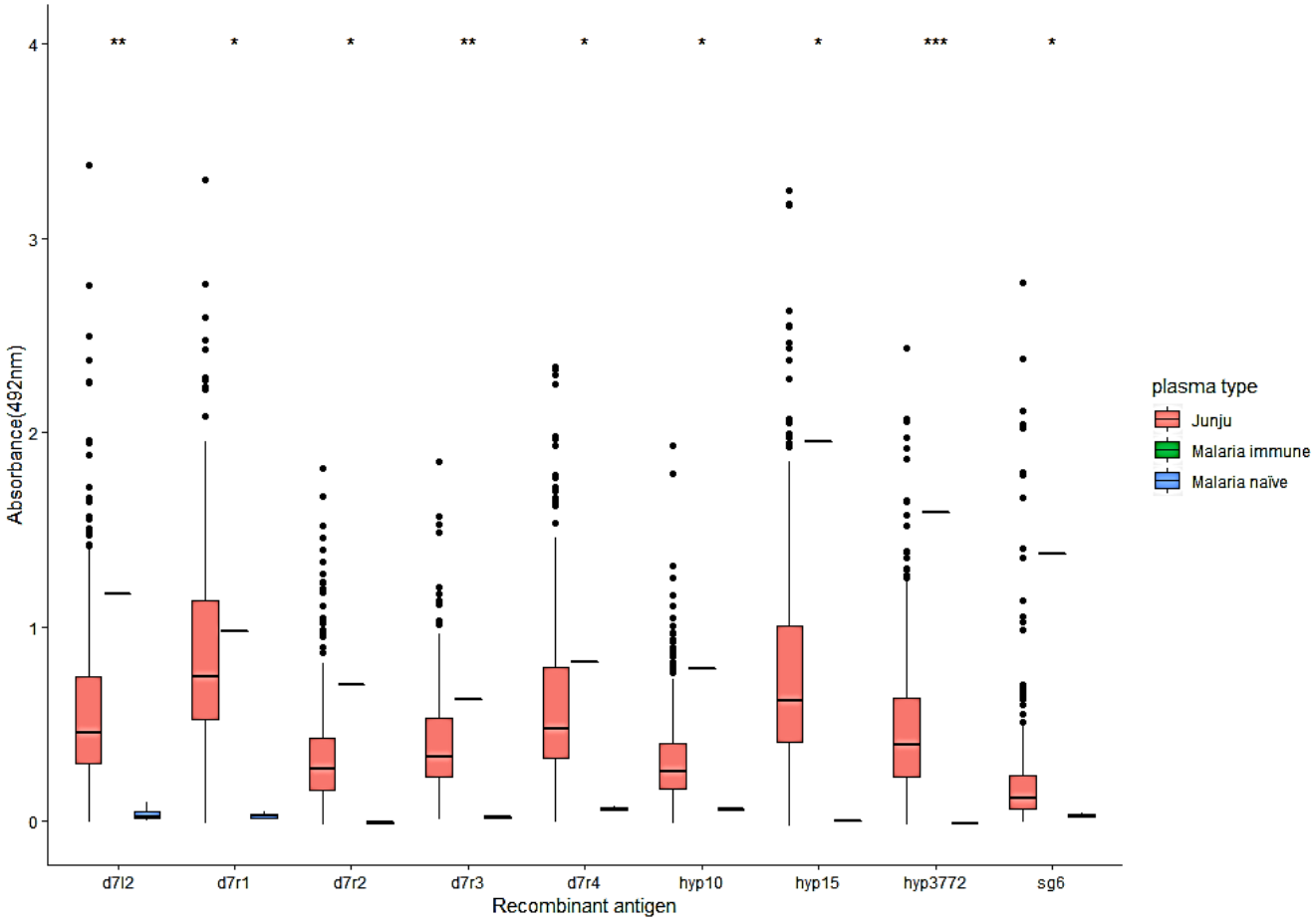

Figure 8. IgG responses according to the level of malaria exposure.

Plasma IgG levels to salivary antigens were compared in malaria immune African adults, malaria naïve European adults and a cohort in Junju, Kilifi. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

4.6.2 Antibody responses in temporal variation in transmission intensity

In order to determine whether antibody responses to the mosquito salivary gland antigens reflect temporal variation in malaria transmission intensity, ELISA was carried out. IgG responses to the recombinant mosquito salivary gland antigens were compared when malaria transmission was moderate (2008) and when it was low (2014) in Junju, Kilifi county. Antibody responses to D7r2, D7r3, D7r4, Hyp 10 and SG6 were significantly lower in 2014 as compared to 2008 (Figure-9). Antibody responses to D7r1 were not significantly different in 2008 and 2014. Antibody responses to Hyp 15 and Hyp 37.7-2 were significantly higher in 2014 as compared to 2008.

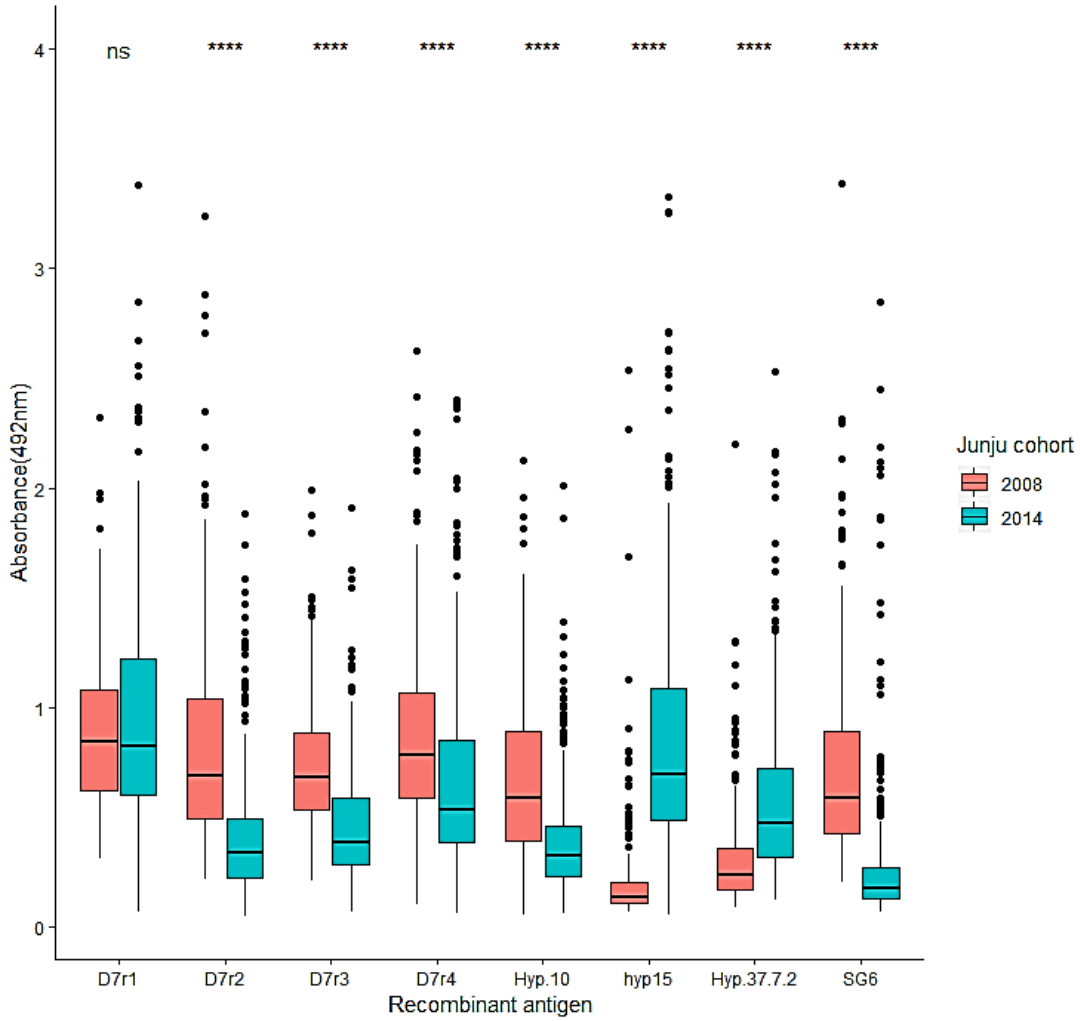

Figure 9. IgG responses according to temporal variation in transmission intensity.

Antibody responses to recombinant salivary antigens at a period of moderate transmission (2008) and low transmission (2014) in Junju, Kilifi. Antibody responses to recombinant salivary antigens predict temporal variation in transmission intensity D7r2, D7r3, D7r4, Hyp 10 and SG6. (ns, not significant; ***, $p < 0.001$).

4.6.3 Antibody responses according to malaria infection outcome

IgG responses were compared in individuals who did not get infected with malaria with those who got infected with malaria in the follow-up period. Individuals who were not infected with malaria in the follow-up period had higher antibody responses to SG6, D7l2 and D7r2 at the cross-sectional bleed (Figure-10). These results strongly suggest that SG6, D7l2 and D7r2 are predictors of malaria infection outcome.

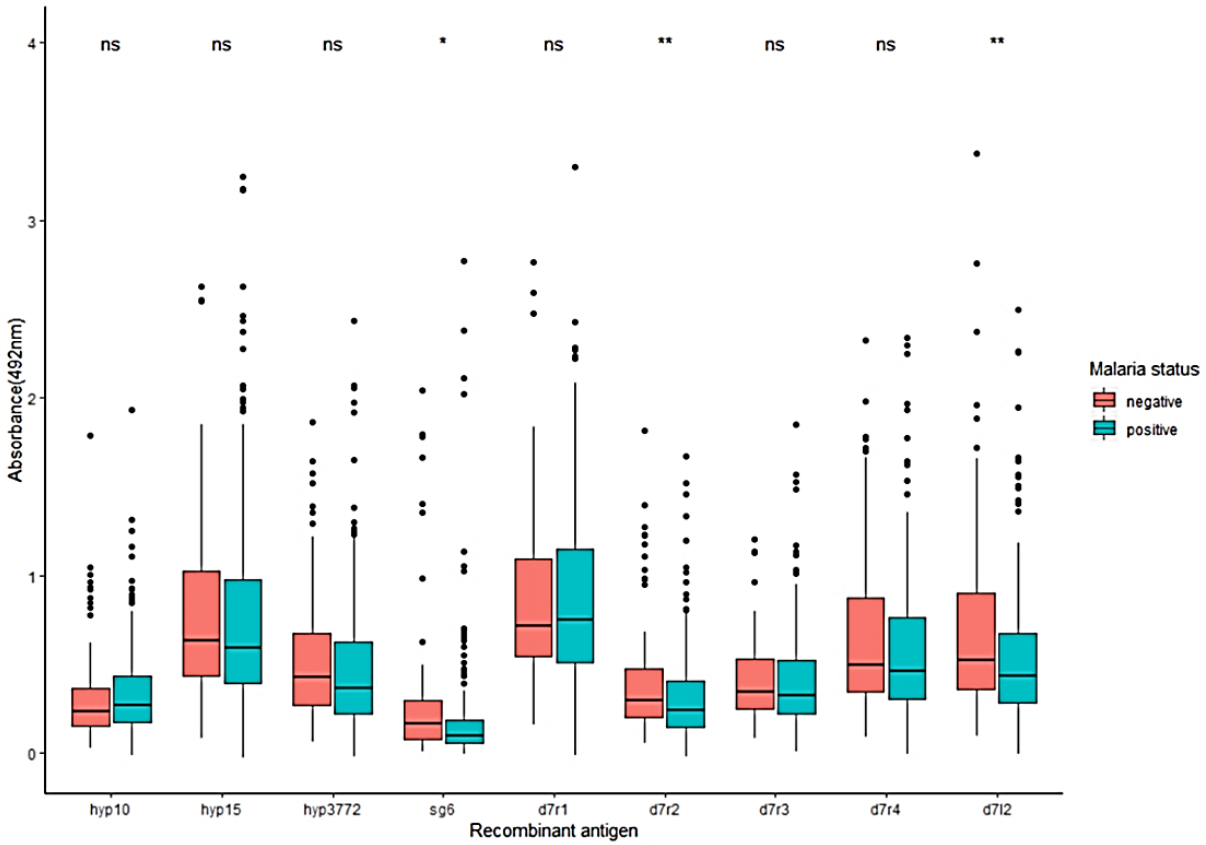

Figure 10. IgG responses to recombinant salivary antigens according to malaria infection outcome.

Only SG6, D7r2 and D7l2 showed significant differences in IgG responses to them between the infected and none malaria-infected groups. (ns, not significant; *, $p < 0.05$; **, $p < 0.01$).

4.7 Mosquito Salivary Gland Antigens as Tools to Distinguish Infectious from Non-Infectious Bites

In order to determine whether individuals were bitten by infected or none-malaria infected mosquitoes, ELISA was carried out. Antibody responses were compared in individuals who had malaria parasites at the cross-sectional bleed and those who did not. Antibody responses to recombinant SG6, D7r2 and D7l2 were significantly higher in individuals who had malaria parasites at the cross-sectional bleed (Figure-11).

