

UNIVERSITY OF GHANA

**PUTATIVE, SECRETED OOKINETE PROTEIN (PSOP24) PEPTIDES AS
A MARKER OF INFECTIOUS BITES AND MALARIA TRANSMISSION
ASSESSMENT**

BY

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DECLARATION

I Bernard Tornyigah, hereby declare that except for references to other people’s work, which have duly been acknowledged, this thesis is the result of my own research conducted at the Immunology Department, Noguchi Memorial Institute for Medical Research, supervised by Dr. Kingsley Badu and Dr. Michael Ofori both of Immunology Department, Noguchi Memorial Institute for Medical Research. Neither all nor parts of this thesis have been presented for another degree elsewhere.

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DEDICATION

I dedicate this work to the Almighty God for His unimaginable grace and provisions through which I have been able to complete this work. I also dedicate this work to my supervisors, Dr. Kingsley Badu and Dr. Michael Ofori for their guidance and support. I finally dedicate this work to my family and all who helped in one way or the other.



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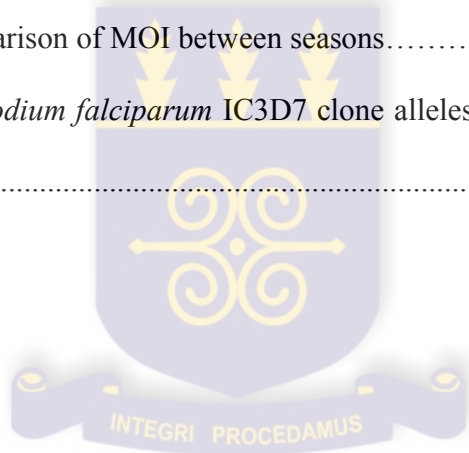
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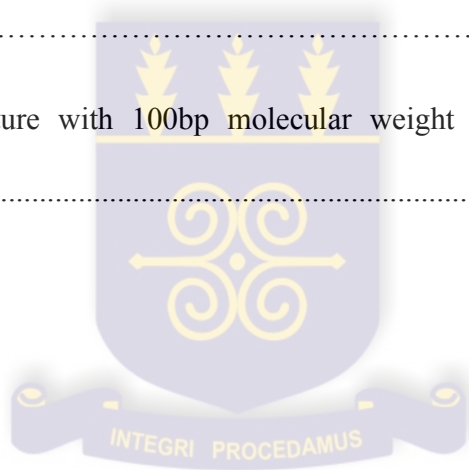
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LIST OF ABBREVIATIONS

AMA 1	Apical Membrane Antigen 1
CSP	Circumsporozoite Protein
CellTOS	Cell-Transversal Protein of Ookinete and Sporozoite
CDC	Center for Disease Control and Prevention
DALY	Disability Adjusted Life Years
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
EBA	Erythrocyte Binding Antigen
EIR	Entomological Inoculation Rate
EST	Expressed Sequence Tag
gSG6	<i>Anopheles gambiae</i> salivary gland protein 6
gSG6-P1	<i>Anopheles gambiae</i> salivary gland derived peptide 1
GLURP	Glutamine-Rich Protein
IRB	Institutional Review Board
ITN	Insecticide Treated Net
LLIN	Long Lasting Insecticide Nets
IPT	Intermittent Preventive Treatment
MSP 1	19kda Fragment of Merozoite Surface Protein 1

MSP 2	Merozoite Surface Protein 2
MDG	Millennium Development Goal
MOI	Multiplicity of Infection
PSOP24	Putative, Secreted Ookinete Protein
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RPM	Revolution per Minute
RNA	Ribonucleic acid
TMB	3,3',5,5'-Tetramethylbenzidine
UV	Ultra Violet
WSE	Whole Saliva Extracts
WBC	White Blood Cell

ABSTRACT

As reported malaria cases continue to decline, heterogeneity in transmission will become more pronounced and thus more sensitive tools would be required to identify micro-geographic areas of higher risk for targeted interventions. Sero-positivity against antigens expressed at different stages of the parasite life cycle and proteins in the mosquito saliva cocktail have gained relevance as transmission monitoring tool. However, the persistent nature of blood stage antigens and the possible use of Circumsporozoite protein (CSP) as a vaccine has necessitated the search for additional markers that can measure even subtle differences in malaria transmission. Sero-positive against antibodies to synthetic peptides of a ookinete stage marker may be a better alternative since it may show contact to an infectious vector. Thus, the study sought to evaluate further and possibly validate PSOP24 as an infectious bite marker of malaria transmission. Synthetic peptides of PSOP24 were designed to cover the most immunogenic epitopes based on several bioinformatic models. Total IgG antibody response to three of the peptides (PSOP24-374, PSOP24-375 and PSOP24-377), CSP and gSG6-P1 were then determined using an indirect ELISA protocol to antibody eluates from two communities 2 Km apart with low malaria transmission during the dry and wet seasons. Malaria parasites were detected in study participants by both microscopy and molecular techniques.

In this study, a 2-fold decrease in IgG responses to peptides PSOP24-374 and PSOP24-375 was observed between the dry and wet season ($p < 0.005$), whereas a 2-fold increase in IgG response to PSOP24-377 and the vector-antigen gSG6-P1 was observed between the same sample collection periods ($p < 0.0001$). However, CSP showed no significant difference between the seasons when compared. Spatial variation between the study communities also show a 1 fold increase in IgG response to PSOP24-375 while a 2 fold and 1 fold decrease in IgG response to

PSOP24-374 and PSOP24-377 from Ayeigbekorpe to Odumase was observed during the dry season. In the wet season, Odumase had high median IgG responses to PSOP24-374, PSOP24-377 and CSP than Ayigbekorpe while median IgG response to gSG6-P1 was high in Ayigbekorpe than in Odumase. Also, the study found greater proportion (36.4%) of submicroscopic infections in individuals in Odumase compared to Ayeigbekorpe during the dry season ($p=0.02$) while this trend was reversed during the wet season. Generally, there were less multiple infections per individual, described as the multiplicity of infection between the study communities and also between the sample collection times with majority of participants harboring single alleles of the 3D7 strain.

This data shows a high antibody response particularly to PSOP24-377 that was similar to gSG6-P1, corresponding to the different transmissions in the study communities, hence can be exploited as additional marker for assessing transmission in low transmission settings.

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Malaria is one of the most severe vector-borne diseases in the world with a high case fatality rate in sub-Saharan Africa and Asia. The disease is caused by the protozoan *Plasmodium*, of which there are five species that affect humans (Garcia, 2010). The parasite varies greatly in endemicity based on the presence of its vectors across the continent and the world as a whole. Of these, *Plasmodium falciparum* is the most widely spread and the most virulent, causing an estimated 90% of all reported malaria cases globally, with children under 5 years and primigravida women being the most vulnerable (World Health Organization, 2013b).

According to the World Health Organization (2013b), malaria control efforts have yielded substantial progress towards reducing the burden of the disease as a result of the numerous preventive and control measures employed. This has led to a reduction in the mortality rate associated with the disease by 42% globally and 49% in Africa between 2000 and 2012 (World Health Organization, 2013b). Of these, 90% of the deaths prevented are estimated to be in children below 5 years in sub-Sahara Africa. During the same period, malaria incidence rate also reduced by 25% globally and by 31% in the Africa region. Despite these achievements, an estimated 3.4 billion people are still believed to be at risk of the disease (World Health Organization, 2013b). Yet, these successes together with continuous funding for malaria programs, has provided optimism for malaria elimination and eradication (Alonso *et al.*, 2011).

The continuing decline in morbidity and mortality associated with the disease has been attributed in part to the scaling up of control efforts such as rapid diagnosis and effective treatments,

insecticide-treated nets, and other vector control strategies. These strategies have also provided both adequate personal and community protection when used well. High reduction in malaria burden leads to heterogeneity in the intensity of transmission especially at the community level (Beer *et al.*, 2013; Bousema *et al.*, 2012; Giardina *et al.*, 2014.; Noor, 2012). This then has necessitated research with the sole objective of developing an effective tool for estimating transmission risk.

Presently, the reference procedure for measuring malaria burden is by clinical cases reported by health facilities, entomological and parasitological methods (Endeshaw *et al.*, 2008; Oesterholt *et al.*, 2006). However, these methods have shown a decrease in sensitivity. They are expensive and laborious during low transmission such as during dry seasons or through vector control. In particular, entomological inoculation rate (EIR), the gold standard for malaria transmission intensity estimation, depends on the number of infectious *Anopheles* bites per person per unit time. Despite its relevance in providing important information on vector diversity (Naranjo-Diaz *et al.*, 2013; Smith *et al.*, 2010), it has several limitations. Large sample size is required to evaluate *Anopheles* exposure and risk of the disease by entomological tools such as traps, indoor residual spray and human landing catches (usually done by adult volunteers and may be limited by ethical constraints and extrapolation to children). In addition, these methods are only sensitive at the population level but fail to allow measurement of heterogeneity of individual exposure. Also, these methods can be laborious, expensive and give inaccurate estimation in low transmission settings (malERA Consultative Group on Drugs, 2011; Smith *et al.*, 2004). Parasite prevalence estimation by microscopy being the other malariometer is also limited by reduced detection sensitivity, in low transmission areas, and areas with reduced parasite prevalence

(Corran *et al.*, 2007; O'Meara *et al.*, 2007). Therefore, there is the need to develop less laborious tools with improved reliability and sensitivity for estimating malaria transmission intensity.

Parasite genotyping using polymorphic block regions of certain genes has emerged as one of the possible tools for assessing transmission (Kobbe *et al.*, 2006). This is through the computation of multiplicity of infection (MOI) which refers to the number of different parasite clones infecting a single host. The polymorphic regions of merozoite surface protein (*mSP*) genes have been the standardized tools for *Plasmodium falciparum* genotyping and this is due to the high polymorphic length and sequence of these genes (Assefa *et al.*, 2014; Snounou *et al.*, 1999).

Another encouraging method, centered on the concept of immune responses to both parasite and vector antigens have shown potential as transmission monitoring tools. This has been shown to confer age and exposure dependent semi-immunity to infected individuals while being an indication of parasite and vector exposure. The prevalence of antibodies against *Plasmodium* antigens such as apical membrane antigen 1(AMA1), the 19 kDa fragment of merozoite surface protein 1 (MSP1₁₉), merozoite surface protein 2 (MSP2) have all gained relevance as transmission biomarkers over the century (Bousema *et al.*, 2010; Drakeley *et al.*, 2005), correlating strongly with other measures of transmission including EIR and clinical cases. However, the persistence of such antibodies long after transmission has ceased represent a weakness in this approach, especially for predicting seasonal or short-term changes in transmission (Druilhe *et al.*, 1986).

In that sense, the recombinant protein *Anopheles gambiae* salivary gland protein 6 (gSG6) which was specific for the *Anopheles* was isolated and purified for serological study and was found to elicit immune responses in travelers exposed to the vector temporarily, thus an indicator of exposure, making it an ideal prospect for recent vector exposure (Rizzo *et al.*, 2011). Continuous

search identified a synthetic peptide derived from the *An. gambiae* salivary protein (gSG6-P1), which is reported to be more specific to *Anopheles* species and antigenic as a possible marker for vector exposure. Further validation established it as a more robust bio-marker of *Anopheles* exposure because of its ability to differentiate between hypo, meso and hyper endemic areas and the non-cumulative nature of antibodies against gSG6-P1 (Badu *et al.*, 2012; Poinsignon *et al.*, 2008). However intense mosquito exposure may not necessarily mean a high malaria exposure if mosquitoes are not infected, it is critical to obtain additional mosquito-staged sporozoite protein biomarker that will indicate exposure to sporozoite infected bites.

Unlike merozoites, the sporozoite stage are exposed to the immune system for only short periods after inoculation, and anti-sporozoite antibodies would most commonly be detected in individuals with frequent or recent exposure. Circumsporozoite surface protein (CSP), is viewed as one of the best serological tools is less immunogenic and show short term variations in transmission (Kusi *et al.*, 2014; Wong *et al.*, 2014), but its use in the RTS,S vaccine represents a challenge: should it succeed as a malaria vaccine as transmission estimation tools based on this antigen will rather be measuring vaccine induced antibodies instead of natural exposure. Thus, a parasite marker based on the sporozoite stage would be valuable in assessing this changes and also for increasing the serological toolbox for transmission monitoring.

