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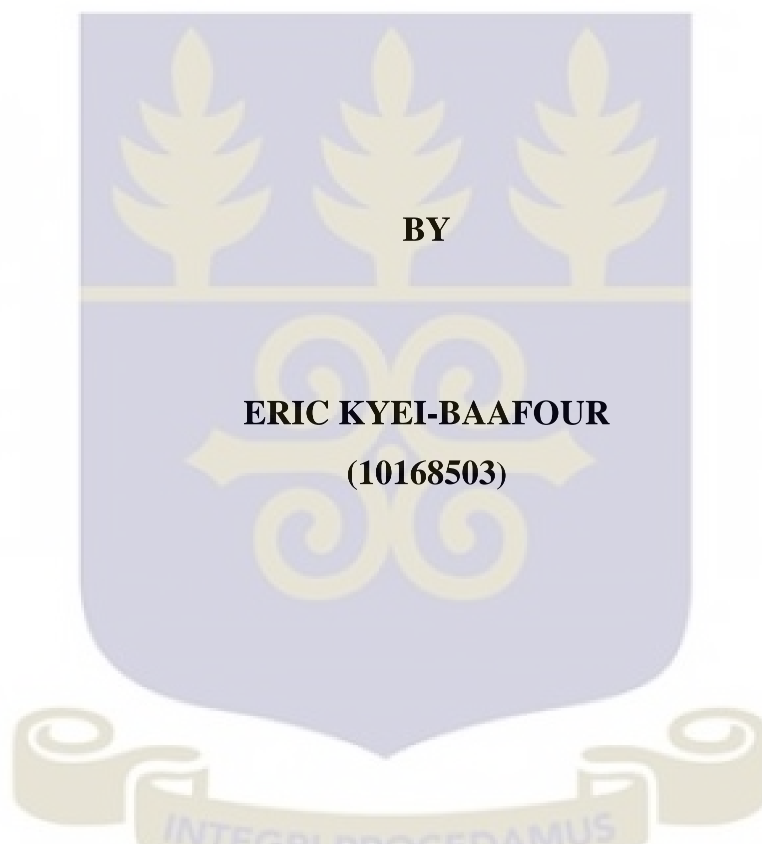
EFFECT OF PARASITE DIVERSITY ON THE LEVELS AND QUALITY OF ANTIBODY RESPONSES TO *PLASMODIUM FALCIPARUM* IN AN AREA OF SEASONAL MALARIA TRANSMISSION



BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULLFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY (MPHIL) DEGREE IN ZOOLOGY

JULY, 2015

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DECLARATION

I Kyei-Baafour Eric, 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, and at the Department of Animal Biology and Conservation Science, supervised by Prof. Ben A Gyan, and Dr. Kwadwo Asamoah Kusi both of Immunology Department, Noguchi Memorial Institute for Medical Research, and Dr. Langbong Bimi of the Department of Animal Biology and Conservation Science, University of Ghana. Neither all nor parts of this thesis have been presented for another degree elsewhere.

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DEDICATION

I dedicated this work to The Lord God Almighty. I also dedicate it to the three wonderful women in my life, my wife Sandra, my mother Alberta, and my daughter Elizabeth.