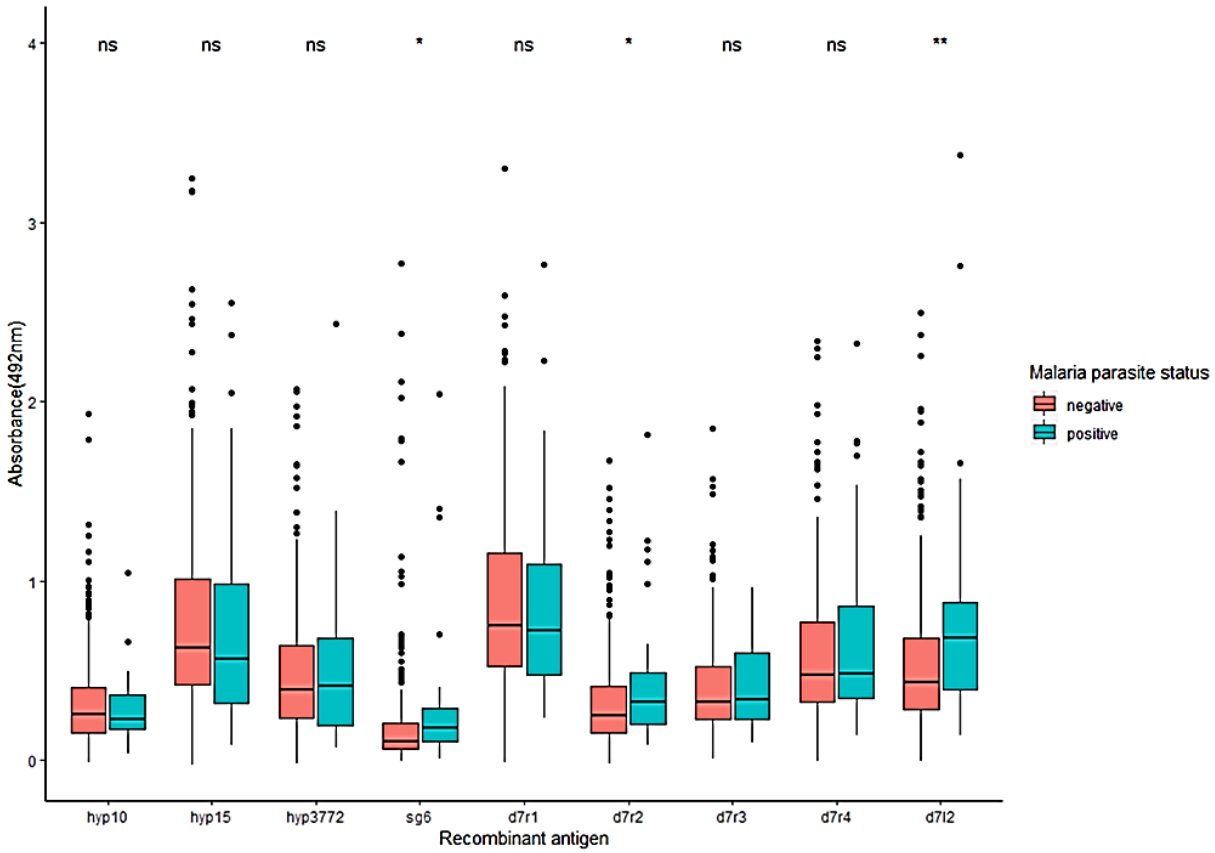

Figure 11. IgG responses according to malaria parasite status at cross-sectional bleed.

Antibody responses to SG6, D7r2 and D7l2 were significantly higher in individuals who had malaria parasites. (ns, not significant; *, $p < 0.05$; **, $p < 0.01$).

4.8 Mosquito Salivary Gland Antigens as Tools To Evaluate The Effectiveness of Bed-Net Use as a Vector-Control Strategy

Use of bed-nets as a vector control strategy is dynamic in terms of how long the bed-net has been in usage, the place where the bed-net was acquired and whether the bed-net was treated or not. IgG responses to the recombinant salivary gland antigens were compared in individuals who possessed bed-nets and those who did not. Individuals who did not possess bed-nets had higher antibody responses to SG6, D7r1, D7r2, D7r4 and D7l2. However, IgG responses were only statistically significant to D7l2 (Figure 12).

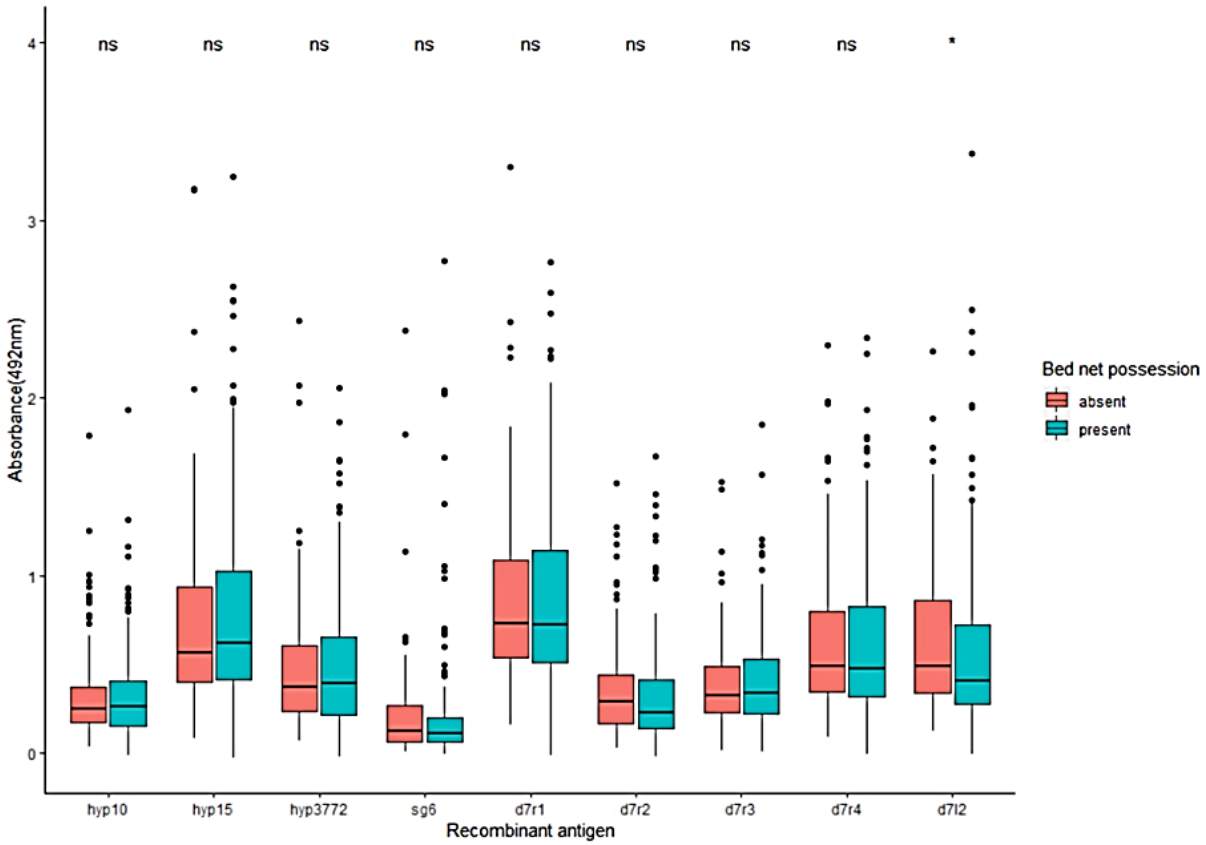

Figure 12. IgG responses to recombinant salivary antigens according to bed net possession.

IgG responses to recombinant D7l2 was significantly lower in individuals who possessed bed-nets. (ns, not significant; *, $p < 0.05$).

Antibody responses to the recombinant salivary gland antigens were also compared in individuals who slept under bed-net the previous night before the cross-sectional bleed and those who did not. Individuals who did not sleep under bed-net had higher responses to SG6, D7r1, D7r2, D7r4 and D7l2 with a significant difference seen only in immune responses to D7l2 (Figure 13).

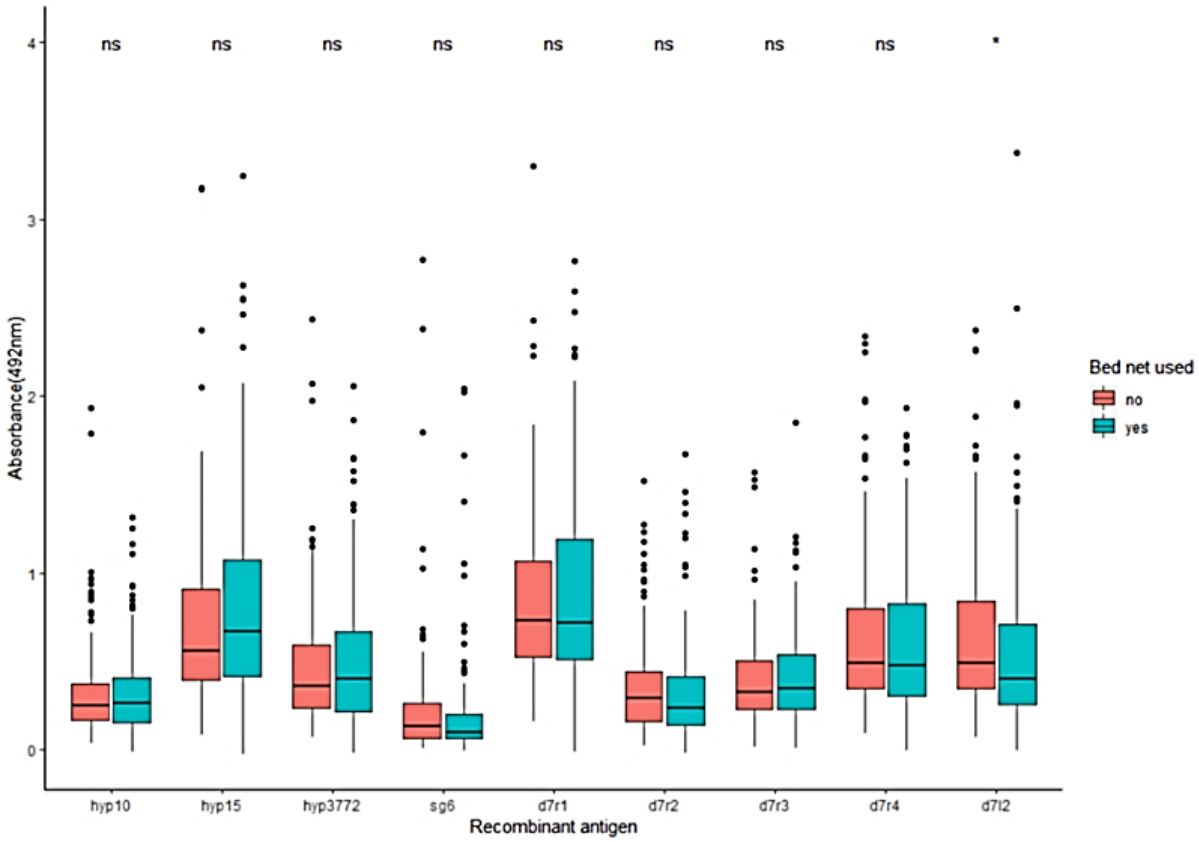

Figure 13. IgG responses according to bed-net usage.

IgG responses to D7l2 was lower in individuals who slept under bed-net. (ns, not significant; *, $p < 0.05$).

The time when the bed-net was acquired (ranging from more than 5 years ago and the last 3 months) had no significant impact on the immune responses to the recombinant salivary gland antigens (Figure 14).

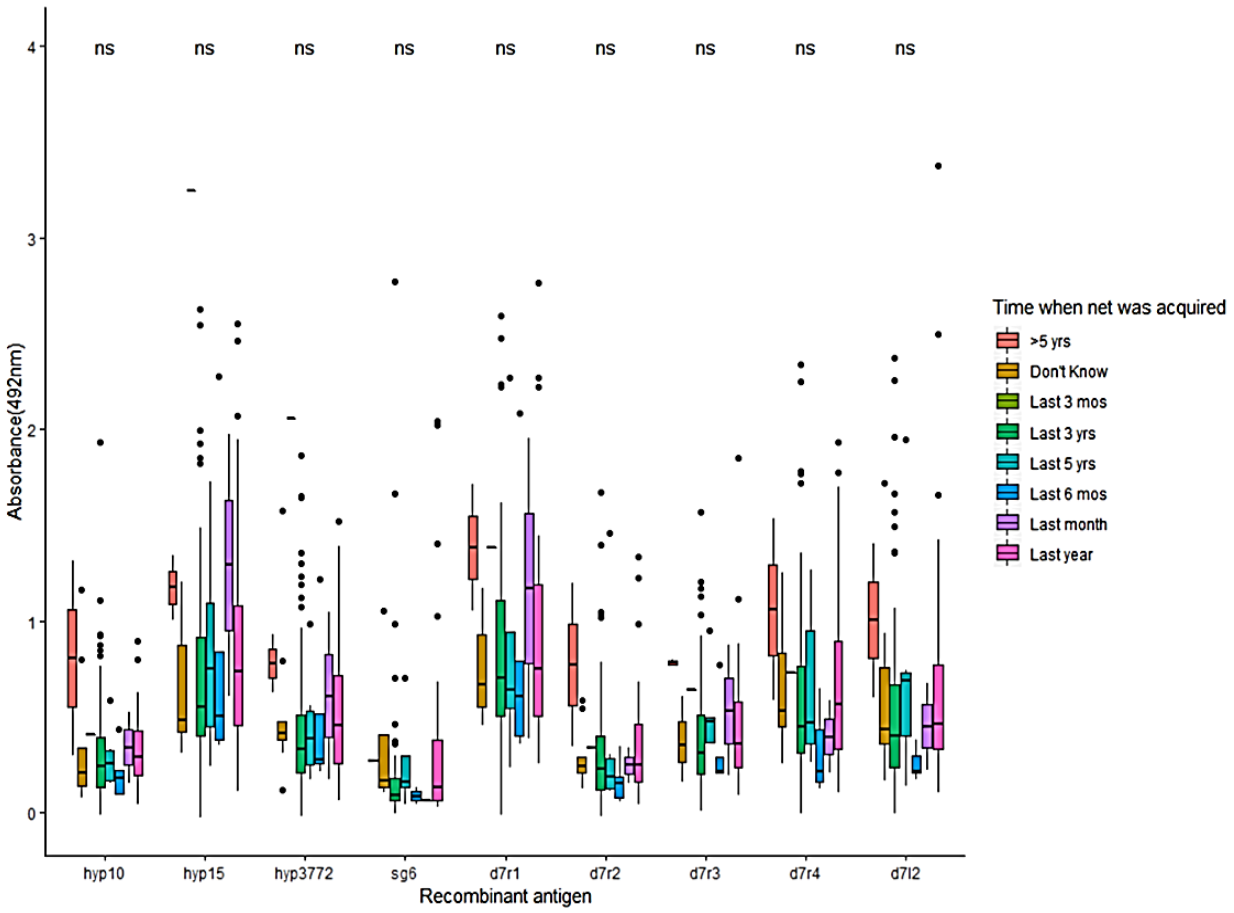

Figure 14. IgG responses to recombinant salivary antigens according to the time when bed-net was acquired.