Plasmodium falciparum Putative, secreted ookinete protein (PSOP24) is a unique candidate for the sero-epidemiological estimation of transmission. PSOP24 is a novel putative antigen that is believed to be expressed by both ookinetes in the mosquito mid-gut and in the sporozoite stage (Ecker *et al.*, 2008). It is a conserved antigen which showed variations in transmission based on a statistical model from a protein microarray protocol (Baum *et al.*, 2013). Additionally, Ecker *et*

al. (2008) identified six proteins including PSOP24 in *P. berghei* that were important for the transversal of the ookinete by parasite, thus suggest it's function as a malaria blocking protein.

1.1.2 Objective/aim

To determine whether IgG antibody response to the derived peptides of Putative, secreted ookinete protein (PSOP24) is an immune-epidemiological marker of exposure to *Plasmodium falciparum* and transmission assessment.

1.1.3 Specific Objectives

1. To determine antibody responses to PSOP24 peptides, CSP and gSG6-P1 antigens between seasons and among age stratified groups
2. To determine the spatial and temporal variation in parasite-specific IgG responses between two communities with low malaria transmission
3. To determine parasite multiplicity of infection (MOI) as a proxy of malaria transmission intensity in the study communities

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

Malaria is among six other diseases that impose great socio-economical and disability effect in the world with a high fatality records observed in sub-Sahara Africa and Asia. The disease is caused by a protozoan belonging to the genus *Plasmodium* of which there are over hundred (100) species that infect a variety of hosts, including reptiles, humans, birds, rodents and other vertebrates (Cox, 2010; Garcia, 2010). *Plasmodium* species have long been known to be host specific. Nevertheless, this notion has recently been challenged by the discovery of a simian parasite known to naturally infect macaques, now identified to also infect humans (Cox-Singh & Singh, 2008), suggesting a zoonotic form of the disease. Thus, five species currently account for all human malaria infections namely; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and now *Plasmodium knowlesi* (Cox-Singh & Singh, 2008; Garcia, 2010) with *P. falciparum* being the most widely spread and the most virulent (World Health Organization, 2013b). These species differ with regards to their morphology, clinical manifestations and detailed life cycles of the individual parasites.

Severity of the disease is influenced by both parasite and host factors such as parasite species involved and the genetic disposition and immunity of the host (Ganz & Ebert, 2010), of which children under 5 years, primigravida women and travelers from non-endemic areas being the most vulnerable. However, these groups obtain some degree of clinical immunity upon continuous exposure which affects the severity of later infections.

2.2 *Plasmodium* life cycle

The *Plasmodium* life cycle is one of the most complex known, characterized by an asexual stage in the vertebrate host and a sexual stage in an *Anopheles* mosquito vector. This begins when the infective form of the parasites (sporozoites) are introduced under the skin of the host by an *Anopheles* mosquito during a blood meal (Yamauchi *et al.*, 2007), from which some enter the lymphatics, while others enter the blood stream and invade liver hepatocytes (Frevort, 2004; Yamauchi *et al.*, 2007). The sporozoites then undergo an asexual pre-erythrocytic replication and develop into hundreds of merozoites. However, in *P. vivax* and *P. ovale*, some of the sporozoites develop into a dormant form called hypnozoites, which are activated at a later time into erythrocytic forms leading to relapses of the disease (Mueller *et al.*, 2009). Depending on the infecting parasite species, it has been reported that it takes about 5-16 days for the hepatocytes to be ruptured, releasing merozoites into the peripheral blood of the host.

Few seconds after merozoite release, they recognize certain ligands on the surface of the erythrocyte and actively invade it (Perkins, 1984). The merozoites that had successfully invaded erythrocyte undergo a period of development and replication, characterized morphologically by a ring shape known as trophozoites. The trophozoites ingest the host erythrocytic hemoglobin to produce amino acids for the parasite and heme as a by-product (Francis *et al.*, 1997). This development and replication results in increased number of merozoites which rupture the erythrocyte, releasing all the erythrocytic contents including the merozoites into the peripheral blood stream which further goes on to invade other erythrocytes and begin another round of erythrocytic development and replication. Also, parasite metabolites, which are the other components of the ruptured infected erythrocytes, are responsible for the periodic fever paroxysms that accompany malaria infections (Bartoloni & Zammarchi, 2012). Nevertheless, not

all trophozoites go through erythrocytic development and replication. Some trophozoites undergo a sexual cycle by terminally differentiating into gametocytes. Gametocytes (micro and macro gametocytes) are ingested by a female *Anopheline* mosquito during a blood meal from an infected person and fuse to form a zygote in the midgut of the mosquito (Aly *et al.*, 2009). The zygote develops into an ookinete, penetrates the gut wall and mature into oocyst. After a period of 8-15 days, the oocyst bursts, releasing large numbers of infectious sporozoites which migrate to the salivary gland of the mosquito where they are injected during another blood meal to continue the transmission of the parasite (**Figure 2.1**).

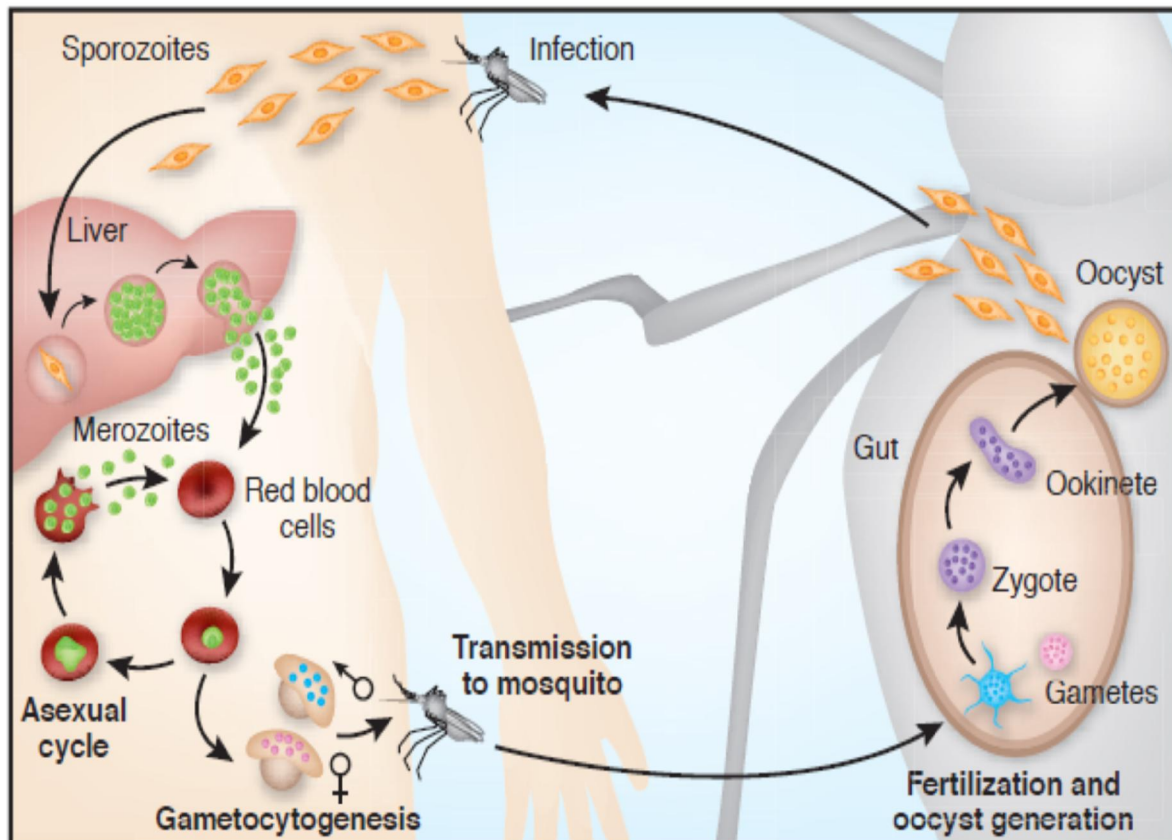


Figure 2.1: Life-cycle of *Plasmodium falciparum*

Source: (Pasvol, 2010)

2.3 Global malaria burden

Malaria continues to be a major health burden in many parts of the world with children under the age of five, pregnant women and non-immune individuals being the most vulnerable. Accumulating evidence over the past decade (2000-2012) shows a remarkable achievement towards the reduction of malaria incidence and malaria-associated mortality coinciding with expanding coverage of malaria control and prevention interventions (World Health Organization, 2013b). Despite these achievements chalked over the past decade, an estimated 207 million cases and 627,000 malaria deaths were estimated to have occurred in 2012 with majority occurring in endemic countries in sub-Saharan Africa, where it is estimated a child dies every minute either directly or indirectly as a result of the disease (World Health Organization, 2013b).

Socio-economically, the disease exerts an enormous toll on the life of affected individuals and their corresponding countries through loss of manpower which has been measured through the disability adjusted life years (DALY) (the number of healthy life years lost on account of a disease) (Abdalla *et al*, 2007; Breman *et al.*, 2005). This metric (DALY) is an acceptable form of measuring disease morbidity and mortality expressed in time with malaria leading the race with 85.7% DALY as of 2000 (Breman *et al.*, 2005). This can result in poverty for affected families and subsequently loss of revenue for the country. In some endemic countries, the disease accounts for up to 40% of public health expenditures; 30% - 50% of inpatient hospital admissions; up to 60% of outpatient health clinic visits are consequences of malaria (Okwa, 2008) (**Figure 2.2**)

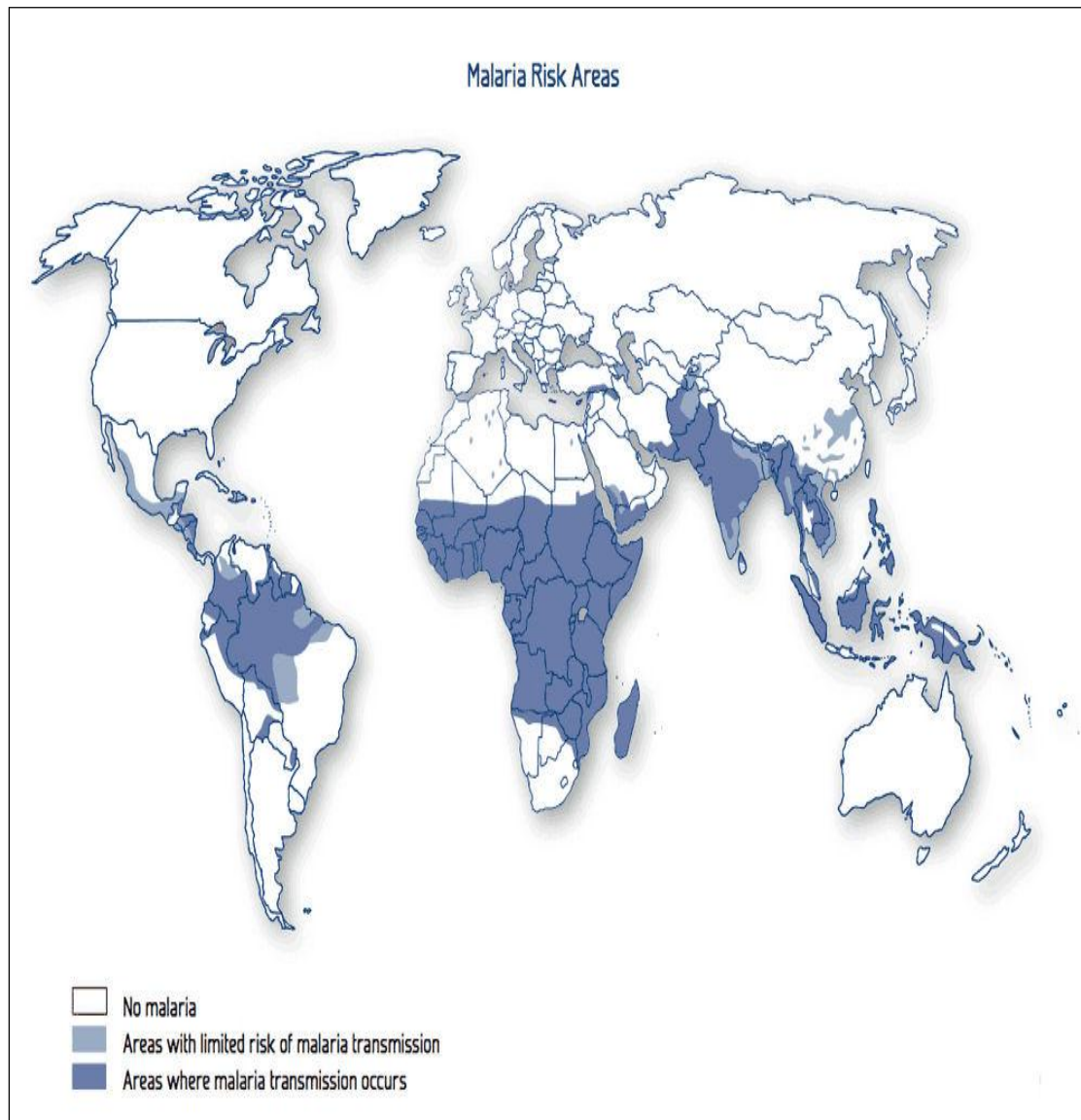


Figure 2.2: Global Malaria Risk Areas

Credit: www.who.int/malaria/travellers/

2.4 Malaria control and prevention strategies

Despite the tremendous health challenges posed by malaria globally and its connection to poverty, substantial progress has been made towards the fight against the disease. This progress has been achieved through several control and prevention interventions adopted and implemented by individual countries and supported by the expansion of global funds towards disease eradication under the Roll back malaria interventions (Greenwood, 2008; O'Meara *et al.*, 2010; Snow & Marsh, 2010; World Health Organization, 2013b). This has resulted in a wide-scale reduction in malaria incidence and mortality, while allowing some countries to achieve the Millennium Development Goal 4 (MDG) of reversing under-five malaria incidence and mortality by 75% by 2015 (World Health Organization, 2013b).