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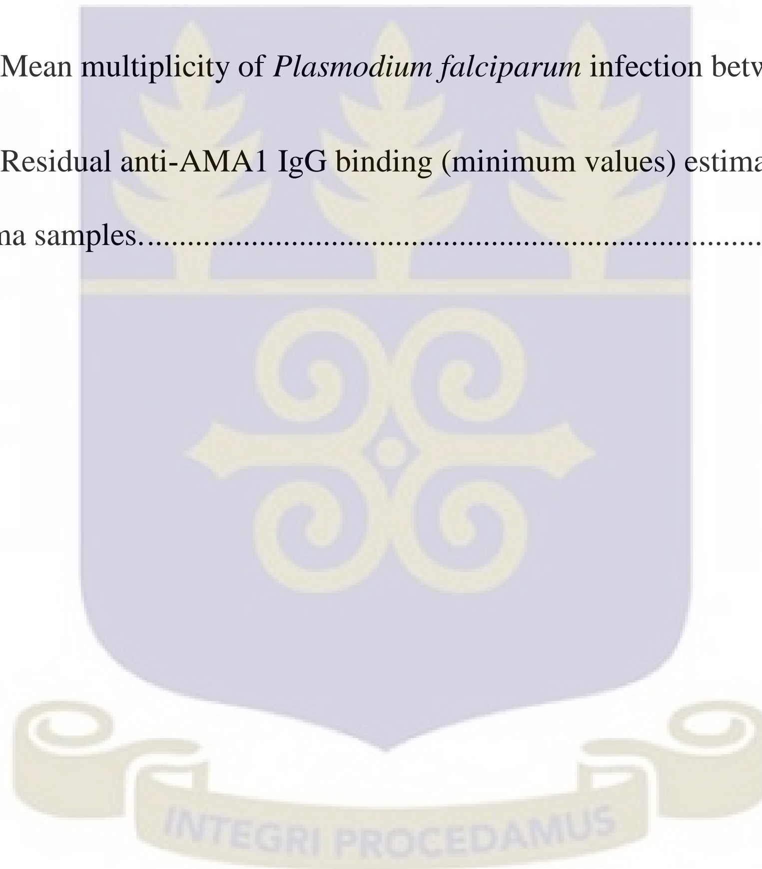

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List of Abbreviations

ADCI	Antibody Dependent Cellular Inhibition
AMA1	Apical Membrane antigen-1
APC	Antigen Presenting Cells
AU	Antibody units
BC	Before Christ
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CelTOS	Cell Traversal Protein for Ookinetes and Sporozoites
CSA	Chondroitin Sulphate A
CSP	Circumsporozoite Protein
DDT	Dichlorodiphenyltrichloroethane
DiCo	Diversity Covering
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleotide triphosphate
EBA	Erythrocyte Binding Antigen
EGF	Epidermal Growth Factor
G-6PD	Glucose-6-Phosphate Dehydrogenase
GDP	Gross Domestic Product
GHS	Ghana Health Service
GMEP	Global Malaria Eradication Program
GPI	Glycosylphosphatidyl inositols
HbS	Haemoglobin S
HBs	Hepatitis B surface antigen
HLA	Human Leukocyte Antigen
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E

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IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IPTp	Intermittent Preventive Therapy for pregnancy
IRB	Institutional Review Board
iRBC	infected Red Blood Cell
IRS	Indoor Residual Spraying
kDa	kilo Dalton
LLIN	Long Lasting Insecticidal Nets
LSA	Liver Stage Antigen
mAB	monoclonal antibodies
MDA	Mass Drug Administration
MHC	Major Histocompatibility complex
MOI	Multiplicity of Infection
MSP	Merozoite Surface Protein
NHRC	Navorongo Health Research Center
NK cells	Natural Killer cells
nPCR	Nested Polymerase Chain Reaction
OD	Optical Density
PAM	Pregnancy Associated Malaria
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
*Pf*EMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
RBC	Red Blood Cell
RBM	Roll Back Malaria
RDT	Rapid Diagnostic Test
RIFINs	Repetitive Interspersed Family Protein
STC	Scientific and Technical Committee
STEVOR	Subtelomic Variable Open Reading frame