IgG responses to recombinant mosquito salivary antigens were independent of the time when the bed-net was acquired. (ns, not significant)

Individuals whose bed-nets were treated with insecticide had lower antibody responses to all the recombinant mosquito salivary gland antigens. Antibody responses to all the nine recombinant antigens were lower in individuals whose bed-nets were treated. However, only Hyp 15, Hyp 37.7-2, SG6, D7r1 and D7r3 were significantly lower (Figure 15).

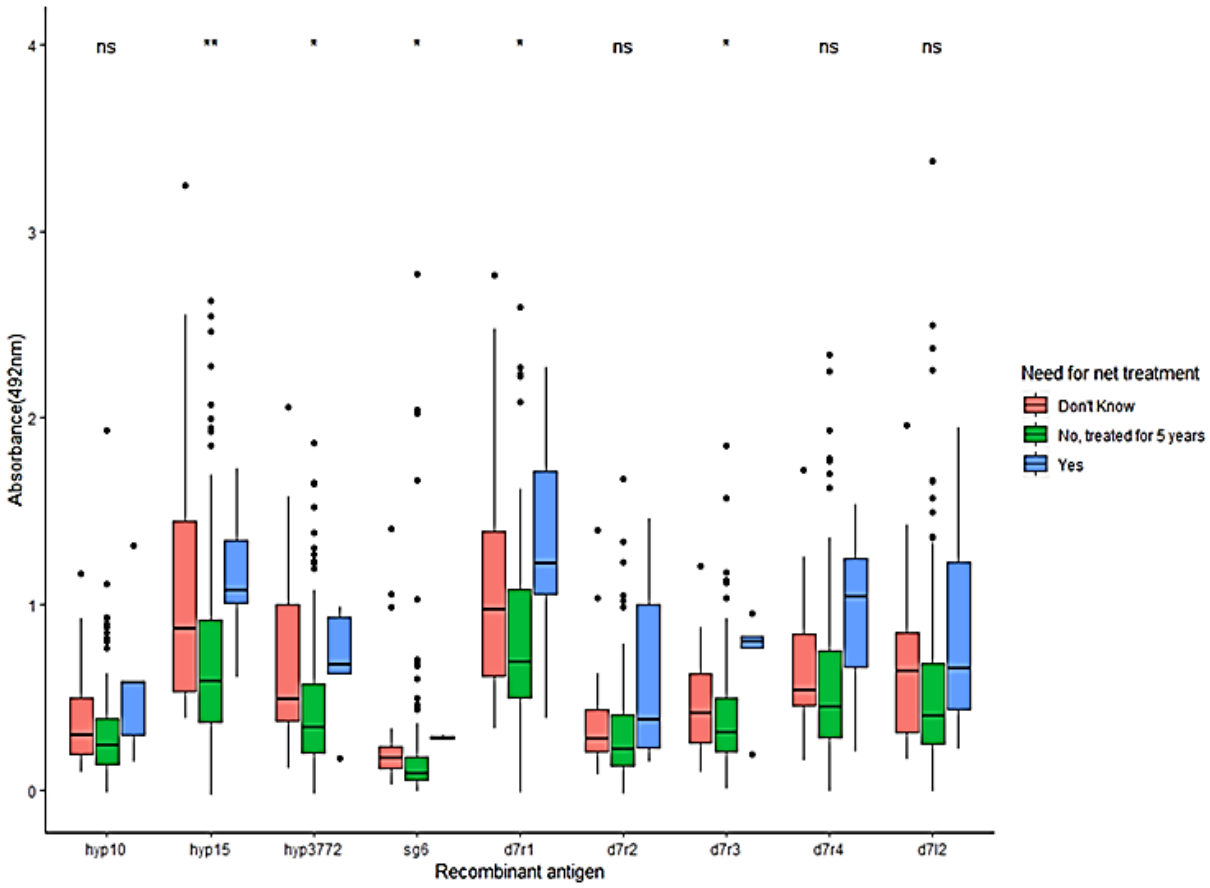

Figure 15. IgG responses according to bed-net treatment status. Antibody responses to Hyp 15, Hyp 37.7-2, SG6, D7r1 and D7r3 were significantly lower in individuals whose bed-net was treated. (ns, not significant; *, $p < 0.05$; **, $p < 0.01$).

To determine whether the origin of the bed nets had an effect on the immune responses to the recombinant antigens, an ELISA was carried out as described earlier. It was established that the place where the bed-net was acquired did have a significant impact to the immune responses to recombinant D7r4. Individuals whose bed nets were from government facilities at a cost had the lowest antibody responses to D7r4 while those whose bed-nets were obtained free of charge from campaigns had the highest responses to D7r4 (Figure 16).

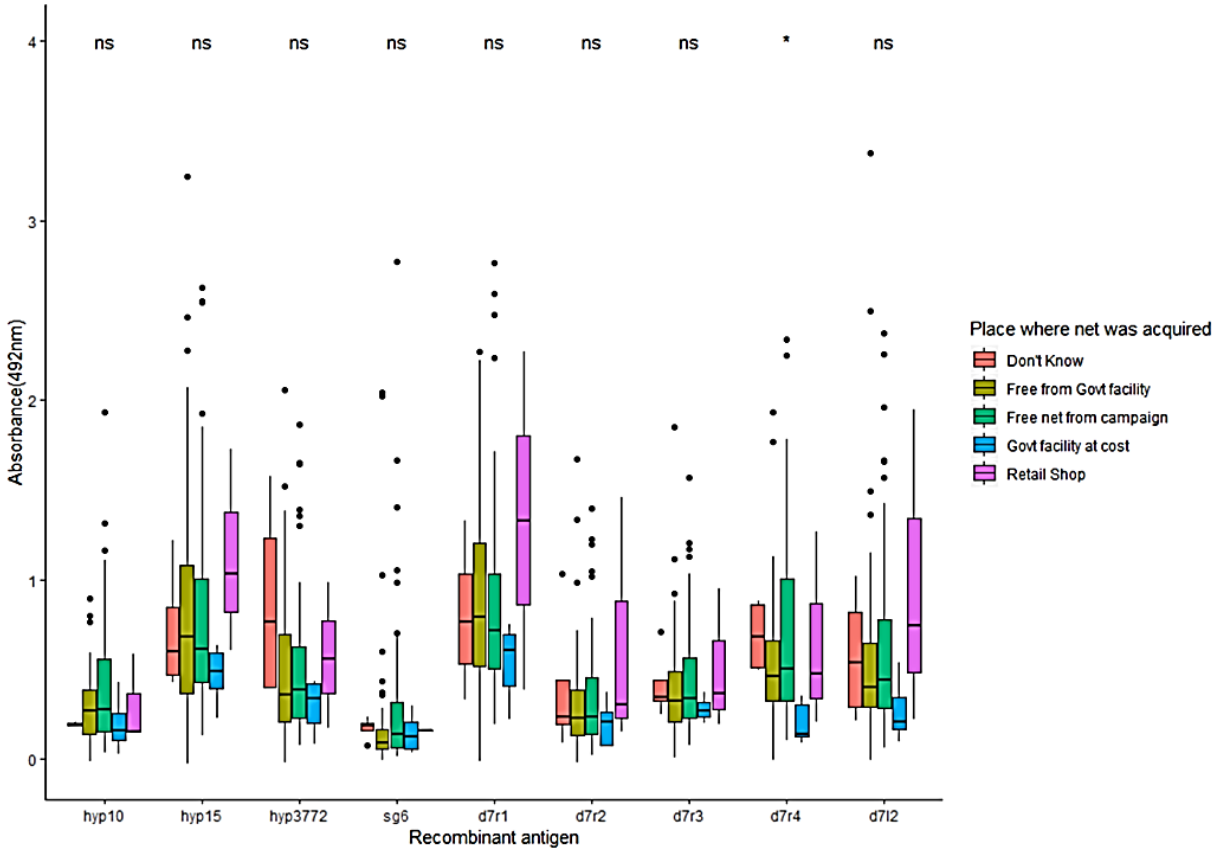

Figure 16. Antibody responses according to the place where bed-net was acquired. Antibody responses to D7r4 was lower in individuals who acquired bed-net from government facility at a cost. (ns, not significant; *, $p < 0.05$).

4.9 Correlations of Antibody Responses to the Recombinant Mosquito Salivary Gland Antigens

Correlations of IgG responses to the recombinant mosquito salivary gland antigens were determined by Spearman method (Figure-17). The antibody responses to the recombinant mosquito salivary gland antigens were all significantly correlated with each other with the exception of SG6. Antibody responses to recombinant SG6 were not significantly correlated with antibody responses to recombinant Hyp 15, Hyp 37.7-2, D7r1

and D7r2. Moreover, antibody responses to recombinant SG6 were significantly correlated with antibody responses to Hyp 10, D7r3, D7r4 and D7l2.

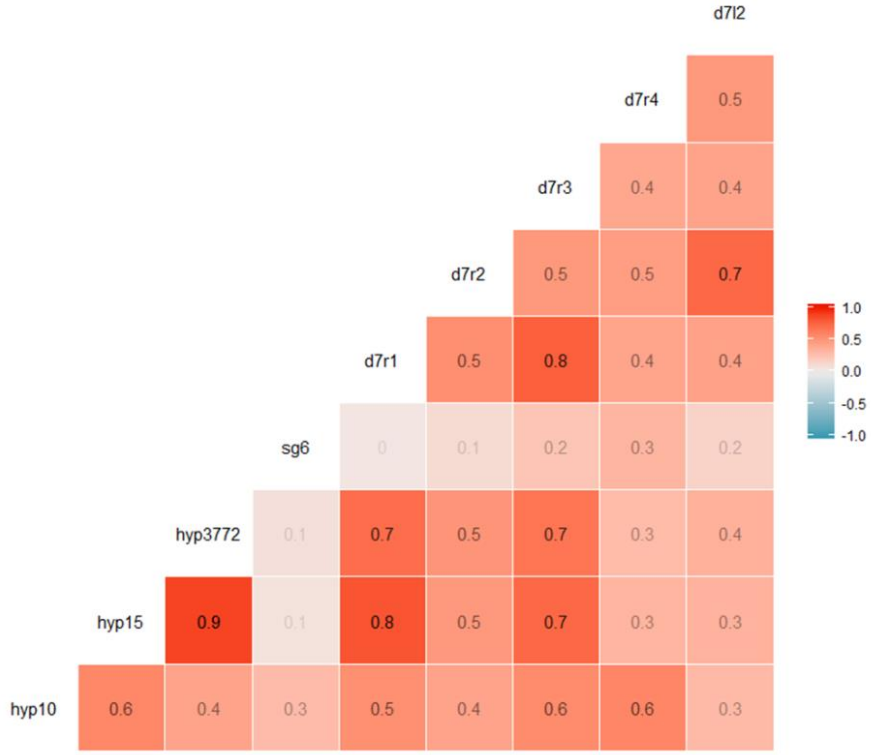

Figure 17. Correlations of antibody responses to the recombinant mosquito salivary gland antigens. Antibody responses to the recombinant mosquito salivary gland antigens significantly correlated with each other.

CHAPTER FIVE

DISCUSSION

5.1 Effects of Age and Sex on Antibody Responses to Recombinant Mosquito Salivary Gland Antigens

Increase with age caused a significant increase of antibody responses to recombinant D7r2 and D7l2. Increase of antibody responses with age could be attributed to gradual acquired immunity to mosquito saliva as an individual grows older (Drame *et al*., 2010b). This phenomenon is also supported by an earlier study which showed that surface area exposed to vector bites increased with age and this could lead to increase of antibody responses with age (Bryan 1980). Interestingly, age increase caused a significant decrease in antibody responses to Hyp 15 and D7r1 from ages two years to greater than seven years. This decrease of antibody responses with age could be due to desensitization of the immune response with continued exposure to the particular antigens. Similar findings of the effect of antibody responses with age were documented by (Buezo *et al*., 2020). In their study, an increase in age caused a decrease in immune responses to *Aedes albopictus* salivary gland protein extract and al34k2 salivary antigen. The effect of age on antibody responses signifies that immune responses to the recombinant antigens are sensitive to age.

Females had significantly higher antibody responses to recombinant SG6 as compared to males. This is consistent with a study carried out which showed that females had higher antibody responses to gSG6-P1 though it was not statistically significant (Drame *et al*., 2012). The higher antibody responses in females could be as a result of estrogens promoting immune responses in the females while androgens suppress the immune responses in the males (Giefing-Kröll *et al*., 2015). Females having higher antibody responses signify that immune responses to recombinant SG6 are sensitive to sex.