This successes are due to the up-scale coverage of insecticide treated net (ITNs) or long lasting insecticide nets (LLINs), indoor residual spraying campaign launch by some countries, intermittent preventive treatment for pregnant women (IPTp) and infants (IPTi), through prompt diagnosis either with rapid diagnostic test kits or microscopy and treatment with effective chemotherapies such as artemisinin combination therapies and public education on the disease. Also, easy access to health care and malaria surveillance is contributing to the reduction of the disease as seen now (Fegan *et al.*, 2007; Lindblade *et al.*, 2015; Okiro *et al.*, 2010).

2.5 Malaria surveillance indicators

For the sustainability of malaria interventions and reduction in the incidence and deaths associated with the disease, malaria surveillance has become one of the key diagnostic tools put up by the Rollback Malaria program in the fight against the disease (Roll Back Malaria Annual Report 2013). It generates information on the distribution and trends of malaria incidence and

deaths that are critical for the design and implementation of malaria control programs. Although the capacity of malaria surveillance varies with disease endemicity, its objectives are to identify areas or population groups most affected by the disease, trends in cases and deaths that require adjustments in the scale or blend of interventions and also to assess the impact of control measures (Access Malaria & Testing, 2011). With this information, it enable various stakeholders in malaria control programs to direct resources to populations most in need, assess the impact of an on-going program and respond to unusual trends. As a result, progress in malaria control can be accelerated and wastage of resources avoided as well as knowing the malaria trends in the population. In general, malaria surveillance has been carried out using incidence and mortality rate, entomological surveys, parasite density and also by serological surveys.

2.5.1 Malaria incidence and mortality rate

Globally, malaria incidence and mortality rates have been one of the oldest indexes of measuring malaria burden and gauging the risk of the disease. This involves the diagnosis, treatment and reporting of malaria cases on patients seeking treatment for illness at a health post, clinic, or hospital (O'Meara *et al.*, 2010) and this has served as a good proxy for national trends in malaria morbidity and mortality (Snow *et al.*, 1999). While the disease incidence or mortality rates varies according to the consistency of the diagnostic procedure use, availability of report by health facilities have an impact on the interpretation of data (Baird *et al.*, 2002).

Over the past decade, the sensitivity of this method as an index of measuring malaria risk and malaria burden has become unreliable especially in sub-Saharan Africa. Firstly, because malaria dominates as a cause of febrile illness, incidence rates tends to be overestimated as a result of

presumptive diagnosis by healthcare providers in areas where diagnosis are either inadequate or unavailable (Koram & Molyneux, 2007). Secondly, because self-medication often relieves symptoms and there are a lot of unreported deaths and presumptive autopsies, the true burden of the disease in a community may be under reported (Chaturvedi *et al.*, 2009; Rowe *et al.*, 2006). Thirdly, inaccurate diagnosis (Hay *et al.*, 2010) and the progress in the reduction in malaria transmission to near elimination levels in several countries globally (World Health Organization, 2013b), is also making this index as a means of malaria surveillance difficult and insensitive. However, the World Health Organization (WHO) continues to use this method to estimate malaria burden in countries where surveillance data are inadequate or unavailable. Based on the millennium development goal indicators, malaria incidence rate is computed as the number of new malaria case per 100,000 of the population per year while the mortality rate is the number of deaths associated to malaria per 100,000 of the population per year.

2.5.2 Entomological surveys

The magnitude of malaria, especially in developing countries is not due to the poverty of its citizens, but rather the vector range, behavior and ecological parameters that support them (Gallup & Sachs, 2001). Vector capacity correlates with malaria incidence (Beier *et al.*, 1999), necessitating interventions that control the disease by targeting vectors through vector control intervention strategies. Thus, various entomological indicators have been used to monitor and evaluate these interventions through estimation of the entomological inoculation rate (EIR), which is the product of the human biting rate and the proportion of mosquitoes that are infectious (Stoute *et al.*, 1997).

2.5.2.1 Human biting rate

Perpetuation of malaria involves blood meal taken by *Anopheles* vectors which is important for the development and maturation of the *Plasmodium* parasite and also for the survival of the vector. Knowledge from several investigations into the biology and feeding behavior of *Anopheles mosquitoes* (Parous, nullparous, sporozoite infected and non-infected) (Koella *et al.*, 1998; Wekesa *et al.*, 1992) have been exploited in the design or development of several entomological techniques such as the human-landing catches, CDC light trap, knock-down catches, exit trap in the study of disease dynamics in a population. However, the quality and quantity of catches depends on technique used, weather conditions and location of the technique (Mboera, 2005).

Human-landing catch method is currently the golden standard, largely based on its validation over the decade to represent natural human-vector contact and has been globally used in several studies to estimate the EIR (Kilama *et al.*, 2014; Mboera *et al.*, 1998; Sithiprasasna *et al.*, 2004). This method is designed to replicate normal human behavior, where humans acting as both baits and catchers sit at vantage points, aspirating mosquitoes landing on their exposed limbs (Dia *et al.*, 2005; Krockel *et al.*, 2006). It has an advantage of directly measuring the biting rate of mosquitoes considered to be representative of the vector population responsible for malaria transmission (Davis *et al.*, 1995). On the contrary, *Anopheles* mosquitoes are attracted to individuals at different rates, as well as the different skills of the catchers introduces some bias in the use of this method for EIR estimation (Lindsay *et al.*, 1993; Mboera, 2005). Also, this method is faced with ethical issues in connection with increased risk of collectors to *Anopheles* exposure, extrapolation to children, as well as the cumbersome and time consuming nature of this method are some of the short-falls of this method (Kusi *et al.*, 2014; Mboera, 2005).

In search for a substitute, development and application of light traps by the Center for Disease Control and Prevention (CDC) in the USA have been used for sampling of arthropod vectors such as *Aedes taeniorhynchus*, *Ae. nigromaculis*, *Culex tarsalis*, *Cx. t. surmmorus* and *Anopheles* mosquitoes, and have been exceptional good (Kusi *et al.*, 2014; Mboera, 2005).

Of the various light trap designs, the standard CDC miniature light trap has been found to be fairly good for sampling *Anopheles* mosquitoes that feed late in the night (indoor feeders). This method has been deemed useful in obtaining the *Anopheles* fauna in an area as it catches more mosquitoes than human-landing catch (Mboera *et al.*, 1998). However, this method catches more mosquitoes than would normally be attracted to an average human host, with high numbers infected with the malaria parasite due to the fact that it attracts resting mosquitoes leading to overestimation of the sporozoite rate (Mbogo *et al.*, 1993). Thus, although light traps has been useful in some ways, it does not represent the true human biting mosquito population.

Another common method used for sampling indoor mosquitoes is knock-down catches (World Health Organization, 2013a). This method is based on knowledge that several *Anopheles* mosquitoes rest indoors before and/or after feeding. Thus, collection has been done usually by knockdown with insecticide (pyrethrum) spray catches, although exit traps or aspirators have also sometimes been added (Kilama *et al.*, 2014; World Health Organization, 2013a).

Although collections by these methods give a good estimate of the mean house density in a given area (World Health Organization, 2013a), they may not necessarily give a good estimate of EIR. This is due to the fact that sampling indoor-resting mosquitoes tends to miss the mosquitoes that leave the house immediately after feeding, and may include those that enter after feeding outdoors on other hosts. Thus indoor resting-catches can give rise to a false impression of effectiveness of a control measure.

2.5.2.2 Sporozoite rate determination

Determination of the presence of *Plasmodium* sporozoites in mosquitoes continue to remain an important component in understanding transmission dynamics and assessing vector control interventions in population specific epidemiological study (World Health Organization, 2013a). Mosquito infection rate in addition to human biting rates provide important parameters for estimation of entomological inoculation rates and transmission intensity (Kilama *et al.*, 2014; Smith *et al.*, 2010). This serves as a measure of the proportion of the population at risk of the disease and the infection status is usually assessed by the presence or absence of *Plasmodium* sporozoites in the salivary gland of mosquitoes. It can also be determined by gene detection (Beier *et al.*, 1999).

Traditionally, microscopy has been used for visualization of mosquito infection status through the dissection of mosquito salivary glands (World Health Organization, 2013a). However, the requirement of skilled microscopist, and inability to determine *Plasmodium* species remains the main limitation for this method, thus being supplemented with a rapid immunological technique which is based on the detection of specific antigens in either the thoracic salivary gland or the oocyst of infected mosquitoes using an enzyme-linked immunosorbent assay (ELISA) (Beier *et al.*, 1990; Wirtz *et al.*, 1989). Circumsporozoite protein (CSP) has been the preferred antigen due to its implication in the journey of sporozoites from the oocyst to the salivary gland and also in the invasion of hepatocytes in the host. Also, because CSP is conserved in all *Plasmodium* families it is able to identify *Plasmodium* species addressing the limitation of the dissection method (Charlwood *et al.*, 2015; Fontenille *et al.*, 2001). Although, this method is cheap, sensitive and robust, a number of studies have shown that CSP ELISA overestimates true salivary gland sporozoite rates. This overestimation is linked to shedding of CSP during the

journey of the sporozoites through the mosquito (Beier *et al.*, 1990; Fontenille *et al.*, 2001). Also, most investigators concentrate on detection of CSP from one or two *Plasmodium* species base on its prevalence in the study area.

With recent advances in molecular biology, there is a shift to polymerase chain reaction (PCR) based assays which are sensitive and specific than ELISA, for sporozoite detection in mosquitoes. This method utilizes primers designed against species specific regions in the sequences encoding the small subunit ribosomal RNA (ssRNA) to detect all four *Plasmodium* species (Snounou *et al.*, 1993). To further enhance the sensitivity of this method, a nested approach using two rounds of PCR have been employed. However, to address the problem of separate PCR reactions for detection of each species, more advanced methods based on high-throughput assays such as real-time PCR have been developed for detection and quantification of sporozoites in the mosquito (Bass *et al.*, 2008).

2.5.3 Parasitological survey

Malaria prevalence is simply the proportion of persons found to be infected with malaria parasites out of the total number of persons sampled and assessment of an individual parasite load determines the level of infection. Normally, prevalence studies involves taking thick and thin blood smears from volunteers, staining with Giemsa, and microscopically examining the stained slides to detect and count parasites (Freaan, 2010). This method allows investigators to identify and quantify which *Plasmodium* species is prevalent in the study area and has mostly been conducted through either a cross-sectional survey or longitudinal survey of specific study populations. Data obtained from this method has been useful in mapping and evaluating patterns of endemicity (Hay *et al.*, 2009), as well as evaluation of implemented control interventions and

chemotherapy effectiveness (Greenwood & Armstrong, 1991). However, this method as an indicator of malaria transmission intensity has inherent limitations.

Parasite prevalence estimation by microscopy has a reduced detection sensitivity, especially at low transmission settings, as well as by naturally acquired immunity and drug intake that reduce parasite prevalence (Corran *et al.*, 2007; O'Meara *et al.*, 2007).