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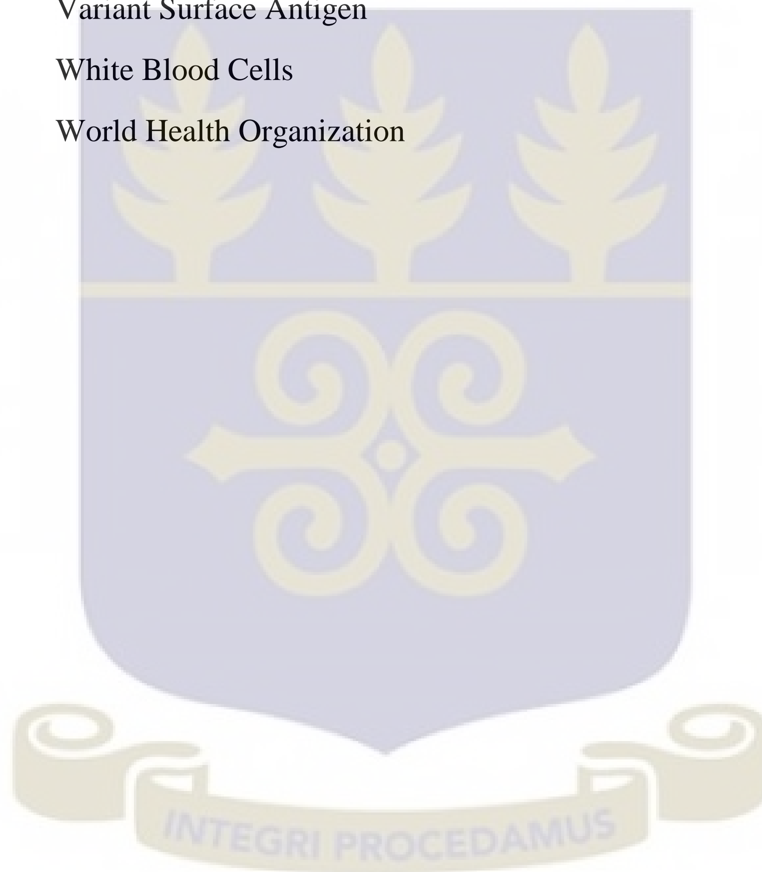
SURFIN	Surface-associated interspersed protein
Tc cells	T-cytotoxic cells
TGF-β	Transforming Growth Factor beta
Th cells	T-helper cells
TNF-α	Tumor Necrosis Factor
Tregs	T-regulatory cells
UV	Ultra violet
VSA	Variant Surface Antigen
VSA	Variant Surface Antigen
WBC	White Blood Cells
WHO	World Health Organization



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ABSTRACT

The important role of antibody-mediated mechanisms in protection from clinical malaria has been demonstrated by passive transfer experiments but the targets of protective immunity are not clearly defined. A number of antigens are however in various stages of testing as possible vaccine candidates. Polymorphism in these antigens, which has been reported to be an immune evasion mechanism, has hampered the development of these antigens as vaccines since antibody responses against one allelic form of an antigen have been shown to be less effective against parasites that express a different allele of the same antigen. In animal studies, immunization with a mixture of allelic antigens induced cross-reactive antibodies that had greater and broader *in vitro* inhibition capacity compared to antibodies induced against the respective single antigens.

This study therefore sought to determine the effect of parasite diversity on the levels and quality of antibody responses to *P. falciparum* in individuals living in an area of seasonal malaria transmission. Indirect ELISA was used to determine total IgG responses to AMA1-3D7, AMA1-FVO, $MSP1_{19}$, CSP, and CelTOS in stored plasma samples taken at two sites, one close to a dam and the other at least 20km away from the dam during the wet and dry season. Competition ELISA was used to determine the relative proportions of cross-reactive and stain-specific anti-AMA1 antibodies. Malaria parasites were detected in participant samples by both microscopy and molecular methods.

The study found greater proportion of parasitaemic individuals at the dam site compared to those away from the dam during the dry season (*p=0.0061*), while proportions were similar in the rainy season. Generally, there were more multiple

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infections per individual, described as the multiplicity of infection (MOI) in the wet season (60% of participants) compared to the dry season (40.3%, *p=0.001*). A similar trend was observed when MOI was compared between seasons for the non-dam site (*p=0.001*), but MOI was similar between the wet and dry seasons at the dam site Antibody levels to sporozoites antigens (CSP and CelTOS) were higher at the dam site compared to the non-dam site, irrespective of the season. No differences between sites were however observed for the blood stage antigens (AMA1 and $MSP1_{19}$). Antibody specificities to multiple AMA1 alleles were observed at sites with MOI greater than 1 and specificity to only the 3D7 allele was observed at sites with single infections. This data generally shows high levels of clinical immunity that is observed in high transmission areas may be associated more with infection by multiple parasite strains (hence a wider breadth of antibody responses) rather than high parasite burden. Consequently low levels of clinical immunity in low transmission areas may be the result of infection with one or a few parasite strains that may induce responses that is not as broad as is seen in high transmission areas.