5.2 Salivary Gland Antigens as Markers That Distinguish Infectious from Non-Infectious Anopheline Bites

The role of a mosquito bite in malaria infection cannot be underestimated. In a controlled human malaria infection model, it was shown that individuals who got infected via mosquito bites developed more adverse events as compared to their counterparts who were infected intravenously (Alkema *et al*., 2022).

Distinction of infectious from non-infectious bites is key in studies involving malaria immunity. This is because in areas with malaria transmission, some individuals might be identified as immune yet it is because they have not been exposed to infectious bites. It is

interesting to note that infectious bites are not majorly responsible for a seasonal surge in malaria infections but uninfected bites on human reservoir of infection (Paul *et al*., 2004). One would assume that all sporozoite infected mosquitoes would cause an infection if they bit an individual. This is not the case as only mosquitoes harboring more than ten thousand sporozoites in their salivary glands have been shown to be more likely to initiate a malaria infection (Aleshnick *et al*., 2020; Graumans *et al*., 2020).

In this study, individuals who had malaria parasites at the cross-sectional bleed had higher antibody responses to SG6, D7r2 and D7l2. Malaria parasitaemia could be as a result of a recent bite exposure to a sporozoite infected mosquito or the individuals would simply be human reservoirs of the parasite. The concept of human reservoirs of *Plasmodium falciparum* parasites has been scientifically and in proven. A good example was shown in northern Ghana where more than forty percent of the population was shown to harbor multi-genome malaria infections despite vector control and chemotherapy being largely adapted in the area (Tiedje *et al*., 2022). Similar findings in Kombewa and Marani , Western Kenya whereby more than forty percent of the study individuals had parasitaemia (Ondeto *et al*., 2022). For this study, the parasitaemia is more attributed to exposure to infected mosquito bites as this was backed up by higher antibody responses to SG6, D7r2 and D7l2 salivary gland antigens. These findings could be due to higher expression levels of these antigens by a *Plasmodium*-infected mosquito hence more of the antigens are released during a blood meal leading to higher antibody responses. Higher expression level of some mosquito salivary gland antigens after *Plasmodium falciparum* infection has been shown in other studies. Using Serial Analysis of Gene Expression (SAGE) four salivary gland antigen genes were found to be upregulated during invasion by *Plasmodium falciparum* sporozoites while five genes encoding secreted proteins displayed strong induction patterns (Rosinski-Chupin *et al*., 2007).

It would be interesting if the antibody responses would predict the time of exposure to the mosquito bites. This would play a critical role on monitoring the success of vector control strategies or predict the outbreak of vector-borne diseases as recent exposure would easily be identified. The time of exposure to the mosquito bites would most likely be at the beginning of the malaria transmission season. This is backed up by a study carried out in southeastern French individuals whereby average levels of IgG responses to *Ae. caspius* salivary gland extracts increased during the peak of exposure to the mosquito bites. However, the IgG

responses returned to base level after four months implying that the antibody responses were short lived (Fontaine *et al*., 2011).

These results suggest that SG6, D7r2 and D7l2 are predictors of parasitaemia hence have potential to be biomarkers of infectious bites. This is consistent with the findings in another study whereby expression level of SG6 and long form D7 were upregulated in the salivary glands of *P. falciparum* infected *A. gambiae* mosquitoes (Marie *et al*., 2014). However, lack of entomological data on exposure was a limitation in this study.

5.3 Salivary Gland Antigens as Markers of Malaria Exposure

Antibody responses to mosquito salivary gland have previously been used to assess exposure to malaria vector bites (Kearney *et al*., 2021) . Antibody responses to recombinant gSG6 were shown to be sensitive enough to show variation in vector density (Rizzo *et al*., 2011). In this study, antibody responses to mosquito salivary antigens were evaluated according to the level of malaria exposure, temporal variation in malaria transmission intensity and malaria infection outcome.

IgG responses to the Hyp 10, D7r4, SG6, D7r3, D7l2, D7r1, Hyp 37.7-2, D7r2 and Hyp 15 recombinant mosquito salivary gland antigens were predictors of malaria exposure as malaria exposed individuals had significantly higher responses as compared to malaria naïve individuals. The malaria exposed individuals were malaria immune African adults and African children from a malaria endemic region (Junju, Kilifi County) while the malaria naïve individuals were European adults. These findings are in agreement with a similar study where individuals from Senegal (malaria endemic region) had significantly higher IgG responses to SG6 and 5ˈnucleotidase as compared to those from Marsaille, France (non-malaria endemic region) (Ali *et al*., 2012). These results also emphasize the specificity of the antibody responses to the salivary gland antigens on the species in regards to vector bite exposure. Within the three groups, the levels of antibody responses were also variable hence showing distinction of the responses at the individual level. Measurement of antibody responses to Hyp 10, D7r4, SG6, D7r3, D7l2, D7r1, Hyp 37.7-2, D7r2 and Hyp 15 salivary gland antigens is a potential tool for screening of travellers who have made recent visits to malaria endemic regions. Similar findings were shown in Eighty-eight French soldiers who had made a five months' journey to tropical Africa. In the study, forty-one percent of the individuals showed significantly higher antibody responses to mosquito saliva after the five month journey (Orlandi-Pradines *et al*., 2007). Antibody responses to Hyp 10, D7r4, SG6, D7r3, D7l2, D7r1, Hyp 37.7-2, D7r2 and Hyp 15 mosquito salivary gland antigens therefore

have potential to be used as tools to measure malaria exposure at both the population and individual levels.

The core vector control measures against malaria are insecticide treated nets and indoor residual spraying. Application of larvicidal strategies is recommended as supplementary vector control measures (WHO, 2019). Given the significant role played by vector control as a tool in the fight against malaria, measuring the effectiveness of these vector control measures needs a time-sensitive tool. Studies aiming to monitor individual risk of exposure to malaria are carried out so as to evaluate effectiveness of a vector control measure. Establishing the spatio and temporal variation in malaria transmission risk is a key tool in malaria surveillance. The use of antibody responses to mosquito salivary antigens so as to assess temporal variation in malaria exposure has been applied. In this study, antibody responses to Hyp 10, D7r4, SG6, D7r3, D7r1, Hyp 37.7-2, D7r2 and Hyp 15 were compared in 2008 and 2014 in Junju, Kilifi County. Antibody responses to D7r2, D7r3, D7r4, Hyp 10 and SG6 were significantly lower in 2014 as compared to 2008. These results suggest that in 2014 there was lesser human-vector contact as compared to 2008 which might be attributed to increase in bed-net usage in 2014 (Mogeni *et al*., 2016). However, it is interesting to note that malaria cases were higher in 2014 as compared to 2008 (Mogeni *et al*., 2016). This may be attributed to slower acquisition of immunity in low transmission set up hence higher probability of presentation with symptomatic malaria when children get older (Snow *et al*., 1997). Antibody responses to D7r2, D7r3, D7r4, Hyp 10 and SG6 therefore have potential to be used as tools for monitoring change in malaria transmission intensity within the same region at different time points. In a similar study, antibody responses against *A. gambiae* salivary gland protein 6 peptide 1 (gSG6-P1) along the Thailand-Myanmar border varied according to the season. The rainy season recorded significantly higher antibody responses as compared to the cool and hot season (Ya-Umphan *et al*., 2017).

In another study carried out in the northern highlands of Tanzania, antibody responses against *A. gambiae* salivary gland protein 6 peptide 1 (gSG6-P1) effectively showed a temporal variation in malaria transmission intensity. In the study carried out in 2019, the gSG6-P1 seroprevalence intensified from 18.8% during the dry season in March to 25.0% during the rainy season in June followed by a significant drop to 11.0% during the next dry season in September. In the same study, the area that had the highest number of study participants using bed nets also had the lowest antibody responses to the salivary antigens despite the largest number of mosquitoes being collected in that area (Kassam *et al*., 2021). It

is important to note the use of antibody responses to mosquito salivary antigens in serosurveillance needs to be in line with the dominant species of mosquitoes in the area. This is backed up by a study in the Solomon Islands whereby only 11% of the individuals had high anti-gSG6 antibody titers despite exposure to up to 190 bites of *A. farauti* per night (Pollard *et al*., 2019). In our study, the dominant mosquito species in the area were *A. gambiae* (O'Loughlin *et al*., 2016), from whom the recombinant salivary antigen genes were sourced.

The role of mosquito saliva or its antigens in disease transmission is an area that has received keen interest from malariologists and other arthropod vector researchers. The area of most interest is whether arthropod saliva enhances or suppresses disease transmission. Understanding the role of vertebrate immune responses to arthropod saliva or its antigens is also vital. Salivary proteins play an important role in modifying pathogen transmission, suppressing vertebrate immune system, formation of an infection and severity of the disease (Demarta-Gatsi & Mécheri, 2021; Guerrero *et al*., 2020; Marín-López *et al*., 2023; Olajiga *et al*., 2021; Wang *et al*., 2023). In a particular study, *A. aegypti* venom allergen-1 (*Aa*VA-1) salivary protein was shown to enhance host cell immune autophagy in mice. This was achieved by *Aa*VA-1 intracellularly interacting with a dominant negative binder of Beclin-1. The release of Beclin-1 resulted in initialization of downstream autophagic signaling (Sun *et al*., 2020). In another study, *A. aegypti* recombinant D7 salivary protein were shown to inhibit Dengue Virus infection through direct interaction with the virions (Conway *et al*., 2016). In this study, individuals who did not get infected with malaria in the follow-up period had higher antibody responses to SG6, D7l2 and D7r2 at the cross-sectional bleed. These results further suggest that SG6, D7l2 and D7r2 are predictors of malaria infection outcome. It would have been expected that individuals who got malaria to have higher antibody responses to the recombinant antigens but it was the opposite. This implies that antibody responses to SG6, D7l2 and D7r2 may have some protective role against clinical infection with malaria. This potential protection was also suggested in a study with mice malaria models where a protective role of mosquito saliva was demonstrated by pre-exposure of mice to mosquito saliva, and after analysis the mice were found to exhibit lower liver parasite burden of *P. yoelii* after infection (Donovan *et al*., 2007).

A similar outcome was reported in another study where pre-exposure of mice to a protein in *Anopheles* saliva named AgTRIO produced a significant reduction in liver *P. berghei* burden (Agunbiade *et al*., 2018). Another study showed a contrasting result whereby mice immunized with salivary protein D7 succumbed faster to West Nile Virus as compared

to the controls (Reagan *et al*., 2012). In another study, RNAi was used to silence AgTRIO in *Anopheles gambiae* mosquitoes and the *Plasmodium bhergei* sporozoites from those mosquitoes were less effective in colonizing mice liver as compared to the controls (Chuang *et al*., 2019). Further studies need to be carried out to evaluate the protective role of mosquito salivary antigens as this will contribute to the development of a multifactorial or arthropod-based vaccine against malaria. Studies on evaluation of arthropod salivary proteins as vaccine candidates have been undertaken (Olajiga *et al*., 2021). *A. gambiae* saliva vaccine (AGS-v), a peptide based vaccine, underwent phase 1 trials whereby vaccinated individuals had increased levels of vaccine specific IgG antibodies and IFN-γ (Manning *et al*., 2020).

5.4 Mosquito Salivary Gland Antigens as Tools to Evaluate Effectiveness of Bed-Net Use as a Vector Control Strategy

Bed-nets are used as vector control strategy through preventing physical contact of an individual with mosquito while asleep, especially at night. The role of bed-nets in the control of malaria has been mathematically modelled (Agusto *et al*., 2013). In their study, malaria has potential to be eliminated if 75% of the population used bed-nets. However, the following aspects have to be considered while evaluating the impact of bed-net use as a vector control strategy: access to the bed-nets, use of the bed-nets (correctness and consistency), bio efficacy of the insecticide used and the durability of the bed-net (Lindasy *et al*., 2021). Evaluating the effectiveness of bed-net as a vector control strategy is an area that still has a huge potential for exploitation. Recently, two hundred households in South western Ethiopia were assessed on the effectiveness of bed-net and other vector management strategies after three years of implementation. In their study, a standard questionnaire was used and their findings included a decline in malaria cases (Asale *et al*., 2019). In a similar study carried out in Vietnam, malaria transmission was still ongoing despite usage long-lasting insecticidal nets (LLINs). Using a cross-sectional behavioral and net-coverage survey, they established that the malaria transmission is ongoing due to individuals sleeping in their farm hats and forest huts, which had no bed-nets.

These findings were backed up by entomological surveys where no anopheline species were captured in the village but *A. dirus* and *A. maculatus* were captured in the farm and forest huts (Edwards *et al*., 2019). Combining these entomological, behavioral and net-coverage surveys with sero-surveillance will definitely result in better findings as effectiveness of a vector control strategy at an individual level would be assessable. In this study, individuals who possessed bed nets and slept under them had significantly lower

antibody responses to recombinant D7l2 as compared to those individuals who neither possessed bed nets nor slept under them. A higher antibody response to D7l2 proteins is partially attributed to its blood feeding role in the female mosquito hence it is released into the human skin in substantial quantities during blood feeding. The D7 family of proteins which is expressed abundantly in blood-feeding Nematocera play an anti-hemostatic, anti-inflammatory and anti-immune role during blood feeding (Alvarenga & Andersen., 2023; Ribeiro & Francischetti, 2003; Smith *et al*., 2022;). These findings imply that D7l2 has potential to be used as a serological tool to evaluate the effectiveness of a vector control strategy such as bed-net use.