2.5.4 *Plasmodium falciparum* genotyping as a measure of transmission intensity

Polymerase chain reaction based parasite genotyping using highly polymorphic loci blocks of certain surface antigens, have gained relevance in epidemiological studies over the past century (Agyeman-Budu *et al.*, 2013; Assefa *et al.*, 2014; Greenhouse & Smith, 2015). This is because of the knowledge it sheds on infection dynamics, parasite diversity, transmission intensity, drug efficacy and immune development (Assefa *et al.*, 2014; Magesa *et al.*, 2002). In malaria endemic areas, parasite genotyping has provided useful indicators of disease dynamics based on number and frequency of parasite strains or mixture of genetically distinct clones in a host (Babiker *et al.*, 1999). In malaria epidemiology, multiplicity of infection (MOI), referring to the number of different parasite genotypes of each strain co-infecting a single host have added value to the other surveillance tools including serology, entomological monitoring and malaria incidence measurements (Agyeman-Budu *et al.*, 2013; Assefa *et al.*, 2014). This involves genotyping of the genes that encodes the merozoite surface proteins (MSPs) (that is *msp 1* and *msp 2*) and the *glurp* gene, which are the standard genes accepted by the WHO for *Plasmodium falciparum* genotyping (Venture & Venture, 2007). However, *msp-1* and *msp-2* have received the most attention because its genes are highly polymorphic in length and sequence (Agyeman-Budu *et al.*, 2013; Ntoumi *et al.*, 1995). In line with this, several studies on transmission intensity estimation base on *msp2* genotyping shows a positive correlation between increase in

transmission and the average number of parasite clones or genetically distinct clones per host (Babiker *et al.*, 1997; Ghanchi *et al.*, 2010; Haddad *et al.*, 1999).

2.5.5 Sero-epidemiological assessments of malaria transmission

With the continuous decline in malaria transmission, assessing malaria transmission intensity and evaluating the impact of interventions by the traditional methods (EIR and parasite prevalence) have become laborious and less sensitive. Thus, in recent years, alternative risk indicators estimated from statistical models on antibody responses to parasite and vector antigens have been evaluated mainly by enzyme-linked immunosorbent assay (ELISA) (Bousema *et al.*, 2010; Corran *et al.*, 2007; Schofield, 1990). This stems from the observation that, exposed individuals develop antibodies against parasite and vector antigens for long periods of time in contrast with the life span of vectors or putative infections. Antibody prevalence estimation provides information on cumulative effects of population exposure, while the modelling of this information using the rate of antibody acquisition (seroconversion) and lost (reversion) rates provides important information on disease dynamics in a population (Sepúlveda, 2015). A number of studies conducted using the seroconversion rate (SCR) model have shown a strong correlation with EIR (Bousema *et al.*, 2010; Drakeley *et al.*, 2005).

Over the years, several serological markers have been added to the serological toolbox for measuring malaria transmission and evaluating intervention programs (Kusi *et al.*, 2014; Marie *et al.*, 2014; Poinsignon *et al.*, 2008). These markers are based on the different surface antigens that accompany the different morphologies of the various stages of parasite life cycle and they include the 19 KDa fragment of merozoite surface protein 1 (MSP1₁₉), Merozoite surface protein 2 (MSP2), Apical membrane antigen 1 (AMA1), cell-transversal protein of ookinete (CelTOS), circumsporozoite protein (CSP), *An. gambiae* salivary gland protein 6 (gSG6) and its peptide

(gSG6-P1), etc (Drakeley *et al.*, 2005; Kusi *et al.*, 2014; Poinsignon *et al.*, 2008). The immunogenicity, half-life (decay rate) and age related acquisitions of these proteins defines their utility or usefulness in a particular endemic settings.

2.5.5.1 Blood stage antigens

Of more than 5,000 proteins expressed by the *Plasmodium* species, very few have been investigated as potential biomarkers for sero-surveillance (Florens *et al.*, 2002; Hall *et al.*, 2005). Among those studied, a sizable percentage are blood stage antigens that include merozoite surface protein 1 (MSP1), MSP2, apical membrane antigen 1 (AMA1), erythrocyte binding antigen (EBA), glutamine-rich protein (GLURP) (Cook *et al.*, 2012; Elliott *et al.*, 2014). However, to date, antibody responses to MSP1-19 and AMA1 are the most studied markers of exposure and modeling based on MSP1-19 antibodies have shown strong correlation with other indicators of transmission intensity; EIR, parasite rate, malaria incidence, and altitude (Bousema *et al.*, 2010; Corran *et al.*, 2007; Drakeley *et al.*, 2005). Antibodies to different blood stage antigens are acquired at different rates relative to exposure, therefore, choice of antigens have been decided based on the immunogenicity, polymorphism longevity of elicited antibodies and the targeted population (Elliott *et al.*, 2014). Base on this properties, AMA1, a highly immunogenic antigen, has been shown to saturate at moderate-to-high endemicity making it inappropriate at this settings, while it could be more appropriate than MSP1-19 antibodies for serosurveillance in very-low-transmission settings (Bousema *et al.*, 2010; Corran *et al.*, 2007; Drakeley *et al.*, 2005). Antibodies against blood stage antigens are known to be persistence even long after transmission has ceased. This phenomena could be due to the continuous antigenic stimulation resulting from chronic, low-density parasite carriage or the slow decay of antibodies

(Bousema *et al.*, 2010; Corran *et al.*, 2007; Drakeley *et al.*, 2005). This therefore, represents a weakness in the use of blood stage antigens, especially for predicting seasonal or short term changes in transmission.

2.5.5.2 Vector antigens

Measuring of human antibodies to vector antigens have been an alternative concept for estimating *Anopheles* exposure (Drame, *et al.*, 2010b). This is linked to previous studies that showed that the anti-saliva antibody response could be a potential marker of exposure to vector-borne diseases in individuals exposed to bites of arthropod vectors, such as ticks (Lane *et al.*, 1999), phlebotomine (Barral *et al.*, 2000) and *Triatoma* (Nascimento *et al.*, 2001). Later, Remoue *et al.* in 2006 demonstrated that IgG responses to whole saliva extracts (WSE) of *An. gambiae* represented a marker of exposure to *An. gambiae* bites. However, due to the distribution of some families of salivary proteins between blood sucking vectors (Poinignon *et al.*, 2008; Ribeiro & Francischetti, 2003) and the cross reactivity between common epitopes on immunogenic salivary proteins, WSE was rejected as a relevant marker of *An.* exposure.

Advances in molecular biology helped to identify 71 secreted salivary proteins (Arcà *et al.*, 2005), of which *An. gambiae* salivary gland protein 6 (gSG6) and its associated peptide (gSG6-P1) was indicated to be immunogenic and IgG levels to this protein correlated with direct mosquito exposure measurements (Poinignon *et al.*, 2008; Rizzo *et al.*, 2011). Also, levels and prevalence of antibodies to gSG6 and gSG6-P1 have shown varying degrees between regions with different transmission and seasons (Drame *et al.*, 2010a; Poinignon *et al.*, 2008; Rizzo *et al.*, 2011).

In addition, IgG levels to vector antigens (gSG6) have also been shown to wane quickly as compared to the blood stage antigen (Poinsignon *et al.*, 2008; Rizzo *et al.*, 2011). Thus, this class of sero-markers has been viewed as ideal candidates for determining recent exposures. However, intense mosquito exposure may not necessarily mean a high malaria exposure if mosquitoes are not infected, suggesting over estimation based on this marker (Personal communication, Dr. Kingsley Badu, 19th November, 2014).

2.5.5.3 Sporozoite antigens

Unlike the vector and blood stage antigens, antigens from the sporozoite stages of *Plasmodium* are exposed to the immune system for only short periods after inoculation. Also, they are less immunogenic, and antibody levels to these antigens have been shown to be relatively non-persistent while being able to indicate exposure to parasites (Druilhe *et al.*, 1986; Kariu *et al.*, 2006). It is for this reason why antigens belonging to this class have been proposed as the ideal candidates for transmission assessment. Like blood stage antigens, only a limited number of sporozoite antigens have been investigated as potential sero-surveillance markers. This include circumsporozoite protein (CSP), cell-transversal protein for ookinetes and sporozoites (CelTOS) (Druilhe *et al.*, 1986; Kariu *et al.*, 2006; Kusi *et al.*, 2014) with CSP being the most studied and its potential as a sero-surveillance indicator demonstrated.

2.5.5.4 Putative, Secreted Ookinete protein (PSOP24)

Studies using protein microarrays to profile antibody responses have identified some promising candidate antigens (Baum *et al.*, 2013; Crompton *et al.*, 2010; Doolan *et al.*, 2008). Recent studies by Baum *et al.* (2013) identified several promising antigen-specific responses that discriminate between regions with high and low *P. falciparum* transmission, including

Plasmodium falciparum putative, secreted ookinete protein (PSOP24). The PSOP24 is a novel antigen conserved among the *plasmodium* family, which is also expressed by ookinetes in the mosquito mid-gut. Hence, will be a unique candidate for sero-epidemiological estimation of malaria transmission. Based on Baum *et al.* (2013), PSOP24 was found to be immunogenic and corresponded to the transmission levels of the study communities.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 MATERIALS

3.1.1 REAGENTS

Casein from Bovine Milk, Phenol Red, Tween 20, Sodium azide, Hydrochloric acid, Sulfuric acid, Saponin, Chelex, Agarose, Gel red, Magnesium Chloride, each Deoxy-nucleotide primers and DNA *Taq* polymerase were all obtained from Sigma-Aldrich (St. Louis, MO USA), Phosphate Buffered Saline was obtained from Invitrogen (UK), Sodium Hydroxide was obtained from VWR International (Belgium), Horseradish Peroxidase (HRP) Conjugate of goat anti-human IGg, NUNC Immuno plates and 3,3',5,5'-Tetramethylbenzidine (TMB) Ultra were obtained from Thermo Scientific (USA), Low binding 96 sero-well plates were obtained from Bibby Sterilin (UK), Plate sealer was obtained from Greiner Bio-One (Belgium), 50x Tris acetic EDTA (TAE) was purchased from National Diagnostics (USA), Nuclease free water (DEPC Treated Water, Invitrogen, CA, USA), Biotek Elx 405 Automated ELISA Plate Washer And Biotek EL 808 ELISA Plate Reader; All Obtained From Biotek Instruments (Winooski, VT; USA).

3.1.2 Putative, Secreted Ookinete protein (PSOP24) peptide design

The PSOP24 peptides were designed by Dr. Anne Poinson from the Institute de Recherché pour le Development (Senegal) and Dr. Kingsley Badu from Noguchi Memorial Institute for Medical Research (Ghana) using *in silico* approach. The peptide design was based on identifying potential immunogenic peptides, predicted by algorithms and to investigate the specificity of

these peptide sequences compared to expression sequence tag (EST) or genome libraries of other organisms.

The putative peptides were identified with bioinformatics tools (BcePred, BepiPred and AbcPred) used for identifying and predicting immunogenic epitopes. The MHC class 2 binding region was also identified using ProPred and the sequences were aligned using BLAST program in UniPot to compare predicted PSOP24 peptides with known genome or expressed sequence tag (EST) libraries. Peptides were synthesized and purified (>70%) by ChinaPeptides (China). All peptides were shipped lyophilized and reconstituted in 1mL filtered double distilled water and stored in aliquots at -80°C until ready to use with details shown in Table 3.1.

Table 3.1: Predicted peptide sequences of PSOP24

Peptide sequences	Peptide name
DVHNFLLVDKEMERRKE	PSOP24-372W
KYPDIYGFFLIILILYILKYVFKKMMYKLY	PSOP24-373W
LLKRILYNVEKKKVLSNYESILQDILENT	PSOP24-374W
IKYFFEVITEILIVIRLGLIYRY	PSOP24-375W
LENVEMVCVTFLSIHKFGRE	PSOP24-376W
IGVFTYISTQALKKINH	PSOP24-377W
EGSFSYAPPPYQGVEN	PSOP24-378W

3.1.2 Ethical approval

This current study used archived blood filter blots from a previous cohort study that was conducted between 2012 and 2014. Ethical approval for the parent study was granted by the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana. Written informed consent were obtained from all participants or their parents/legal guardians before enrolment into the study. For this part of the study selection of archived samples was done blindly.

3.1.3 Study site and population

The study was conducted in two communities near Dodowa; Odumase and Ayigbekorpe, all in the Shai Osudoku district of the Greater Accra region. The district is an urban setting with Dodowa as its administrative capital. The district lies between latitude 5° 53' north and 6° 19'south and longitude 0° 20' east and 0° 5' west of the equator and is bounded to the east by Ada West, to the north by Yilo Krobo, Menya Krobo and Asuogyaman districts, to the south by Ningo-Prampram and to the west by Akwapim Nouth and Tema Metropolitan respectively. The study sites are 2 Km apart and unlike the district capital, they have a rural setting with the majority of houses made of clay. Like most other communities in the district, Dodowa has two peak rainfall seasons with the major peak occurring between May and July and the minor season between October and December which coincides with the main malaria transmission periods. This is then followed by the dry season from January-March. However, Dodowa like Asutuare has a low malaria transmission of 21.9 bites/man/year due to the numerous malaria control

interventions rolled out in the district (Badu *et al.*, unpublished). The 2010 population size of then Dangme East district estimated by the 2010 population and housing census was 130,795 (Gss, 2012) with majority of the inhabitants being Dangmes and Christians. Inhabitants in the district engage mainly in farming and petty trading with a lot of the men migrating to Tema and Accra for other livelihoods. The Dodowa sub district has several public and private health facilities with the district hospital close to the study communities (Gyapong *et al.*, 2013).