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1 CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is an infectious disease caused by a unicellular Haemosporozoan parasite of the genus *Plasmodium*, of which four species, namely *P. vivax, P. malariae, P. ovale and P. falciparum* cause disease in humans. *Plasmodium knowlesi* and *P. cynomolgi* which are known simian malaria parasites have recently been shown to also cause life-threatening human malaria (Cox-Singh *et al.*, 2008, Luchavez *et al.*, 2008, Ta *et al.*, 2014). These parasites are transmitted to humans through the infective bite of a female *Anopheles* mosquito, which serves as the vector.

The most severe forms of the disease such as cerebral malaria and severe malarial anaemia are however mainly associated with *P. falciparum* infection (Suh *et al.*, 2004). High-risk groups include individuals with less developed immune systems, children under 5 years, and un-exposed travellers to malaria-endemic regions, pregnant women, especially during their first and second pregnancies, and those in endemic areas but are rarely exposed to infection(Miller *et al.*, 1994). Infections with *Plasmodium falciparum* can cause a wide spectrum of illness ranging from apparently symptomless infections to severe forms and ultimately death. Some form of non-sterile clinical immunity is developed but only after repeated exposure to the parasite (Bull *et al.*, 2002).

About half of the world's population is at risk of infection; with almost 198 million clinical cases, and about 584,000 deaths recorded worldwide in 2013 with *P. falciparum* infection accounting for about 91% of cases in sub-Saharan Africa (World Health Organization., 2014). Malaria is also a major cause of illness and death,

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particularly among children under 5 years of age, and primigravid pregnant women in Ghana accounting for about 34% of all outpatient illnesses, in 2010 (Ghana Health Service., 2010), with *P. falciparum* being the most prevalent parasite in Ghana. (Asante *et al.*, 2011b, Ahmed, 1989).

Current strategies to control malaria involve preventive measures that target the vector by the use of insecticides to spray breeding grounds, insecticide treated bed nets, and chemotherapeutic approaches where drugs are used to target the parasites. However the spread of drug-resistant parasite strains and insecticide-resistant mosquitoes have greatly hampered control efforts (Yadouleton *et al.*, 2010, Marfurt *et al.*, 2010), and a recent report linking climate change to the increased incidence of malaria in areas previously known to be malaria-free, such as higher altitudes, has also compounded the problem(Siraj *et al.*, 2014). Vaccines are the most appropriate and cost effective means of controlling and eventually eliminating malaria as this has been used to effectively control or eliminate other diseases such as polio (Good, 2001), but this has not been successful due to a host of factors including: complex host-parasite interactions, polymorphism and antigenic variation, HLA restrictions, and the lack of suitable and potent adjuvants that would induce high-titre antibody responses(Good, 2001).

In disease-endemic areas, immunity to malaria develops in a slow manner, with children under 5 years being very susceptible to clinical disease while adults beyond age 15 become semi-immune to malaria following repeated infection with the parasite. Immunity to malaria is mediated by components of both the innate and adaptive immune responses. The adaptive immune response is mediated by both cellular and