Individuals whose bed nets were treated had significantly lower antibody responses to recombinant Hyp 15, Hyp 37.7-2, SG6, D7r1 and D7r3. This implies that individuals whose bed-nets were treated had lesser contact with the vector since the insecticide impregnated in the bed nets served to repel and/or kill the vector. This is consistent with the findings in another study whereby insecticide treated bed-nets were a better vector control strategy as compared to a none-treated bed-nets (Alessandrol & Bennett, 1995). In another study, the direct and indirect benefits of insecticide treated bed-nets were compared. Interestingly, as more users applied insecticide treated nets the direct benefit of the treated net diminished as number of mosquitoes attempting to enter houses increased as fewer bites were successful. However, the relationship with usage is less pronounced as the number of mosquitoes being repelled are fewer as more are killed (Unwin *et al*., 2023). In general, insecticide treated bed-nets do play a significant role in reduction of malaria cases as shown in South West Cameroon where there was a significantly low malaria prevalence after a mass distribution campaign of treated nets (Bongajum *et al*., 2023). The concentration of pyrethrin in an insecticide treated net is also an important factor to consider while evaluating a bed-net's effectiveness as vector control strategy. In a particular study, washing five times reduced the mosquito knock down effects hence recommending impregnation of the bed-net after five washes so as achieve best defense from mosquito bites (Aung *et al*., 2023).

An interesting finding was individuals whose bed-nets were obtained from government facility at a cost had significantly lower antibody responses to D7r4. Those who acquired the bed-nets free from campaigns had significantly higher antibody responses to D7r4. This was the general trend from all the other antigens though not statistically significant. The higher antibody responses when a bed-net was obtained free of charged might be attributed to alternative uses of the bed-net as compared when the bed-net was

purchased (Moscibrodzki *et al*., 2018). Misuse of free insecticide treated bed-nets were observed in Zambia whereby individuals used the issued bed-nets for fishing hence resulting in fishery decline (Welsh *et al*., 2023).

Time when the bed-net was acquired ranging from a month to more than five years had no significant impact to the mosquito salivary antigens. These results suggest that the efficacy of a bed-net is not affected by its age as long as it is used correctly and consistently. The idea of production of local durable, non-insecticidal and biodegradable bed-nets in Africa has been suggested so as to avail affordable and readily available bed-nets in Africa (Okumu 2022). It is important that bed-net use should be consistent in endemic regions despite perceived low-risk of malaria or prevailing environmental conditions such as heat (Rek *et al*., 2020).

5.5 Correlations of Antibody Responses to the Recombinant Mosquito Salivary Gland Antigens

The antibody responses to the recombinant D7r1, D7r2, D7r3 and D7r4 were all significantly correlated with each other. The good correlation in responses between the D7r is in agreement with the another study where the D7r showed a similarity ranging from 53% to 73% (Arca *et al*., 2002). In addition, antibody responses to all the D7 family proteins were significantly correlated with each other and this could be attributed to their similar function as scavengers of biogenic amines such as histamine and serotonin produced by the host (Jablonka *et al*., 2019). These findings imply that any of the D7 family of proteins can be used as a serological tool due to their good correlations.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i) Among the salivary glands antigens assessed in this study, only SG6, D7r2 and D7l2 were demonstrated to be potential biomarkers of exposure to infected Anopheline bites hence a valuable addition to serological tools of estimating human-vector contact.

ii) Antibody responses to the recombinant Hyp 10, Hyp 15, Hyp 37.7-2, SG6, D7r1, D7r2, D7r3, D7r4 and D7l2 are potential tools for estimating temporal variation in malaria transmission intensity.

iii) Only D7l2 showed the most potential as a serological tool for evaluating the efficacy of bed-nets as a vector control strategy.

6.2 Recommendations

(i) Further studies need to be carried out to compare expression levels of salivary gland antigens in *P. falciparum* infected versus non-infected *A.gambiae* mosquitoes. This is to improve accurate estimation of malaria transmission risk.

(ii) These salivary gland antigens need to be further evaluated as tools for estimating spatial variation in malaria transmission intensity.

(iii) More studies need to be carried to evaluate D7l2 as a tool for evaluating the effectiveness of vector control strategies. This could be done by comparing an area before and after the implementation of a vector control strategy.

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APPENDICES

Appendix A. Ethical Approval Letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
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KEMRI/RES/7/3/1 **October 15, 2018**

TO: **PROF. FAITH OSIER,**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CGMR-C**
KILIFI.

Forwarded: 19/10/18 For:

DIRECTOR
CENTRE FOR GEOGRAPHIC MEDICINE
RESEARCH, COAST

Dear Madam,

RE: **SERU 3139 (*REQUEST FOR ANNUAL RENEWAL): DEFINING* THE MEROZOITE TARGETS OF PROTECTIVE IMMUNITY AGAINST PLASMODIUM FALCIPARUM MALARIA THROUGH MULTI-CENTRE COHORT STUDIES.**

Thank you for the continuing review report for the period **October 29, 2017** to **September 14, 2018.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **October 30, 2018** through to **October 29, 2019**. Please note that authorization to conduct this study will automatically expire on **October 29, 2019**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **September 17, 2019.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the SERU for review prior to initiation.

Yours faithfully,

ENOCK KEBENEI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

KEMRI WELLCOME TRUST RESEARCH PROGRAMME P.O. Box 230 19 OCT 2018 DIRECTORS OFFICE KILIFI

Appendix B. Proteome Discoverer Files

(i) Results of Hyp 10 mass spectrometry raw files searched against proteome discoverer

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
AGAP008307-PA hy	3R:8811666-8812005:1|gene:AGAP008307\ [protein_coding	-7933.92	69.23	1	5	6	263	91	10.1	6.27
ENSMUSP00000099	pep:known chromosome:GRCm38:11:99385254:99389364:-1	300.27	9.61	5	4	5	8	562	57.1	5.07
AGAP009623-PA gl	3R:37154051-37155049:1|gene:AGAP009623\ [protein_codi	-195.90	13.51	1	2	3	8	333	35.6	8.32
ENSMUSP00000074	pep:known chromosome:GRCm38:7:30729887:30739407:-1 g	187.23	4.10	3	1	2	9	439	47.5	7.91
ENSMUSP00000007	pep:known chromosome:GRCm38:11:100203162:100207548:	186.64	8.66	8	2	5	6	485	52.9	5.17
PBANKA_132640	organism=Plasmodium_berghei_ANKA | product=glyceraldehy	179.38	7.29	7	2	3	11	343	37.2	7.81
ENSMUSP00000028	pep:known chromosome:GRCm38:2:34772092:34776531:1 g	169.94	2.59	10	2	2	3	656	72.5	5.16
ENSMUSP00000023	pep:known chromosome:GRCm38:15:101810689:101818169:	168.19	4.24	1	2	3	5	708	71.0	8.06

ENSMUSP0000 0023	pep:known chromosome:GRCm38:15:101845428:101850786:	154.5 9	4.86	5	3	4	5	638	65.7	8.15
ENSMUSP0000 0055	pep:known chromosome:GRCm38:13:52504375:52530836:-1	71.84	5.50	1	1	1	2	200	22.6	8.76
PBANKA_1447 70	organism=Plasmodium_berghei_ANKA product=elongation fa	68.52	1.52	1	1	1	3	790	90.1	8.12
PBANKA_1308 40	organism=Plasmodium_berghei_ANKA product=GCN2 alpha-	55.59	1.25	1	1	2	7	1521	177.7	8.40
ENSMUSP0000 0116	pep:putative chromosome:GRCm38:9:65310152:65318737:1	49.30	6.74	3	1	1	1	193	21.1	5.91
PBANKA_0719 40	organism=Plasmodium_berghei_ANKA product=LETM1-like pr	47.96	1.47	1	1	2	10	679	80.1	9.36
ENSMUSP0000 0137	pep:known chromosome:GRCm38:17:89908511:89910449:-1	41.49	1.51	1	1	1	1	530	58.0	5.68
ENSMUSP0000 0002	pep:known chromosome:GRCm38:14:55672235:55678750:1	40.40	3.44	1	1	1	1	349	38.1	7.44
ENSMUSP0000 0126	pep:known chromosome:GRCm38:15:101946004:101954287:	39.96	2.06	1	1	2	2	1069	112.3	7.97
PBANKA_1364 10	organism=Plasmodium_berghei_ANKA product=conserved Pl	37.16	3.74	1	1	1	2	374	43.6	6.18
PBANKA_1221	organism=Plasmodium_berghei_ANKA	29.79	1.49	1	1	2	3	1141	131.0	8.88

60	product=cell division c									
PBANKA_145880	organism=Plasmodium_berghei_ANKA | product=kinesin, puta	28.65	1.53	1	1	2	2	1438	167.5	8.28
ENSMUSP00000037	pep:known chromosome:GRCm38:16:35832874:35871544:-1	26.64	1.10	1	1	2	2	1818	203.8	6.79
ENSMUSP00000125	pep:known chromosome:GRCm38:6:122325555:122336919:-	22.55	15.71	8	1	1	3	70	7.8	8.62
AGAP006003-PA cu	2L:24631950-24632477:-1|gene:AGAP006003\ -[protein_cod	0.00	7.26	4	1	1	1	124	13.6	4.32

(ii) Results of Hyp 15 mass spectrometry raw files searched against proteome discoverer

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
AGAP008307-PA hy	3R:8811666-8812005:1|gene:AGAP008307\ -[protein_coding	235.15	18.68	1	1	1	14	91	10.1	6.27
ENSMUSP00000007	pep:known chromosome:GRCm38:11:100117327:100121566:	68.84	4.11	10	1	2	2	438	47.8	4.86
PBANKA_070470	organism=Plasmodium_berghei_ANKA | product=conserved Pl	42.77	1.81	1	1	1	20	496	58.2	9.54
PBANKA_1127	organism=Plasmodium_berghei_ANKA |	35.7	3.83	1	1	2	2	392	47.4	9.16

30	product=conserved Pl	4								
PBANKA_102030	organism=*Plasmodium_berghei*_ANKA | product=ankyrin, put	23.08	5.56	1	1	1	8	612	71.2	9.33

(iii) Results of Hyp 37.7-2 mass spectrometry raw files searched against proteome discoverer

Accession	Description	Score	Coverage	# Proteins	#Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
AGAP001989-PA hy	2R:13210405-13211178:1|gene:AGAP001989\ -[protein_codi	3707.50	62.02	1	10	13	245	258	28.8	8.31
PBANKA_132640	organism=*Plasmodium_berghei*_ANKA | product=glyceraldehy	181.73	4.66	1	1	2	8	343	37.2	7.81
AGAP009623-PA gl	3R:37154051-37155049:1|gene:AGAP009623\ -[protein_codi	148.10	13.51	1	1	3	5	333	35.6	8.32
ENSMUSP00000023	pep:known chromosome:GRCm38:15:101810689:101818169:	76.84	4.24	1	1	3	3	708	71.0	8.06
ENSMUSP00000099	pep:known chromosome:GRCm38:11:99385254:99389364:-1	72.77	3.91	1	1	2	2	562	57.1	5.07
AGAP000209-	X:3463792-3475668:1|gene:AGAP000209\ -	67.91	0.65	1	1	1	1	1069	112.8	6.13

PA gl	[protein_coding]									
ENSMUSP0000 0023	pep:known chromosome:GRCm38:15:101845428:101850786:	62.19	4.86	5	1	3	4	638	65.7	8.15
PBANKA_1447 70	organism=*Plasmodium_berghei*_ANKA | product=elongation fa	39.29	1.52	1	1	1	2	790	90.1	8.12
ENSMUSP0000 0055	pep:known chromosome:GRCm38:13:52504375:52530836:-1	32.07	5.50	1	1	1	2	200	22.6	8.76
PBANKA_0107 60	organism=*Plasmodium_berghei*_ANKA | product=conserved Pl	0.00	0.49	1	1	4	13	5531	646.9	8.22

(iv) Results of SG6 mass spectrometry raw files searched against proteome discoverer

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
AGAP000150-PA GS	X:2405694-2406041:-1|gene:AGAP000150\ -[protein_coding]	1673.83	44.83	1	5	7	86	116	13.2	5.47
AGAP009623-PA gl	3R:37154051-37155049:1|gene:AGAP009623\ -[protein_codi	153.08	13.51	1	2	3	4	333	35.6	8.32
AGAP008282-PA hy	3R:8559769-8560424:-1|gene:AGAP008282\ -[protein_codin	101.15	23.67	1	1	3	7	169	18.6	5.25
ENSMUSP00000	pep:known	55.81	4.10	3	1	2	3	439	47.5	7.91

074	chromosome:GRCm38:7:30729887:30739407:-1 g									
ENSMUSP00000 116	pep:putative chromosome:GRCm38:9:65310152:65318737:1	52.80	6.74	3	1	1	1	193	21.1	5.91
PBANKA_14477 0	organism=*Plasmodium_berghei*_ANKA | product=elongation fa	48.84	1.52	1	1	1	2	790	90.1	8.12
PBANKA_10196 0	organism=*Plasmodium_berghei*_ANKA | product=conserved Pl	48.65	1.95	1	1	3	7	1383	165.5	8.27
AGAP004583-PA He	2R:57977978-57979236:-1|gene:AGAP004583\ -[protein_co	39.69	3.80	10	1	1	3	421	46.1	7.09

(v) Results of D7r1 mass spectrometry raw files searched against proteome discoverer