3.1.5 Study design

The study was a cross-sectional study, carved from a bigger cohort study, with three follow-ups after the initial recruitment. However, the current study made use of samples from two collection times (February/March, 2013 and October, 2013) corresponding to the dry and wet seasons. The study comprised of individuals from all age groups, except infants below the age of 6 months. During each enrolment, whole blood (300µl collected via finger prick in a EDTA coated microvettes), blood smears (thin and thick films) filter paper blot samples were collected in November, 2012, February 2013, October 2013 and July 2014 just before, during and after the main transmission seasons after obtaining informed consent from participants and parents/guardians of children. A study questionnaire was used to obtain relevant demographic information on study participants during each enrolment to obtain the age, gender, bed net use and clinical history of individual participants. At each sampling time point, samples were collected from both communities.

3.1.6 Sample size calculation

For the current study, 568 samples were used with a minimum sample size of 268 participants per season. This was calculated using the binomial model to estimate the confidence interval (CI). The parasite prevalence of 22.5% was used to estimate the minimum sample size needed to achieve enough statistical power. Sample size was calculated with 95% CI and precision level of 5%.

$$n_o = ZaPq/d^2$$

In the equation above, n is the sample size, Za is the critical value of the standard normal distribution at the 5% level (1.96), P is the estimated parasite prevalence, q=1-P and d is the precision level.

3.2 Methods

3.2.1 Parasite prevalence and density determination

Parasitological examination for *Plasmodium falciparum* identification and counting was performed at each sampling time point using both thick and thin smears obtained by finger-pricking. The smears were stained with Giemsa and parasite quantified by counting 100 microscopic fields. Parasite density defined as the number of *P. falciparum* parasites/ μ l of blood, was estimated using the formula below

No. of parasite/ μ l of blood = Parasite counted/200WBC with the assumption that each μ l of blood contains approximately 8000 leucocytes (WBCs)

3.2.2 Antibody elution from filter paper blots

Antibodies were eluted from filter paper blots using standard methodology by Corran *et al.*, (2008). Briefly, disc of approximately 2.5mm were cut from each filter paper using a puncher. Individual paper discs were transferred to individual wells of a low binding 96-well microtitre plates. At room temperature, antibodies were eluted with 150 μ L PBS containing 0.05% Tween 20 and 0.05% sodium azide by gently mixing the well contents overnight on a rotary shaker at 2 RPM.

3.2.3 Determination of IgG responses to PSOP24 peptides, gSG6-P1 and CSP antigens

Total IgG responses to PSOP24 peptides (Table 3.1) and later PSOP24-374W, PSOP24-375W and PSOP24-377W, the vector antigen gSG6-P1 and the sporozoite antigen CSP were measured using indirect ELISA protocol described by Badu *et al.*, (2012) with minor modifications. Briefly, NUNC immuno plates were coated with 20 μ g/ml of each peptide antigen in 100 μ L PBS

and incubated at 37°C for 2 hrs30 min in a humidity chamber. Plates were then washed four times in a washing buffer (1X PBS with 0.1% Tween-20) with 30 seconds incubation between each wash using the Biotek ELx 405 automated ELISA plate washer. The washed plates were padded dry on an absorbance tissue paper and blocked with 200µL/well of blocking buffer containing 0.5% casein in 1% Tween-20 and incubated at 37°C for 1hr in a humidity chamber. Plates were again washed four times as described above and subsequently incubated overnight at 4°C with 100µL/well of elutes diluted 1:10 in a dilution buffer containing 0.5% casein in 2.5% blocking buffer. A pool of healthy hyper immune plasma samples known to be positive for total IgG to CSP and CelTOS were used as a standard calibrator (diluted 20 times) for inter assay and day-to-day variation in the standard ELISA procedure. Each assay (ELISA plates) had the standard control titrated 2-folds.

Plates were washed four times in a washing buffer and dried as mentioned above. This was followed by addition of 100µl/well of horseradish peroxidase (HRP) conjugated goat anti-human IgG antibodies diluted at 1:10,000 in dilution buffer and incubated at room temperature for 2 hours in a humidity chamber. After incubation, plates were washed again four times as described above and 100µL TMB ultra substrate was added and incubated for 15 minutes for each PSOP24 peptide at room temperature. The reactions were stopped with addition of equal volume of 0.2mM of sulfuric acid. Optical densities (OD's) were measured using a Biotek EL 808 ELISA plate reader at 450nm.

3.2.4 DNA extraction from filter paper blots

To enable the complete use of blood pellets in EDTA coated microvettes, all archived blood pellets from study participants were diluted with 100µl of plain RPMI prior to blotting. The diluted blood pellets were subsequently blotted on 3mm Whatman paper and left overnight for air drying. DNA was extracted from filter paper blot by the chelex method as previously described by Wooden *et al.* (1993). Briefly, approximately 3mm of blood blot disc were incubated in 1000µl of autoclaved 1X PBS and 50µl of 10% saponin solution overnight at 4°C. After centrifugation for 1 min at 13,000 rpm, the PBS-saponin solution was discarded and 1000µl of 1X PBS (without saponin) was added and incubated at 4°C for 30 minutes. The tubes were centrifuged and the supernatant discarded. The filter blots were re-suspended in 100µl of sterile distilled water, centrifuged and the supernatant discarded. The blot pellets were re-suspended again in 70µl of sterile deionized water and 30µl of 20% Chelex-100 resin suspension in deionized water respectively. The tubes were incubated at 95°C for 10 min on a heat block while vortexing at 2 min intervals. After incubation, centrifugation was done at 13,000 rpm for 7 minutes, the supernatants were then recovered and transferred into a new pre-labeled storage tube and stored at -20°C until use for PCR amplification.

3.2.5 *Plasmodium falciparum* genotyping

Polymerase chain reaction (PCR) was performed on the extracted genomic DNA as described by Snounou, (2002) with slight modification. The polymorphic block 3 region of the MSP2 gene was amplified using sequence specific primers; forward primer (5'-ATGAAGGTAATTAACATTGTCTATTATA-3'), and the reverse primer (5'-CTTTGTTACCATCGGTACATTCTT-3') designed from published oligonucleotide sequence by

Snounou *et al.* (1999). PCR product from the primary reaction was amplified using nested primers which are specific to the IC3D7 allelic family: forward- (5'- GCT TAT AAT ATG AGT ATA AGG AGA A -3'), reverse (5' - CTG AAG AGG TAC TGG TAG -3'). Separate reactions were performed for each pair of primary and nested primers. Both primary and nested reactions were done in a final volume of 25 μ l, containing 2.5 μ l 10X reaction buffer, 2 μ M MgCl₂, a 200 μ M dNTP containing each of the four dNTPs, a 300nM concentration of each of the two appropriate primers, and 0.25U of *Taq* DNA polymerase. Both reactions contain 5 μ l of DNA as the template and a genomic DNA from 3D7 laboratory strain which was used as positive control, and molecular grade water as a negative control. The Primary reaction was carried out in a thermo cycler with an initial denaturation at 94°C for 2 min, denaturation at 94 °C for 30 sec, annealing at 55°C for 1 min 30 sec, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. The denaturation, annealing and extension steps were repeated for 30 cycles. The Nested PCR was carried out with an initial denaturation at 94°C for 2 min and 30 cycles of 94 °C for 30 sec, 55 °C for 1 min 30 sec and 72°C for 1 min, followed by a final extension of 72 °C for 5 min.

3.2.5 Agarose gel electrophoresis

PCR amplicons were analyzed with a 2% agarose gel (5g agarose in 250mL TAE-buffer) with Gel red as the nucleic acid staining dye. The loaded gels were subjected to electrophoresis at 100V for 1 hour after which the gels were visualized and image captured with a Toyobo TM-20 UV transilluminator fitted with a camera.

3.2.4 Statistical analysis

The measured optical densities from the ELISA results were converted to weighted concentrations using ADAMSEL software (an Excel-based 4-parameter logistic fit programme). Differences in the levels of antibodies against the five antigens were assessed by comparing the titres. Data for each antigen were split into three age categories: 6 months to 5 years, 6 to 14 years and those greater than 14 years. For each antigen, the Kruskal-Wallis test on ranks was used for comparison of median titres at the different sampling time points, and this was followed by pair-wise comparisons using the Dunn's multiple comparison test where necessary. The non-parametric repeated Mann Whitney U test was used to determine the spatiotemporal variations in antibody titres for all antigens between the communities. Multiplicity of infection (MOI) was calculated as the ratio of samples found to have *m**sp*-2 allelic variants over the total number of samples used in that season. Student t-test was used to compare multiplicity of infection between the study communities and between the transmission seasons.

CHAPTER FOUR

RESULTS

4.1 Study population

The study used blood blot samples from two cross-sectional surveys of individuals living in two communities, Odumase and Ayigbekorpe, in Dodowa which are only 2 km apart. In all, frozen blood blot samples from 567 individuals (mean age = 17, range=0.5-90 years) were analyzed of which 350 (61.7%) were obtained in the dry season and 217 (38.3%) in the rainy/wet season from both communities. A total of 327 (57.7%) of the samples used were from individuals living in Ayigbekorpe of which 200 (61.2%) were females while 240 (42.3%) were from Odumase comprising 119 (49.8%) females. There was significant difference in the gender distribution between the communities ($X^2 = 6.8, p = 0.01$). There was a significantly higher proportion of individuals in Odumase using treated bed nets than in Ayigbekorpe ($X^2 = 124.0, p < 0.00001$ Table 4.1.) There were 33 (10.1%) individuals in Ayigbekorpe who were positive by microscopy for malaria during the study period compared with 28 (11.7%) individuals in Odumase, however, this difference was not statistically significant ($X^2 = 0.21, p = 0.64$). The Welch two sample t-test showed a significant difference in the mean parasite densities between the two communities with the population from Odumase having a higher parasite density as compared to those in Ayigbekorpe ($p = 0.02$) Table 4.1.

Table 4.1: Demographic and clinical characteristics of study population by communities

Characteristics	Community		χ^2	P value
	Ayigbekorpe, N=327 (57.7%)	Odumase, N=240 (42.3%)		
Age (years)				
<5	67 (20.5)	36 (15)	3.6	0.16
6-14	141 (43.1)	102 (42.5)		
>14	119 (36.4)	102 (42.5)		
Sex				
Male	127 (38.8)	121(50.4)	6.8	0.01
Female	200 (61.2)	119(49.6)		
Bed net use				
No	250 (76.5)	70 (29.2)	124	<0.00001
Yes	77 (23.5)	170 (70.8)		
Parasitaemia status				
Negative	294 (89.9)	212 (88.3)	0.21	0.64
Positive	33 (10.1)	28 (11.7)		
Parasite Density (Mean \pm SD)	61.91 \pm 267.56	184.18 \pm 809.11		0.02*

*Welch two sample t-test

4.2 Parasite prevalence and IgG response

Parasite prevalence was generally low with no particular seasonal trends observed between the seasons (Dry season = 9.84%, wet season = 9.82%). Parasite prevalence by microscopy was 11.54% in the less than 5 year group, 14.74% in the 5 to 14 year group and 4.24% in individuals older than 14 years residents in Ayigbekorpe while Odumase had a parasite prevalence of 11.29% in the less than 5 year group, 11.97% in the 5-14 year group and 10.68% in those older than 14 years. There was no differences in parasite prevalence among the age groups when compared ($\chi^2 = 4.904$, $p = 0.086$) Table 4.2. Generally, there was no association between antigen specific seroprevalence and relative antibody levels in the study population. However, the data reveal a significant association between age and parasite status (OR = 0.97, 95%CI = 0.94-0.99, $p = 0.0081$, logistic regression analysis).