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antibody-mediated mechanisms and the crucial role of antibodies was demonstrated in passive transfer experiments that involved treating acutely sick individuals with immunoglobulins that had been purified from semi-immune adults (Cohen *et al.*, 1961, McGregor, 1963, Sabchareon *et al.*, 1991). Antibodies perform such roles as inhibition of red blood cell (RBC) invasion by merozoites, blocking cytoadherence of infected RBCs (iRBCs) to vascular endothelial cells, and enhancing phagocytic activity of monocytes and macrophages (Brown *et al.*, 1982, Bouharoun-Tayoun *et al.*, 1990, Wipasa *et al.*, 2002a, Beeson *et al.*, 2008). These antibodies are induced in response to parasite antigens, a number of which have been identified and are thought to be important targets of immunity. Some of these antigens include: Circumsporozoite surface protein (CSP), the Liver stage antigens (LSA), the Apical membrane antigen-1 (AMA1), the Merozoite surface proteins; MSP1, MSP2, MSP3, MSP4, MSP8, *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), Ring-infected erythrocyte surface antigen (RESA), Variant surface antigen (VSA), Erythrocyte binding antigen (EBA), and *Plasmodium sp* gametocyte surface antigens such as *Pf*g27, *Pf*s16, *Pf*s25 and *Pf*s230.

An understanding of the mechanisms of acquisition of antibodies to these varied parasite antigens is essential for the development of interventions such as vaccines. While some field studies have found associations between antibodies to single antigens, or combinations of these antigens, with protection from clinical malaria (Riley *et al.*, 1994, Dodoo *et al.*, 1999, Cavanagh *et al.*, 2004, Polley *et al.*, 2006, Dodoo *et al.*, 2008), other studies have failed to show these associations (Conway *et al.*, 2000, Perraut *et al.*, 2005). Thus the precise antigenic targets of protective immunity to malaria have not been properly defined and these conflicting findings though may

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reflect differences in design of these studies may also be attributable in part to allelic polymorphisms(Mackintosh *et al.*, 2004).

Antibody responses induced against one allelic form of a particular antigen have been shown to be less effective at limiting the multiplication of parasites that express a different allele of the same antigen (Supargiyono *et al.*, 2013, Perraut *et al.*, 2000). Allelic polymorphism is believed to be an immune evasion mechanism employed by parasites (Hisaeda *et al.*, 2005, Ferreira and Hartl, 2007). Developing a broad based vaccine using the antigenic targets must take into consideration the strains circulating in a particular geographic area. Studies have shown individuals having high allele-specific responses, are mostly under 10 years of age (Cortes *et al.*, 2005b). Another study showed greater antibody cross- reactivity in adults, reflecting a higher level of protective immunity in adults (Terheggen *et al.*, 2014). Thus studying the induction of natural immune responses to these polymorphic antigens in individuals with varying levels of exposure to different parasite strains is necessary for shedding light on the acquisition of naturally acquired immunity to malaria.

1.2 Problem Statement

Individuals in malaria endemic areas have partial immunity to malaria and this immunity is believed to develop rapidly in areas of high transmission but slow in low areas of transmission. Individuals living in low transmission areas are therefore more prone to clinical malaria attacks. Protection from blood stage infection is believed to be mediated mainly by anti-merozoite antibody responses and the functional quality of these responses is believed to be linked with polymorphism in the antigen targets and the frequency of exposure to parasites. It is generally believed that individuals

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accumulate a repertoire of malaria-specific antibodies with age and repeated exposure to different parasite strains over time. It is, however, unclear whether the antibody component of partial clinical immunity in high transmission areas is merely due to high parasite burden or is also dependent on the acquisition of broad antibody immunity to different strains of the parasite.

1.3 Justification:

Allelic polymorphism represents a unique barrier to the development of broad acting subunit malaria vaccine since a potentially effective vaccine would have to induce broad acting immune responses that will be effective against the diversity of parasite strains that are known to circulate within any malaria endemic area. To be able to achieve this, a better understanding of the acquisition of broad clinical immunity especially in areas of high malaria transmission will be required

Currently, obtaining a potent vaccine has been elusive and several factors have been adduced. One important factor is naturally occurring antigenic/allelic polymorphism which has been reported to be an immune evasion mechanism by the parasite (Ferreira and Hartl, 2007). Associating naturally acquired IgG responses to malaria specific-antigens and their alleles with protection from clinical malaria is a very crucial step towards vaccine production. Thus assessing antibody responses to these polymorphic antigens either individually or in combinations in relation to control of malaria infection and parasite multiplication is highly justified. Also, investigating the influence of parasite diversity on the levels of responses in populations with varying transmission intensity.is highly appropriate for vaccine formulators to determine the best strategies for formulating vaccines that can induce broad protective responses.