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
ENSMUSP00000 079	pep:known chromosome:GRCm38:11:100256217:100261029:	245.79	11.98	2	1	7	12	434	48.2	5.06
AGAP008284-PA hy	3R:8562844-8563492:-1|gene:AGAP008284\ -[protein_codin	243.70	26.51	1	1	5	41	166	18.8	9.10
AGAP009623-PA gl	3R:37154051-37155049:1|gene:AGAP009623\ -[protein_codi	191.91	13.51	1	1	3	7	333	35.6	8.32
ENSMUSP00000	pep:known	179.5	5.32	1	1	5	9	470	51.7	5.20

007	chromosome:GRCm38:11:100246091:100248902:	9								
ENSMUSP00000017	pep:known chromosome:GRCm38:11:100262887:100269871:	163.16	11.92	1	1	7	12	453	50.2	5.16
PBANKA_144770	organism=*Plasmodium_berghei*_ANKA product=elongation fa	165.75	1.52	1	1	1	2	790	90.1	8.12
AGAP004583-PA He	2R:57977978-57979236:-1|gene:AGAP004583\ [protein_co	-54.54	3.80	10	1	1	1	421	46.1	7.09

(vi) Results of D7r2 mass spectrometry raw files searched against proteome discoverer

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
AGAP008282-PA hy	3R:8559769-8560424:-1|gene:AGAP008282\ [protein_codin	-9522.16	61.54	1	8	11	421	169	18.6	5.25
ENSMUSP00000099	pep:known chromosome:GRCm38:11:99385254:99389364:-1	199.36	9.43	1	3	6	7	562	57.1	5.07
ENSMUSP00000085	pep:known chromosome:GRCm38:15:101858732:101869705:	130.52	5.41	5	2	3	6	573	61.4	8.02

AGAP009623-PA gl	3R:37154051-37155049:1|gene:AGAP009623\ -[protein_codi	117.21	6.31	1	1	2	3	333	35.6	8.32
ENSMUSP00000023	pep:known chromosome:GRCm38:15:101810689:101818169:	98.88	2.97	1	1	2	2	708	71.0	8.06
ENSMUSP00000079	pep:known chromosome:GRCm38:11:100256217:100261029:	95.63	4.61	2	1	2	2	434	48.2	5.06
PBANKA_071940	organism=*Plasmodium_berghei*_ANKA | product=LETM1-like pr	58.28	1.47	1	1	2	12	679	80.1	9.36
ENSMUSP00000002	pep:known chromosome:GRCm38:14:55672235:55678750:1	47.70	3.44	1	1	1	1	349	38.1	7.44
ENSMUSP00000022	pep:known chromosome:GRCm38:14:16249280:16311926:1	44.90	1.07	1	1	1	1	652	74.3	6.89
ENSMUSP00000055	pep:known chromosome:GRCm38:15:79527707:79546741:-1	33.20	1.84	3	1	1	1	653	72.6	8.68
ENSMUSP00000126	pep:known chromosome:GRCm38:15:101946004:101954287:	20.24	1.12	1	1	1	2	1069	112.3	7.97

(vii) Results of D7r3 mass spectrometry raw files searched against proteome discoverer

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI

AGAP008283-PA hypothetical protein	3R:8561589-8562220:-1|gene:AGAP008283\ - [protein_codin	4542.68	56.47	1	6	7	187	170	18.7	4.88
AGAP009623-PA glyceraldehyde 3-phosphate dehydrogenase	3R:37154051-37155049:1|gene:AGAP009623\ - [protein_codi	164.60	13.51	1	2	3	5	333	35.6	8.32
ENSMUSP00000099420	pep:known chromosome:GRCm38:11:99385254:99389364:-1	150.51	6.05	1	3	3	3	562	57.1	5.07
PBANKA_132640	organism=*Plasmodium_berghei*_ANKA | product=glyceraldehy	101.22	7.58	1	1	3	7	343	37.2	7.81
AGAP008281-PA hypothetical protein	3R:8558236-8558864:-1|gene:AGAP008281\ - [protein_codin	80.26	15.06	1	1	2	3	166	19.4	7.36
ENSMUSP00000023712	pep:known chromosome:GRCm38:15:101810689:101818169:	77.90	2.97	1	1	2	2	708	71.0	8.06
PBANKA_144770	organism=*Plasmodium_berghei*_ANKA | product=elongation fa	70.39	1.52	1	1	1	3	790	90.1	8.12
PBANKA_081890	organism=*Plasmodium_berghei*_ANKA | product=heat shock p	53.21	4.38	10	1	2	2	662	73.3	5.27
AGAP009094-PA cysteine desulfurase	3R:25571979-25573765:-1|gene:AGAP009094\ - [protein_co	45.32	6.71	4	1	2	2	447	49.4	8.13

ENSMUSP00000130491	pep:known chromosome:GRCm38:4:84884465:85131917:1 g	45.10	0.72	2	1	1	3	1397	160.7	7.99
ENSMUSP00000020768	pep:known chromosome:GRCm38:11:5801640:5803733:-1 ge	38.05	4.33	2	1	1	1	254	28.9	8.50

(viii) Results of D7r4 mass spectrometry raw files searched against proteome discoverer

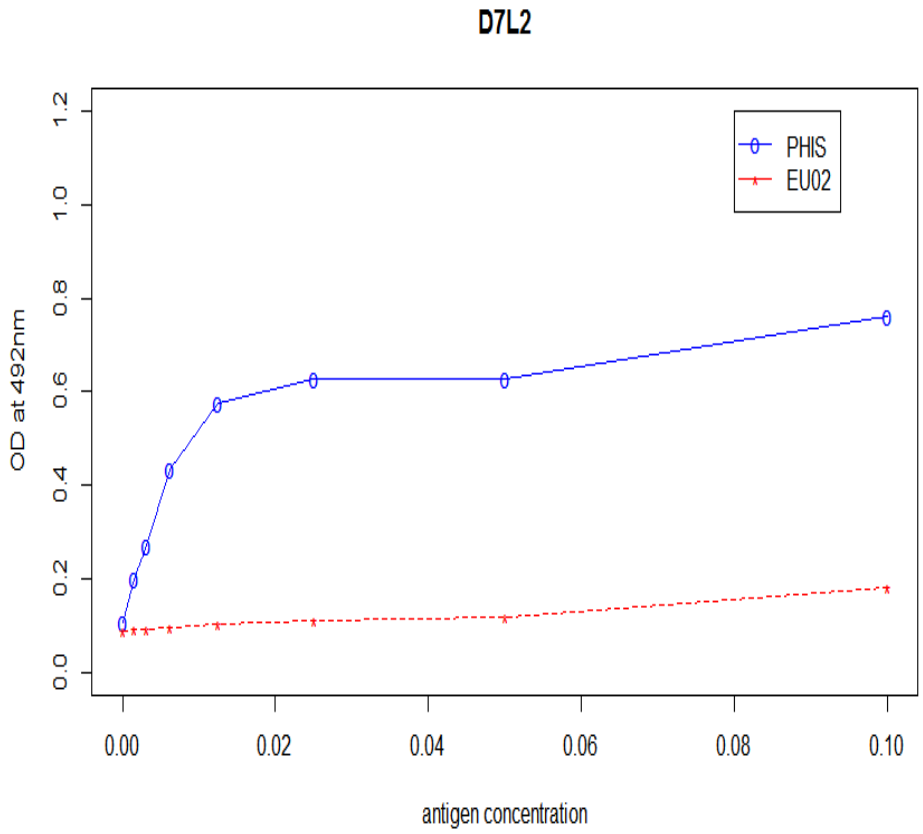
Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
AGAP008281-PA hy	3R:8558236-8558864:-1|gene:AGAP008281\ -[protein_codin	4463.76	63.25	1	10	20	365	166	19.4	7.36
ENSMUSP00000099	pep:known chromosome:GRCm38:11:99385254:99389364:-1	147.87	7.47	1	1	4	7	562	57.1	5.07
PBANKA_132640	organism=*Plasmodium_berghei*_ANKA | product=glyceraldehy	98.26	4.66	1	1	2	4	343	37.2	7.81
PBANKA_122830	organism=*Plasmodium_berghei*_ANKA | product=conserved Pl	22.37	0.38	1	1	3	7	4448	520.1	9.22

(ix) Results of D7l2 mass spectrometry raw files searched against proteome discoverer

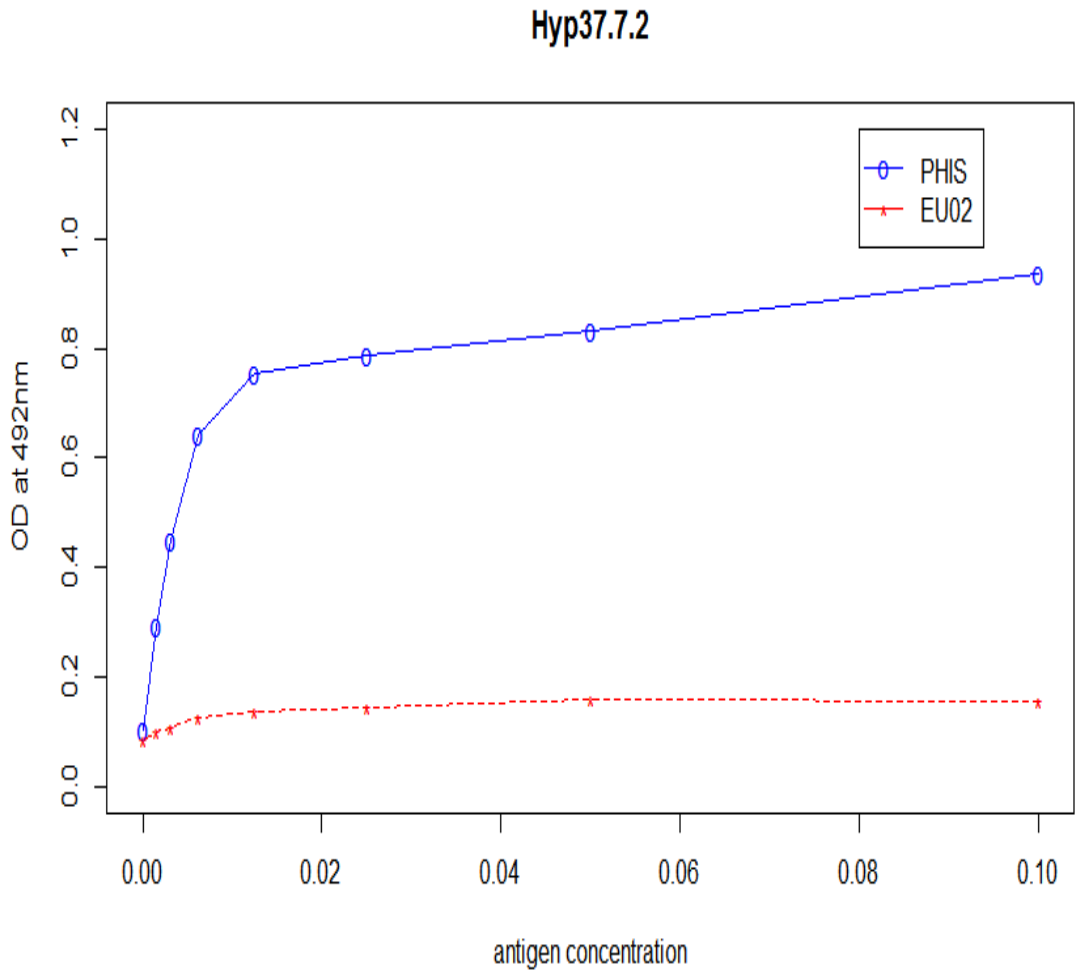
Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
AGAP008279-PA Lo	3R:8545938-8547063:1|gene:AGAP008279\ [protein_coding	-4918.46	68.67	2	19	31	321	316	36.2	7.87
ENSMUSP00000099	pep:known chromosome:GRCm38:11:99385254:99389364:-1	68.50	6.05	1	2	3	3	562	57.1	5.07
AGAP009623-PA gl	3R:37154051-37155049:1|gene:AGAP009623\ [protein_codi	-66.42	6.31	1	1	2	2	333	35.6	8.32
PBANKA_144770	organism=*Plasmodium_berghei*_ANKA | product=elongation fa	32.40	3.16	1	1	2	3	790	90.1	8.12
PBANKA_020145	organism=*Plasmodium_berghei*_ANKA | product=BIR protein,	0.00	5.74	1	1	1	1	209	25.1	9.50

Appendix C. Salivary Antigen Reactivity to Immune Serum to Determine Coating Concentration for ELISA.

Serially diluted salivary antigen tested in pooled hyper-immune serum (PHIS)-malaria immune and malaria naïve European serum.