Table 4.2 Community based parasite prevalence characteristics of study population

Characteristics	Age (years)			P value
	<5	6-14	>14	
communities				
Ayigbekorpe	6 (11.54)	23 (14.74)	5 (4.24)	0.086
Odumase	3 (11.29)	14 (11.97)	11 (10.68)	

4.3 Selection of PSOP24 peptides

For each PSOSP24 peptide, the specific IgG responses were compared according to the seasons within the same randomly selected subsamples on children. A seasonal trend for specific IgG responses were observed for PSOP24-375 ($p = 0.023$) and PSOP24-377 ($p = 0.024$) respectively

Figure 4.1

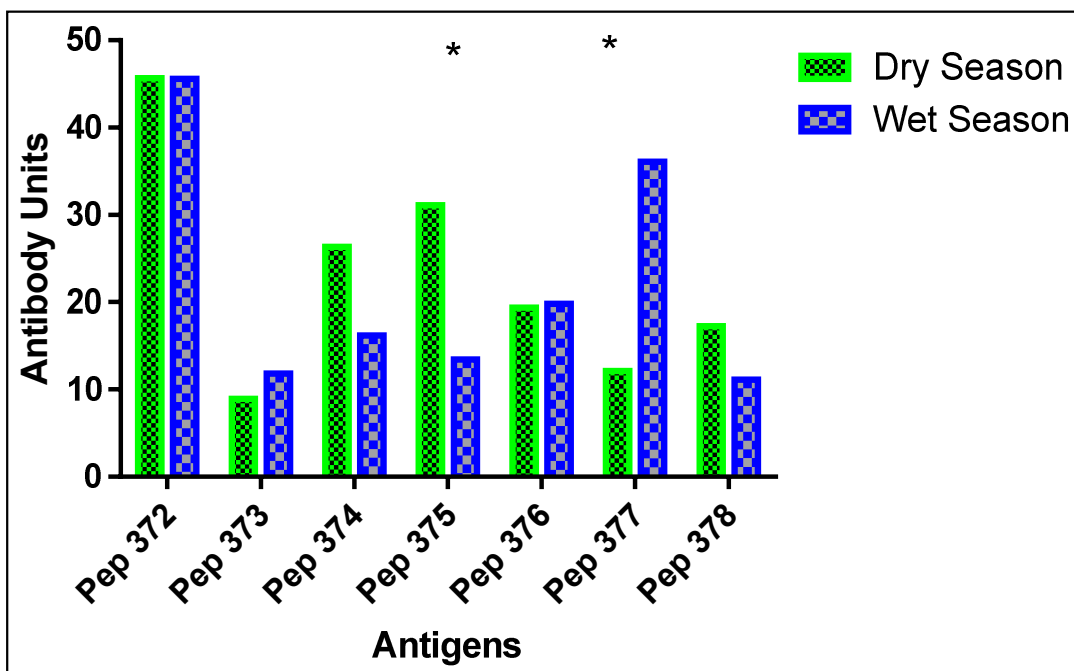


Figure 4.1: Seasonal IgG responses to PSOP24 peptides.

Seasonal trends for each PSOP24 peptide within the same subsample children ($n = 40$). Seasonal trend was only observed for PSOP24-375 and PSOP24-377 with p -values < 0.05 between the seasons. Peptide PSOP24-374 showed some seasonal difference, although not statistically significant (Kruskal Wallis and Dunn's multiple comparison tests).

4.4 Seasonal variation of antigen specific IgG levels

Malaria specific IgG responses to five antigens (PSOP24-PSOP24-374, PSOP24-PSOP24-375, PSOP24-PSOP24-377, CSP and gSG6-P1) were evaluated in blood blot elutes for both the dry and wet seasons. Analysis of the median IgG titres among the study population shows significant differences between the seasons for all the antigens (Mann Whitney U test, $p < 0.0001$). However, no statistical significance between the seasons for the CSP antigen (Mann Whitney U test, $p = 0.613$) (Figure 4.2).

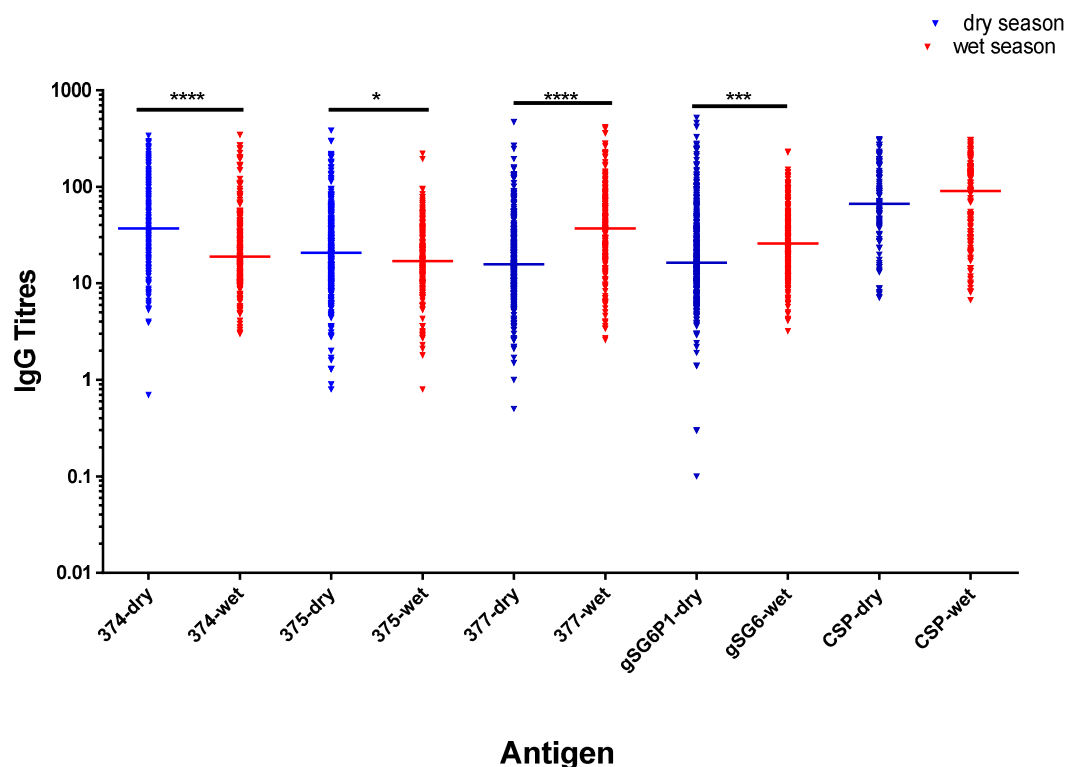


Figure 4.2: comparison of antigen specific IgG responses between the dry and rainy seasons: Comparison of the median IgG titre levels between the seasons to five antigens (PSOP24-PSOP24-374, PSOP24-PSOP24-375, PSOP24-PSOP24-377, CSP and gSG6-P1) shows a significant differences between the seasons for all the antigens (Mann Whitney test, $p < 0.0001$) except CSP which showed no differences between the seasons when compared. Asterisks (*) indicate the level of statistical significant (p value < 0.05) and (***) indicate the level of statistical significant (p -value < 0.0001) between the season.

4.5 Antigens specific comparison within and between age stratified groups

The median IgG titres to peptide PSOP24-374 was significantly different between the dry and the wet seasons in the children less than 5 and between the 5-14 age group with high median IgG titres observed during the dry season but no significant difference was observed in the greater than 14 year group (Table 4.3). However, comparison of median IgG titre to PSOP24-374 shows a significant difference between the less than 5 years group when compared independently to the 5-14 years and the greater than 14 year groups ($p < 0.005$, Kruskal wallis test, Dunn's multiple comparison test).

As was with peptide PSOP24, median IgG titres to peptide PSOP24-375 between the dry and wet seasons differed significantly. However, these levels were comparable between the age groups (Kruskal wallis test). Comparison of the median IgG titres to PSOP24-375 however shows a significant difference between the less than 5 years group and the other age groups when compared independently ($p < 0.005$, Kruskal Wallis test, Dunn's multiple comparison test).

There was 2.5 fold increase in IgG titres to peptide PSOP24-377 from the dry to the wet season in all the age groups (Mann Whitney test) but comparison of the median IgG titres to PSOP24-377 was significant different between the less than 5 years group and the greater than 14 year group when compared independently ($p < 0.005$, Kruskal wallis test, Dunn's multiple comparison test).

Similarly, as were with peptide PSOP24-374 and PSOP24-375, median IgG titres to the antigen gSG6-P1 was significantly different in the children less than 5 and between the 5-14 years group but not in the greater than 14 years group (Mann Whitney test, $p = 0.11$ and $p = 0.779$) between the dry and wet seasons. However, no significant differences was observed between the age

groups when compared independent to the less than 5 years group ($p < 0.005$, Kruskal wallis test, Dunn's multiple comparison test).

Median IgG titres to CSP was significantly different only with in the greater than 14 years group, however, between age comparison shows a significant difference in the 5-14 and the greater than 14 years group when compared independently against the less than 5 year gruoup ($p < 0.005$, Kruskal wallis test, Dunn's multiple comparison test).

Table 4.3 Median antigen specific responses within and between age groups during the wet and dry seasons

Antigen	Age	Seasons		P value
		Dry	wet	
PSOP24-374	<5	24.5	10.8	0.001
	5-14*	31.4	17.4	<0.00001
	>14*	41.6	31.05	0.073
PSOP24-375	<5	13.5	10.2	0.11
	5-14*	19.3	15.3	0.03
	>14*	24.9	24.05	0.779
PSOP24-377	<5	10.7	24.1	0.035
	5-14	15.4	33.05	<0.00001
	>14*	17.7	51.7	<0.00001
gSG6-p1	<5	12.7	24.5	0.0017
	5-14	16.1	26.65	<0.00001
	>14	18	23.15	0.627
CSP	<5	38.05	19.3	0.101
	5-14*	51.6	49.5	0.789
	>14*	96.2	146.5	0.0041

*significant differences between age groups compared by Dunn multiple comparison tests to the less than 5 year's group

4.6 Antigen specific differences between study communities

Spatial variation between the study communities show that IgG responses against peptides PSOP24-374 and PSOP24-375 were statistically significant between the study communities during the dry season with the population at Ayigbekorpe having higher IgG responses than those at Odumase. There was no spatial variation between the study communities when measured against CSP and gSG6-P1. However, a marginal differences was observed for peptide PSOP24-377 ($p = 0.05$, Mann Whitney test) with the population at Ayigbekorpe having a higher IgG levels compared to Odumase (Figure 4.4A). In the wet season, spatial variation was observed between the study communities for PSOP24-374, PSP24-377 and CSP with the population at Odumase having higher median IgG levels compared to Ayigbekorpe. IgG levels against gSG6-P1 show spatial variation between the study communities with the population at Ayigbekorpe having higher IgG median levels compared to Odumase (Figure 4.5B)