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1.4 Main Objective:

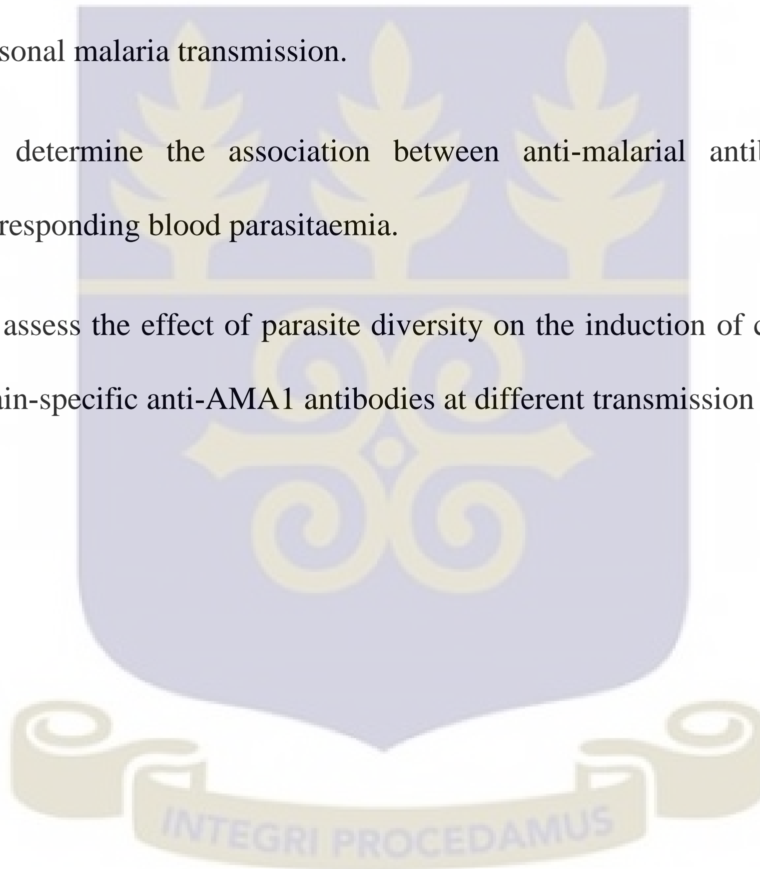
The aim of this study was to investigate the effect of parasite diversity and disease transmission setting on the levels and quality of antibody responses to *P. falciparum* in individuals living in an area of seasonal malaria transmission.

1.4.1 Specific Objectives:

i. To determine and compare specific IgG responses to four malaria antigens (CelTOS, AMA1, CSP and $MSP1_{19}$) in plasma samples collected in an area of seasonal malaria transmission.

ii. To determine the association between anti-malarial antibody levels and corresponding blood parasitaemia.

iii. To assess the effect of parasite diversity on the induction of cross-reactive and strain-specific anti-AMA1 antibodies at different transmission periods.



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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria: The disease

2.1.1 Brief History

The word malaria originated from the Italian word 'mal'aria meaning 'bad air' because of its initial association with the smell from swamps. The disease has been known to humans for over 4000 years with the ancient Chinese describing some of the symptoms around 2700 BC in their writings. The Greek physician Hippocrates also noted most of the symptoms. Malaria devastated and caused decline in populations in many regions of the old world, until the medicinal Peruvian barks were used to treat fevers. The parasites that cause the disease were first discovered by Charles Alphonse Louis Laveran in 1880 (Cox, 2010, Cowman and Duraisingh, 2001).