(i)


Hyp37.7.2
PHIS
EU02
OD at 492nm
0.0
0.2
0.4
0.6
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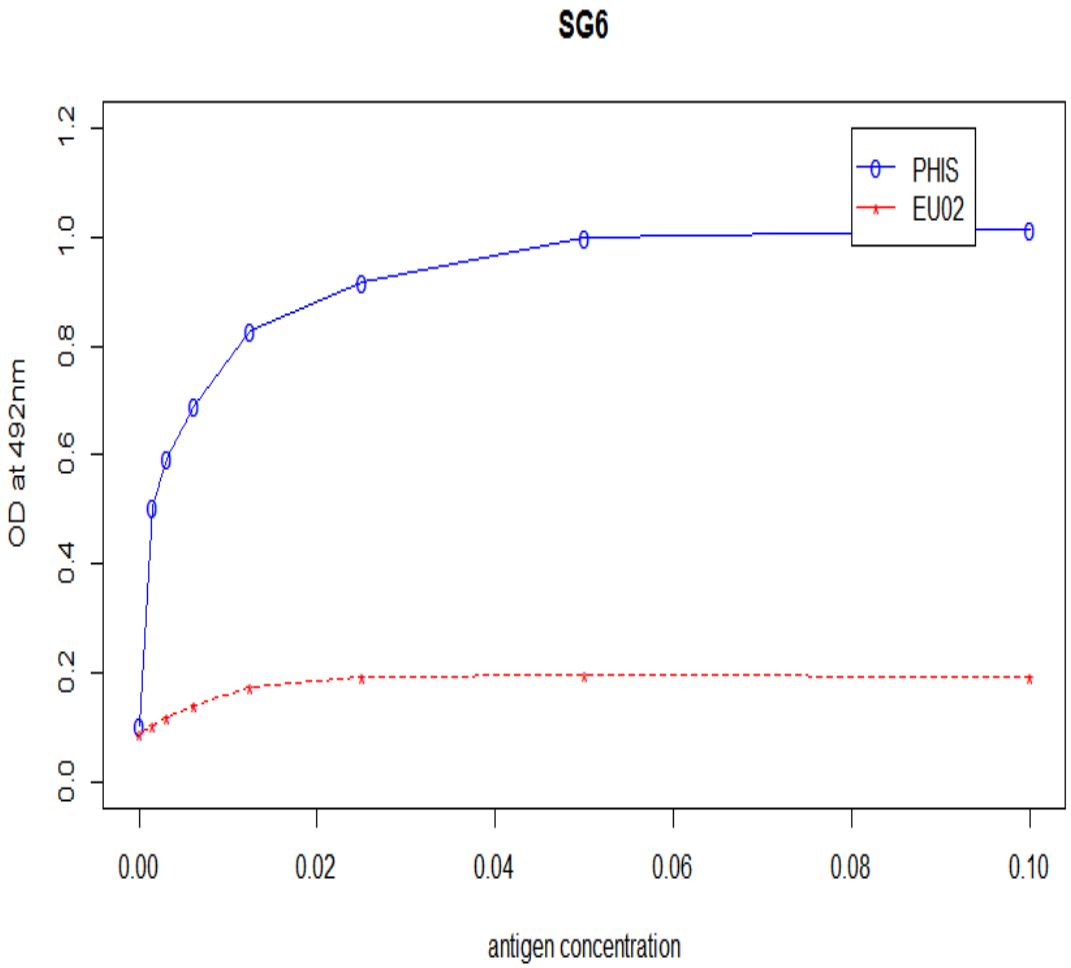
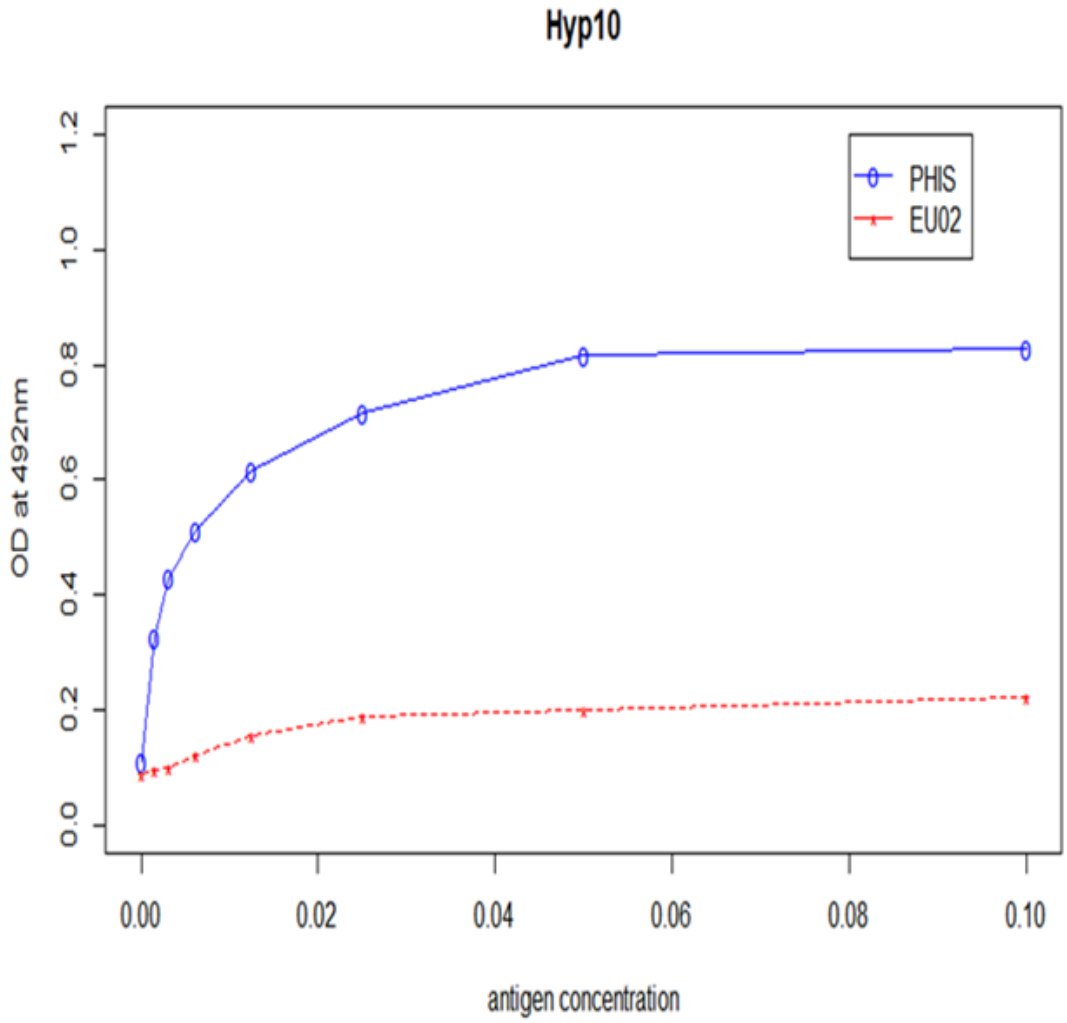
1.2
0.00
0.02
0.04
0.06
0.08
0.10
antigen concentration

(ii)


SG6
PHIS
EU02
OD at 492nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.00
0.02
0.04
0.06
0.08
0.10
antigen concentration

(iii)


Hyp10
PHIS
EU02
OD at 492nm
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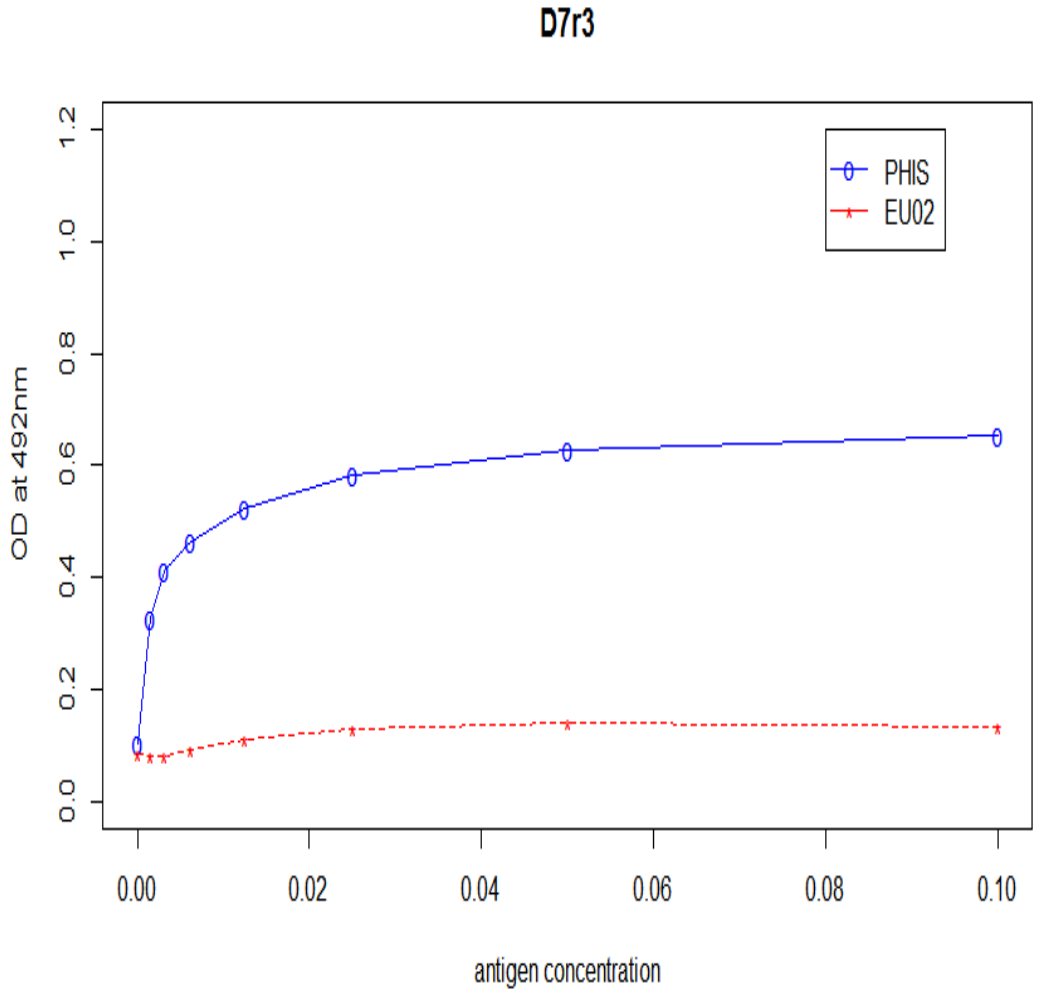
0.2
0.4
0.6
0.8
1.0
1.2
0.00
0.02
0.04
0.06
0.08
0.10
antigen concentration

(iv)


D7r3
PHIS
EU02
OD at 492nm
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0.2
0.4
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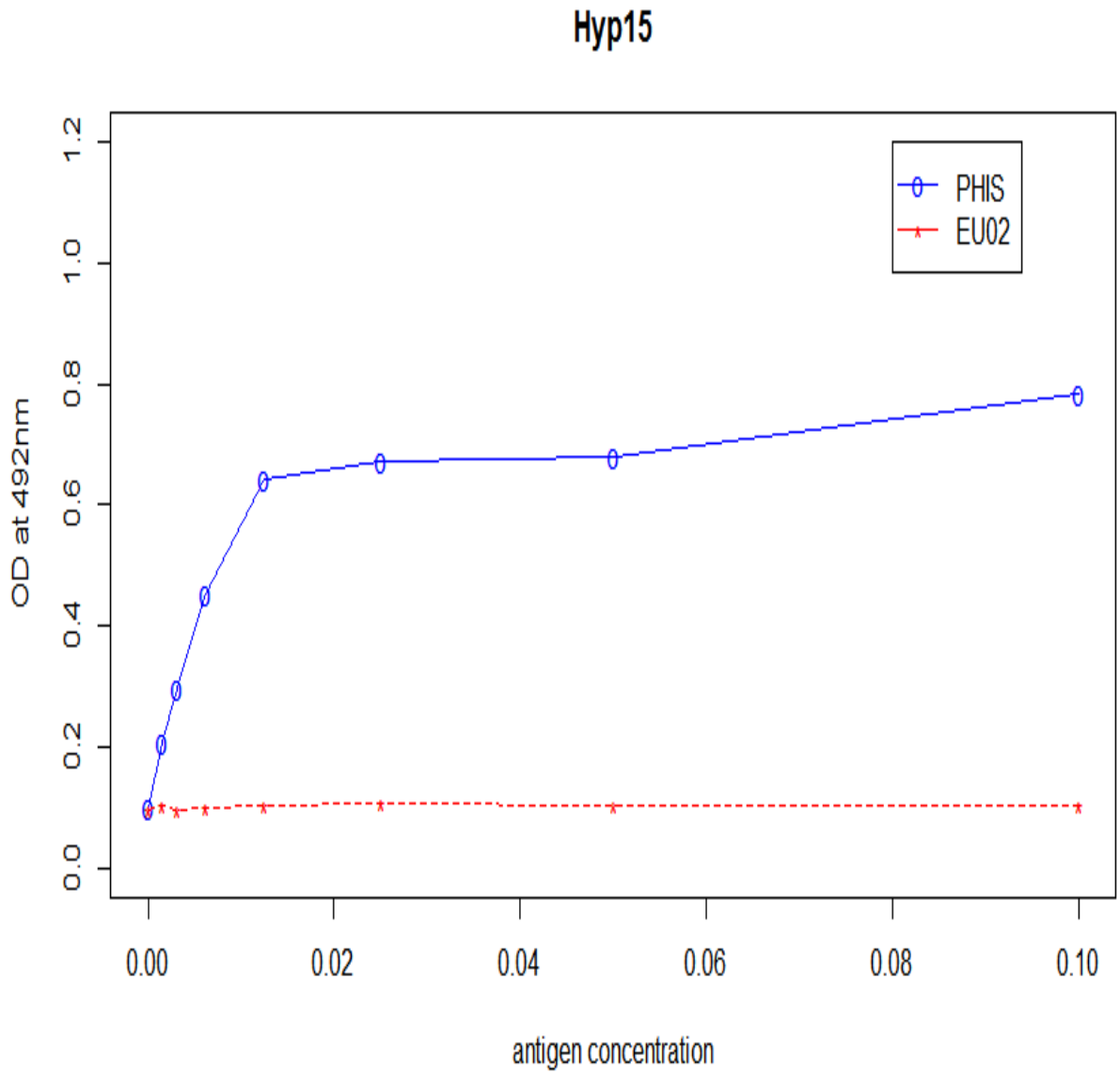
1.2
0.00
0.02
0.04
0.06
0.08
0.10
antigen concentration