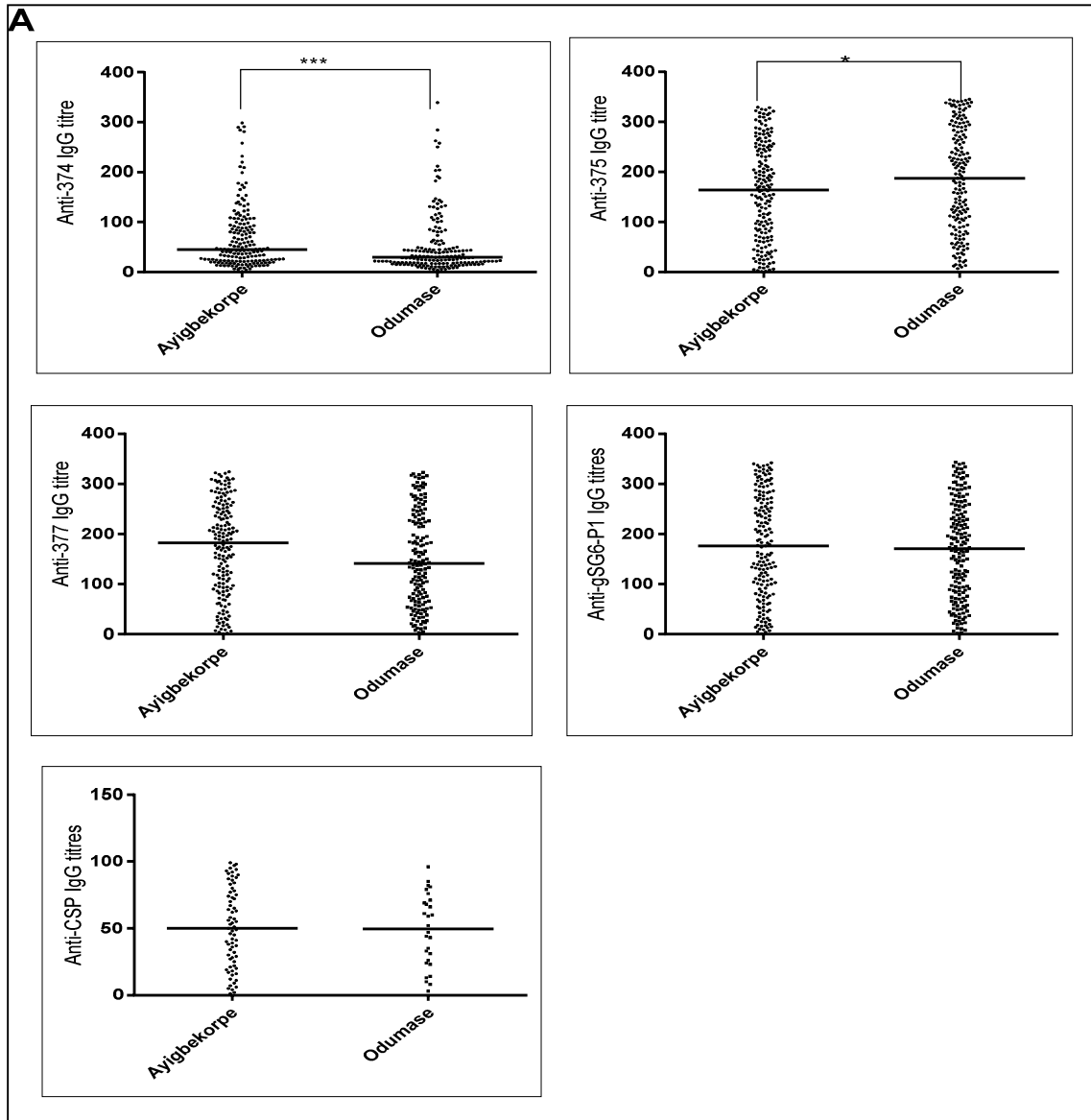


Figure 4.3: Spatial variation in IgG responses to the various antigens between the study communities during the dry season.

Antigen specific median titres from the two communities were compared using Mann-Whitney U-test. Peptide antigens PSOP24-374, PSOP24-375 and PSOP24-377 showed significant differences in malaria transmission between the study communities with the population at Ayigbekorpe having high transmission during the dry season. Scatter plot represents the distribution of the titres. Asterisks (*) indicate the statistical significant (p value < 0.05) and (***) indicate the statistical significant (p value < 0.001) in median between the two communities

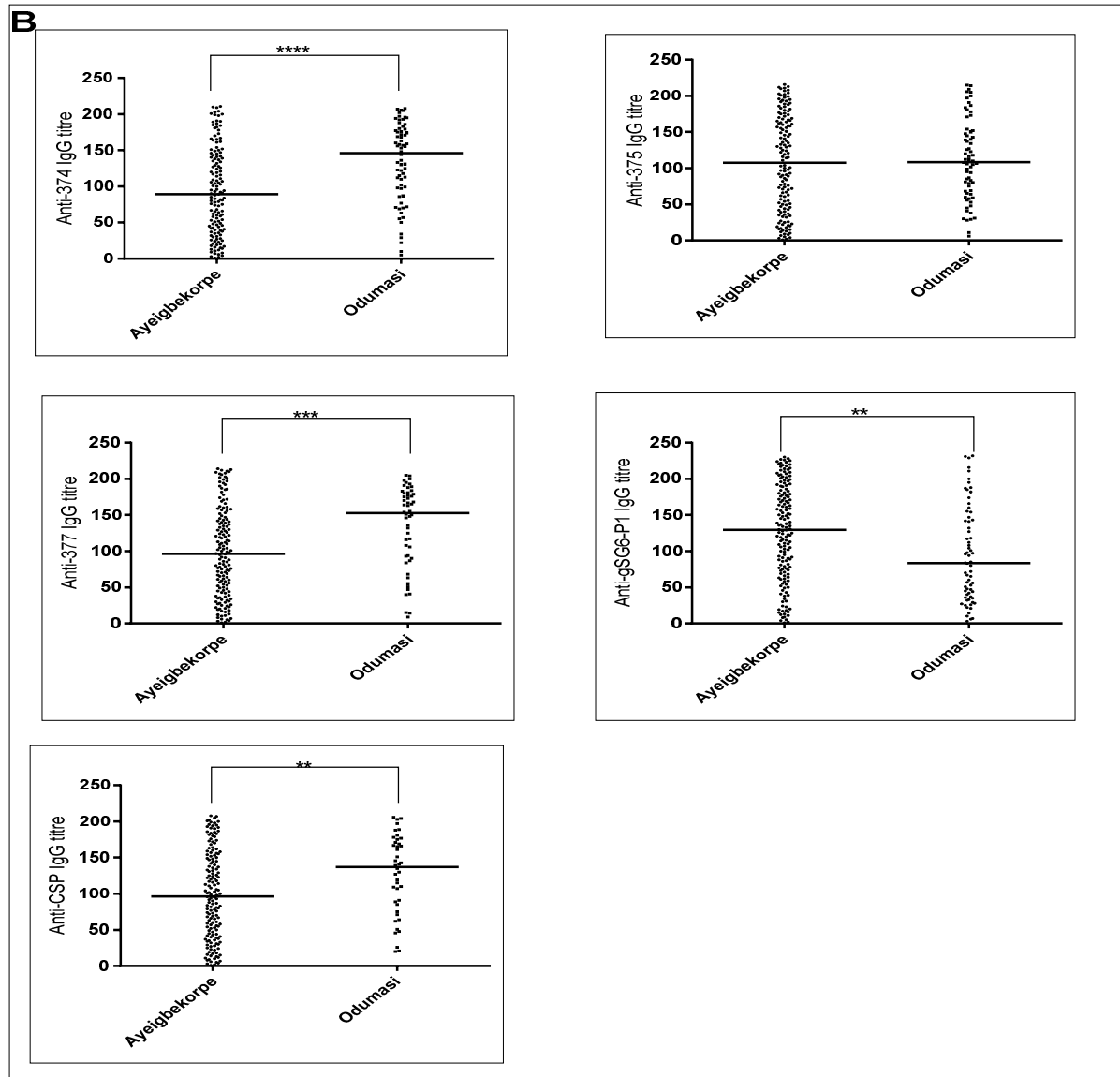


Figure 4.3: Spatial variation in IgG responses to the various antigens between the study communities during the wet season.

Antigen specific median titres from the two communities were compared using Mann-Whitney U-test. Peptide antigens PSOP24-377, gSG6-P1 and CSP antigen showed variation in malaria transmission between the study communities in the wet season. Scatter plot represents the distribution of the titres. Asterisks (*) indicate the statistical significant (p value < 0.05) in median between the two communities

4.7 Parasite prevalence by PCR and multiplicity of infection estimation

Parasite prevalence by PCR in a subset of participants (250) whose blood pellets were available was performed. PCR success was generally low. Parasite prevalence by PCR revealed a 2-2.2 fold decrease in Ayigbekorpe as compared to Odumase during the dry season. However, this trend changed during the wet season as Ayigbekorpe showed a 2 fold increase in parasite prevalence compared to Odumase (**Table 4.4**). Also, a comparative study of PCR success compared to microscopy during the dry and wet seasons showed high submicroscopic parasite prevalence of 44.55% during the dry season and this increased to 62.8% in the wet season **Table 4.5**.

Table 4.4: Submicroscopic parasite prevalence in study sites

Seasons	Communities	
	Ayigbekorpe	Odumase
Dry	18 (16.4%)	40 (36.4%)
wet	65 (46.4%)	34 (24.3%)

Table 4.5: Comparison of microscopy and PCR results of *Plasmodium falciparum* between sample collection times.

seasons	Microscopy	PCR		Total
		Negative	Positive	
Dry	Negative	49 (32.67%)	49 (44.55%)	98
	Positive	4 (3.64%)	8 (7.27%)	12
	Total	53	57	110
Wet	Microscopy	Negative	Positive	
	Negative	38 (27.14%)	88 (62.86%)	126
	Positive	3 (2.14%)	11 (7.86%)	14
	Total	41	99	140

4.8 Multiplicity of infection and IgG levels

Multiplicity of infection (MOI) is the concurrent infection an individual has at any time point. The overall mean MOI of the genotypes per infection between the study sites were 1.26 and 1.19 ($p=0.3$) respectively. When samples were categorized into age groups between the dry and wet seasons, there was no significant differences between the age groups and between the sample collection times although a marginal increase was observed with between the seasons. Figure 4.4 shows a gel electrophoresis picture with DNA bands indicating whether a sample is harboring a single or multiple clones of the IC3D7 parasite strain

Table 4.6: Diversity of *Plasmodium falciparum* IC3D7 clone alleles assessed by MSP 2 marker

Number of PCR fragments	Communities		P value
	Ayigbekorpe	Odumase	
1	95	61	
2	19	16	
3	0	1	
MOI	1.26	1.19	0.3

P value obtained following comparison between study communities by student t-test

Table 4.7: Age stratified comparison of MOI between seasons

Age	Seasons		P value
	dry	Wet	
<5	1.182	1.444	
5-14	1.153	1.16	
>14	1.229	1.214	
MOI	1.18	1.21	0.66
*P value	0.8	0.036	

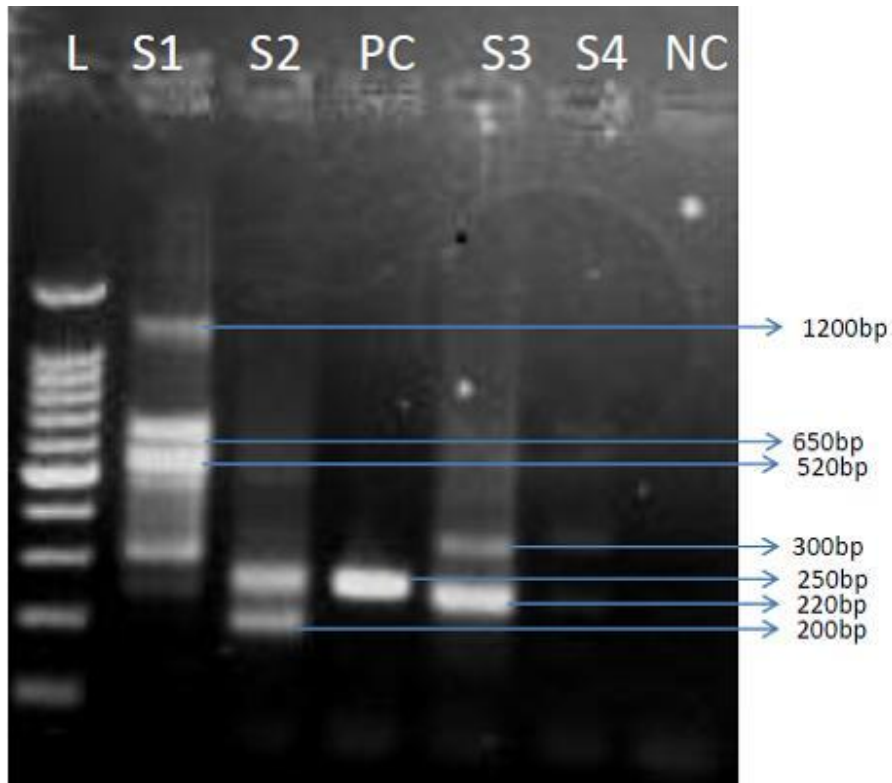


Figure 4.4: Agarose gel photograph with 100bp molecular weight DNA marker indicating multiple infections:

- L 100bp molecular weight marker
- NC Negative control
- S1, S2 & S3 Sample with multiple infections
- PC Positive control

CHAPTER FIVE

DISCUSSION

As malaria prevalence continues to reduce in Africa (O'Meara *et al.*, 2010; World Health Organization, 2013b), heterogeneity in transmission intensity and disease burden will become more pronounced and thus more sensitive tools would be required to identify higher risk micro-geographic areas (hotspot) to enable targeted interventions. The assessment of transmission intensity, the determination of seasonal and spatial variations of transmission and the evaluation of control strategies are of utmost importance for the fight against malaria (Badu *et al.*, 2012; Bousema *et al.*, 2012; Griffin *et al.*, 2010; Oesterholt *et al.*, 2006). Both antibody responses to antigens expressed at different stages of the parasite life cycle and proteins in the mosquito saliva cocktail such as CSP, MSP1, AMA 1, gSG6 and its derived peptide gSG6-P1 continue to be an important transmission monitoring tool (Badu *et al.*, 2012; Bousema *et al.*, 2010; Kusi *et al.*, 2014; Oesterholt *et al.*, 2006; Poinsignon *et al.*, 2008; Remoue *et al.*, 2006; Rizzo *et al.*, 2011). The use of Circumsporozoite protein (CSP) and gSG6-P1 appear as the most promising biomarkers among these for assessing transmission intensity. A number of studies on CSP have shown seasonal dynamics in transmission and also evaluated protective efficacy in transiently exposed travelers to endemic countries (Druilhe *et al.*, 1986; Esposito *et al.*, 1988; Webster *et al.*, 1992), while gSG6-P1 has been shown to be robust in tracking seasonal variation in vector exposure and disease transmission (Badu *et al.*, 2012, 2015; Sagna *et al.*, 2013). However, the fact that not all vector exposure ends up with parasite transmission makes gSG6-P1 marker less useful while the use of CSP in the pre-erythrocytic vaccine RTS,S poses a challenge should it succeed as a malaria vaccine.

In the search for alternative markers, Baum *et al.*, (2013) screened over 800 *P. falciparum* proteins and out of these 15 of them were identified as robust markers of transmission. Of the proteins studied, only the putative, Secreted Ookinete Protein 24 (PSOP24) is expressed in both the ookinete and sporozoite stages of the parasite. Hence the current study sought to further evaluate and possibly validate PSOP24 as an infectious bite marker of malaria transmission. To simplify the experimental set up and make the assay reproducible, the study used synthetic peptides designed to cover the most immunogenic epitopes of the PSOP24 protein. Total IgG antibody response to the peptides was then evaluated as a potential sero-epidemiological marker of exposure to *Plasmodium falciparum* and to assess transmission intensity in the study areas.

Several peptides of PSOP24 were predicted and the most consistent epitopes were generated from three bioinformatics models (BcePred, BepiPred and AbcPred) of which seven were selected (Table 3.1) and tested against 40 randomly selected samples from children less than 14 years of age. The total IgG responses to all the peptides were high with PSOP24-372 showing the strongest immunogenicity. However, there was no significant difference in IgG responses to PSOP24-372, PSOP24-373, PSOP24-376 and PSOP24-378 between seasons which may probably be due to the persistent nature of antibodies to these peptides making their levels stable over seasonal fluctuations in parasite exposure. However, IgG responses to PSOP24-374, PSOP24-375 and PSOP24-377 showed seasonal differences, a property considered as a good marker. This observation was in agreement to previous observations of IgG responses to gSG6, gSG6-P1, MSP1, CSP and AMA1 (Badu *et al.*, 2012; Bousema *et al.*, 2010; Corran *et al.*, 2007; Drakeley *et al.*, 2005; Kusi *et al.*, 2014), thus were selected for further analysis.

In this study, IgG responses to the selected peptides; PSOP24-374, PSOP24-375 and PSOP24-377, CSP and also the vector-antigen gSG6-P1 showed significant differences between the

transmission seasons ($p < 0.05$). However, responses to the peptide PSOP24-377 and gSG6-P1 correlated better with *P. falciparum* exposure which also correlated with increased *An. gambiae* prevalence since IgG levels against these antigens were higher in the wet season than in the dry season (Figure 4.2). It is not clear why IgG levels against PSOP24-374 and PSOP24-375 were higher in the dry season than in the wet season, however it may be due to differences in the profile of IgG subclasses against these peptides compared to PSOP24-377 and gSG6-P1 since subclasses may influence the overall IgG decay (Theisen *et al.*, 1998). In the dry season it is typically expected that malaria transmission will decrease and the consequent lack of exposure to *P. falciparum* should cause the IgG response to wane with time. However, since malaria antigens have been shown to be biased towards different IgG subclasses (Høgh *et al.*, 1993; Maya *et al.*, 2006; Oeuvray *et al.*, 2000; Olesen *et al.*, 2010; Theisen *et al.*, 2001; Theisen *et al.*, 1998), antigens that elicit responses dominated by subclasses with longer half-life may linger even in the absence of more recent exposure and this could explain our findings.

Interestingly, the comparisons of the four tested antigens (peptide PSOP24-374, PSOP24-375, PSOP24-377 and gSG6-P1), the IgG levels were significantly different in the young age groups (<5 and the 5-14 years) between the dry and wet seasons while antibody levels in the older age group (>14) did not change significantly ($p > 0.05$). On the other hand, IgG responses to CSP showed no differences in the young groups (<5 and 5-14 years) but significant differences in IgG levels was observed in the older age cohort. This observation may possibly be due to the fact that less than 10% of the study participants were exposed to the parasite, that is, the prevalence was less than 10% with about 20% submicroscopic infections. Therefore, antibody boosting may possibly be minimal and variation in antibody levels will most likely be seen in the younger age groups with limited pre-existing immunity to the disease in contrast to the adult group who were

expected to have developed semi-immunity following repeated parasite exposure (Baird, 1995; Doolan *et al.* 2009). The observed variation of responses to the gSG6-P1 may possibly be due to tolerance of the immune system to the antigen in adults, a finding supported by studies done by Rizzo *et al.*, (2011) who suggested the possibility of desensitization of the recombinant form of gSG6 with increasing age.

Also, CSP showed seasonal variation with age but this age variations were only significant in the older age group (>14 yrs). This may probably be due to the fact that adults participate in extensive outdoor activities that might increase their risk of exposure.

Malaria incidence have shown considerable variation between communities and even between households with relative short distances and this is now becoming more pronounced as transmission declines and focal hotspots get established (Badu *et al.*, 2012; Mboera *et al.*, 2010; Oosterholt *et al.*, 2006). This has been observed in low transmission areas where clustering of mosquitoes have been seen in certain parts of the community, thus relating to high risk of exposure to few people in the community. In this study, IgG response to PSOP24-374 was observed to be significantly higher in Ayigbekorpe compared to Odumase ($p = 0.0003$). However, IgG responses to PSOP24-375 were significantly higher in Odumase compared to Ayigbekorpe ($p = 0.033$) with no differences observed between the two study communities when assessed by anti-PSOP24-377 during the dry season. The observation with anti-PSOP24-377 was similar to IgG responses to CSP and gSG6-P1 between the study communities. The observed differences between the study communities when assessed against anti-PSOP24-377, anti-CSP and anti-gSG6-P1 may probably be due to the low transmission during that season. This was not surprising because it is consistent with a previous study by Badu *et al.* (unpublished) in the same communities which showed similar EIR estimates from the two communities although a high

mosquito catch was obtained from Odumase compared to Ayigbekorpe. A finding which show a low infectious mosquitoes in both communities.

In the wet season, high IgG responses to peptides PSOP24-374, PSOP24-377 and CSP was observed in Odumase compared to Ayigbekorpe, although no significant difference was observed against anti PSOP24-375 between the study communities ($p = 0.65$). On the other hand, a high IgG response was observed in Ayigbekorpe compared to Odumase. These observations with anti-PSOP24-374, PSOP24-377 and CSP may possibly be due to increase in exposure to infectious bites since these antibodies represent a footprint of parasite exposure (Bousema *et al.*, 2010). However, demographic data from Odumase shows about 90% of the study participants sleep under bed nets. Therefore the observed data may suggest that either the bed nets are not used appropriately or there is a change in mosquito behavior in Odumase. A possibility that has been shown elsewhere (Moiroux *et al.*, 2012; Russell *et al.*, 2011).

However, data on gSG6-P1 as exposure marker, showed a high risk of transmission in Ayigbekorpe compared to Odumase, which is typically expected as a result of the high vector density that accompany the rain and consequently a high vector exposure in that community. Therefore, the observed differences between the communities to anti-gSG6-P1 may possibly be due to a high mosquito exposure and possibly the low bed net usage among the study participants in Ayigbekorpe.

Examination of blood films by microscopy is the main diagnostic tool for detecting malaria infection. However, its sensitivity is restricted as submicroscopic infections, which contribute to the infection reservoir, are most likely to be missed. Therefore, quantifying these submicroscopic infections has become crucial to understanding transmission dynamics and helping in the successful reduction in parasite transmission (Jelinek *et al.*, 1996; Ntoumi *et al.*, 1995; Okell *et*

al., 2009). Generally, parasite prevalence by PCR was consistently higher in both Ayigbekorpe and Odumase compared to microscopy, although this did not correlate with antibody responses. This observation is consistent with findings from several studies showing the sensitivity of molecular techniques (PCR) over microscopy (Coleman *et al.*, 2006; Dal-Bianco *et al.*, 2007; Häscheid & Grobusch, 2002). However, data from this study show similar trends for both microscopy and PCR with prevalence being higher in Odumase during the dry season but higher in Ayigbekorpe during the wet season (Table 4.4). This may possibly be due to bed net usage between the study communities; as bed net usage in Odumase reduced significantly in the dry season and peaked in the wet season. On the other hand, low bed net usage was observed during both seasons in Ayigbekorpe corresponding to the high parasite prevalence in the community during the wet season.

Another interesting finding from this study is the low multiplicity of infection (MOI) found between the study communities and between the seasons with a single clone of IC3D7 being predominant, which confirms finding from other studies performed in low transmission settings (Babiker *et al.*, 1997; Ghanchi *et al.*, 2010; Haddad *et al.*, 1999). These two communities can therefore be considered low transmission areas with a limited genetic diversity as well could be attributed to the numerous interventions rolled out in the district from the 1990s (Agyepong & Manderson, 1999; Ansah *et al.*, , 2001).

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Using antibody levels to PSOP24 peptides (PSOP24-374, PSOP24-375 and PSOP24-377), CSP and gSG6-P1, this study was able to show high antibody responses to PSOP24 peptides, particularly, PSOP24-377 that correspond to the subtle difference in transmissions in the study communities and during the two transmission seasons which was corroborated by gSG6-P1 pattern. As CSP, a constituent of RTS,S awaits its approval as a vaccine, validation of PSOP24-377 could be an additional marker in assessing transmission and for evaluating control measures. No spatial and temporal variations were observed with parasite genotyping due to the low MOI observed between the study communities ($MOI \leq 1.2$) with majority of the study participants harboring single clones indicating low transmission in these two communities.

6.2 Recommendation

1. Further studies should be conducted to validate PSOP24-377 in different transmission settings as a possible marker of transmission assessment.
2. *In-vitro* and *in-vivo* functional studies should be conducted to elucidate the main function of PSOP24.
3. Future studies with PSOP24-377 should include more children under 14 years to see the true picture of PSOP24-377 with age.
4. Genotyping was limited to the use of a single genetic marker (3D7 strain-specific primers) and that future studies must include primers that can be used to pick other strains of the parasite in order to determine the actual circulating parasite clones and how they influence antibody responses.

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APPENDICES

Appendix I: ELISA buffer preparations

1. Preparation of standard solutions and buffers.

Unless otherwise stated, all standard solutions for ELISAs were prepared with double distilled water (dd H₂O), while water in PCR solutions were obtained commercially.

2. Blocking buffer

To prepare a 500ml, 2ml of 5N NaOH was added to 125ml of deionized water and set on a hot plate C for 20 minutes at 300⁰C-400⁰ and stirring at 300rpm. Within the first 5 minutes, 5.00g±0.01g of casein was added to the above solution. 100ml of 10x PBS was added to the boiled casein and topped up to 1L with deionized water to create a solution of 0.5% boiled casein solution. The boiled casein solution was allowed to cool on the bench and 0.02g±0.01g of phenol red was added while still stirring the solution. The pH of the 0.5% casein solution was adjusted with either 10% HCl or 5N NaOH to 7.4±0.02. Using a serological pipette, 5ml of Tween 20 was added to 495ml of the 0.5% casein solution while stirring with a magnetic stirrer.

3. Sample and conjugate dilution buffers

To prepare 500ml of sample diluent, 12.5ml of the blocking buffer was added to 487.5ml of 0.5% boiled casein and mixed by inverting it to obtain homogeneity.

4. Washing Buffer

5L washing buffer was prepared by adding 10 tablets of PBS to a flask containing 5L deionised water and stirred until all is in solution. 5.0ml of Tween-20 was then added while still stirring.

5. 0.2M H₂SO₄ Stop Solution

500.0ml of stop solution, 10.0ml of 10.0M H₂SO₄ was added to 490.0ml of deionised water and the solution shaken to mix. It was then cooled to room temperature and kept in the hood until required.

Appendix II: PCR primer reconstitution

lyophilized primers used in PCR reaction were reconstituted with commercially acquired nuclease free water (DEPC Treated Water, Invitrogen, CA, USA) as instructed by the manufacturer (Eurofins MWG Operon). The reconstituted primers were diluted to a working concentration of 10 μ M using commercially obtained water.