(v)


Hyp15
PHIS
EU02
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1.2
0.00
0.02
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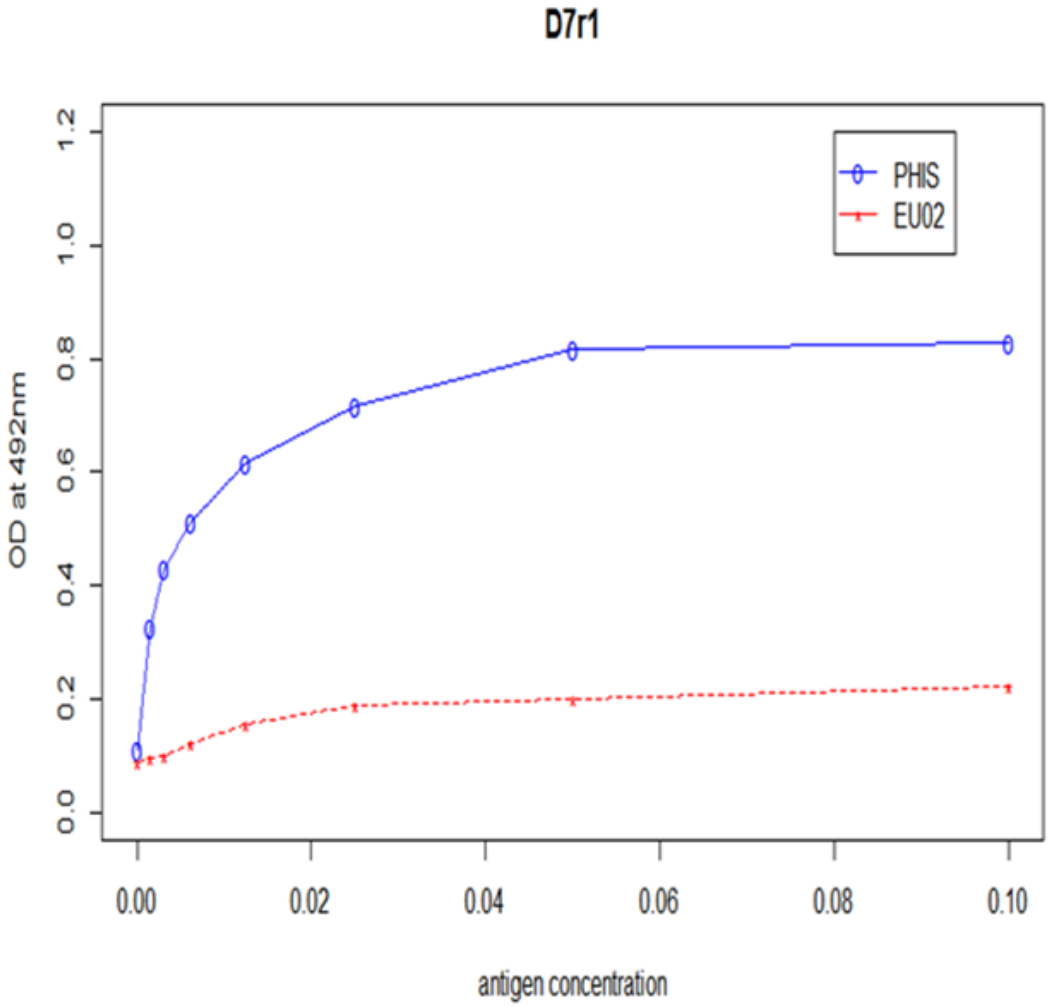
0.06
0.08
0.10
antigen concentration

(vi)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PHIS
EU02
OD at 492nm
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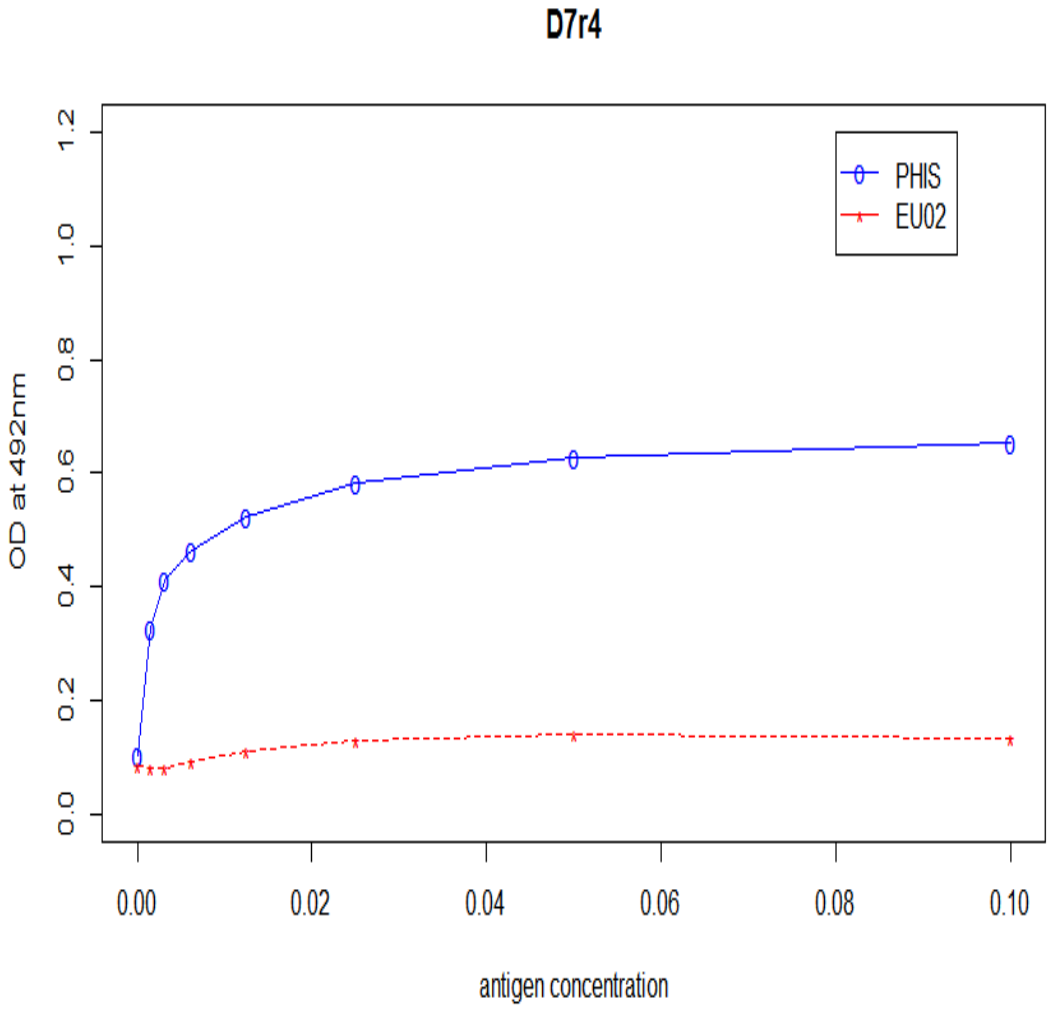
0.2
0.4
0.6
0.8
1.0
1.2
0.00
0.02
0.04
0.06
0.08
0.10
antigen concentration

(vii)


D7r4
PHIS
EU02
OD at 492nm
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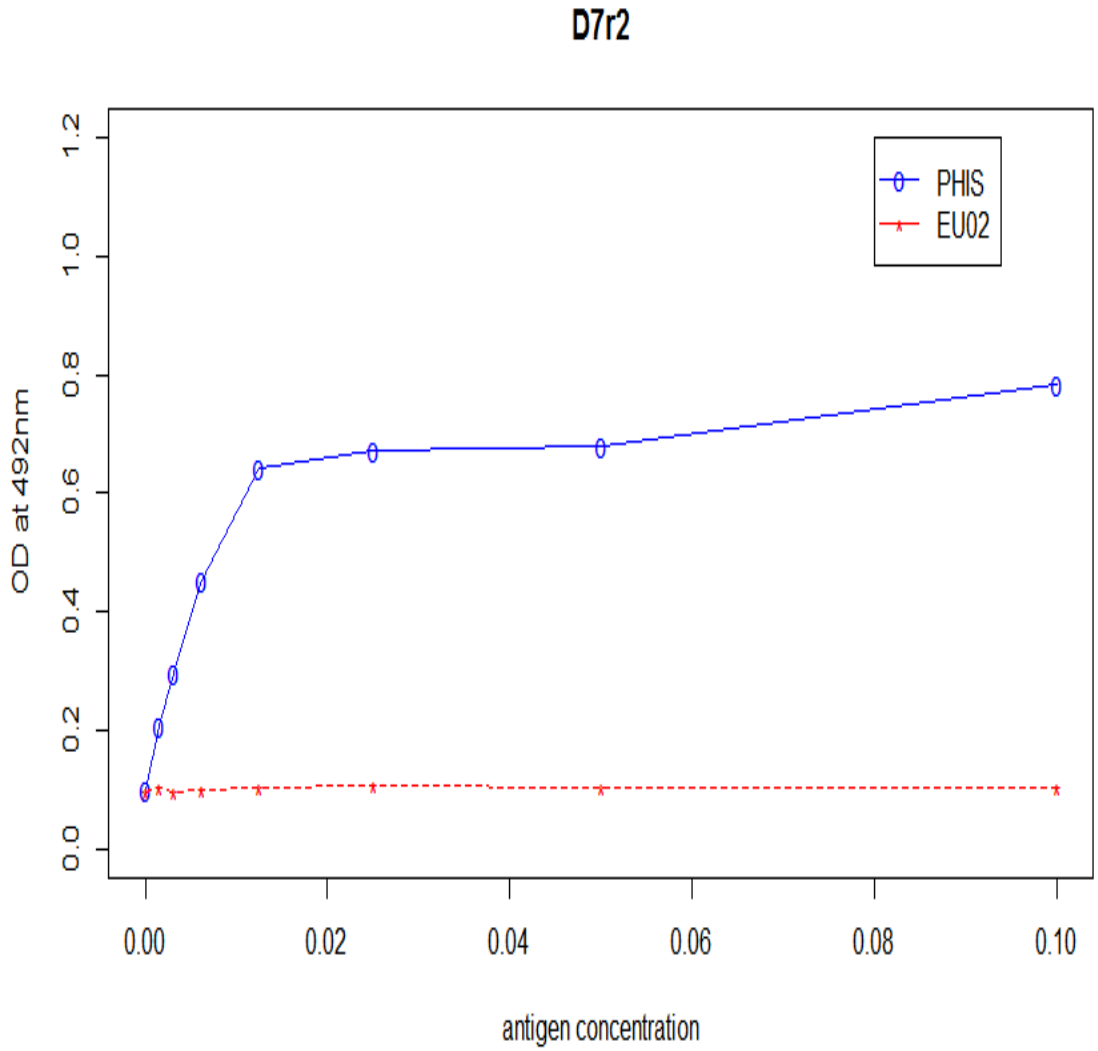
0.2
0.4
0.6
0.8
1.0
1.2
0.00
0.02
0.04
0.06
0.08
0.10
antigen concentration

(viii)



(ix)

Appendix D. List of Publications

Oseno et al. Parasites & Vectors (2022) 15:11
https://doi.org/10.1186/s13071-021-05130-5

Parasites & Vectors

RESEARCH Open Access

Characterization of *Anopheles gambiae* D7 salivary proteins as markers of human–mosquito bite contact

Brenda Oseno[1,2], Faith Marura[1], Rodney Ogwang[1], Martha Muturi[1], James Njunge[1], Irene Nkumama[1,4], Robert Mwakesi[1], Kennedy Mwai[1,5], Martin K. Rono[1,3], Ramadhan Mwakubambanya[2], Faith Osier[1,3,4] and James Tuju[1,3]*

Abstract

Background: Malaria is transmitted when infected *Anopheles* mosquitoes take a blood meal. During this process, the mosquitoes inject a cocktail of bioactive proteins that elicit antibody responses in humans and could be used as biomarkers of exposure to mosquito bites. This study evaluated the utility of IgG responses to members of the *Anopheles gambiae* D7 protein family as serological markers of human–vector contact.

Methods: The D7L2, D7r1, D7r2, D7r3, D7r4 and SG6 salivary proteins from *An. gambiae* were expressed as recombinant antigens in *Escherichia coli*. Antibody responses to the salivary proteins were compared in Europeans with no prior exposure to malaria and lifelong residents of Junju in Kenya and Kitgum in Uganda where the intensity of malaria transmission is moderate and high, respectively. In addition, to evaluate the feasibility of using anti-D7 IgG responses as a tool to evaluate the impact of vector control interventions, we compared responses between individuals using insecticide-treated bednets to those who did not in Junju, Kenya where bednet data were available.

Results: We show that both the long and short forms of the D7 salivary gland antigens elicit a strong antibody response in humans. IgG responses against the D7 antigens reflected the transmission intensities of the three study areas, with the highest to lowest responses observed in Kitgum (northern Uganda), Junju (Kenya) and malaria-naïve Europeans, respectively. Specifically, the long form D7L2 induced an IgG antibody response that increased with age and that was lower in individuals who slept under a bednet, indicating its potential as a serological tool for estimating human–vector contact and monitoring the effectiveness of vector control interventions.

Conclusions: This study reveals that D7L2 salivary antigen has great potential as a biomarker of exposure to mosquito bites and as a tool for assessing the efficacy of vector control strategies such as bednet use.

Keywords: Biomarker of exposure, *Anopheles gambiae*, *Plasmodium falciparum*, Infectious bites, Malaria