2.2 The Parasite

Malaria is a life threatening disease caused by a protozoan parasite of the genus *Plasmodium*. It is transmitted through the bite of the female *Anopheles* mosquito and characterized by fever with symptoms such as chills, headaches, malaise, vomiting, fatigue, and joint pains. These symptoms can result in many clinical manifestations ranging from asymptomatic infection through mild disease to severe complications such as cerebral malaria, and severe anaemia. Four *Plasmodium* species were originally known to cause malaria in humans. However it has recently emerged that two known simian parasites also cause the disease in humans, making a total of six *Plasmodium* species to cause human infections. These are *Plasmodium falciparum, ovale, vivax, malariae*, and the two simian parasites *Plasmodium knowlesi* and *cynomolgi*. (Ta *et al.*, 2014, Cox-Singh *et al.*, 2008, Luchavez *et al.*, 2008).

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The most severe forms of the disease such as cerebral malaria and severe malarial anaemia are mainly caused by *P. falciparum* infection. However, the importance of the other species should not be underestimated, particularly *P. vivax*, which is the most widespread of the species in the world. Infection with *P. vivax* and *P. ovale* result in less severe symptoms. Relapses, however, can occur for up to three years, and chronic disease weakens the patient. The relapses occur due to the fact that the dormant liver stages (hypnozoites) of these parasites may reactivate. *Plasmodium malariae* is also widely distributed, but it is not as common as *vivax* malaria. Aside causing typical malaria symptoms during infection, *P. malariae* can also persist in the blood for very long periods, possibly decades, without ever producing symptoms. A person with asymptomatic *P. malariae*, however, can infect others, either through blood transfusion or mosquito bites. Two or more species of *Plasmodium* can infect a single individual at the same time (World Health Organization., 2010, Miller *et al.*, 1994).

2.3 Life Cycle

Plasmodium has a complex life cycle that involves interaction between the parasite, host, and vector. This cycle is made up of the sexual stage, which takes place in the mosquito, and the asexual stage, which occurs initially in the mosquito and continues in humans. Wipasa (Wipasa *et al.*, 2002a), divided the stages in the human host into three categories namely: the pre-erythrocytic, erythrocytic and the gametocytic stages.

The cycle in humans is initiated with the inoculation of sporozoites through the bite of the female *Anopheles* mosquito during feeding. Sporozoites migrate through the blood stream for some minutes before ending in the liver where invasion of liver cells (hepatocytes) is accomplished. Whilst in the liver they undergo asexual multiplication into schizonts.

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The erythrocytic stage begins with the bursting of schizonts out of hepatocytes as merozoites, to invade circulating erythrocytes or RBCs. The merozoites then develop into trophozoites within a vacuole formed by the internal membrane of the host red cell. The trophozoite feeds on haemoglobin by ingesting small amounts of red cell cytoplasm, which leads to formation of malaria pigment (haemozoin) as polymerized by-product of hemoglobin breakdown.

RBC invasion begins by merozoites attaching themselves to specific receptors on the RBC membrane resulting in invagination of the membrane and causing the merozoites to move into the erythrocyte. While in the erythrocyte, the parasite resides in the parasitophorous vacuole and undergoes further development from the trophozoite stage to the schizont stage after many mitotic divisions. Each schizont releases up to 32 merozoites into the blood stream when the erythrocyte ruptures. Merozoites go through this intra-erythrocytic cycle by reinvading more erythrocytes when they escape the action of the immune system. This stage of the intra-erythrocytic cycle consists of asexual division, in which merozoites develop through ring, trophozoite and schizont stages, and gametogenesis where a small proportion of parasites differentiate into male and female gametocytes.

Gametocytes can be taken up by the mosquito during a blood meal and differentiate into male and female gametes in the mid-gut of the mosquito. Then there is exflagellation forming zygote which further transforms into motile forms known as ookinetes. The ookinetes penetrate the epithelium of the gut and transform into oocysts in the epithelial lining. The oocysts contain large numbers of sporozoites and at maturity the sporozoites burst out and migrate to the salivary glands of the vector and can be transmitted to humans when the mosquito takes another blood meal. There are

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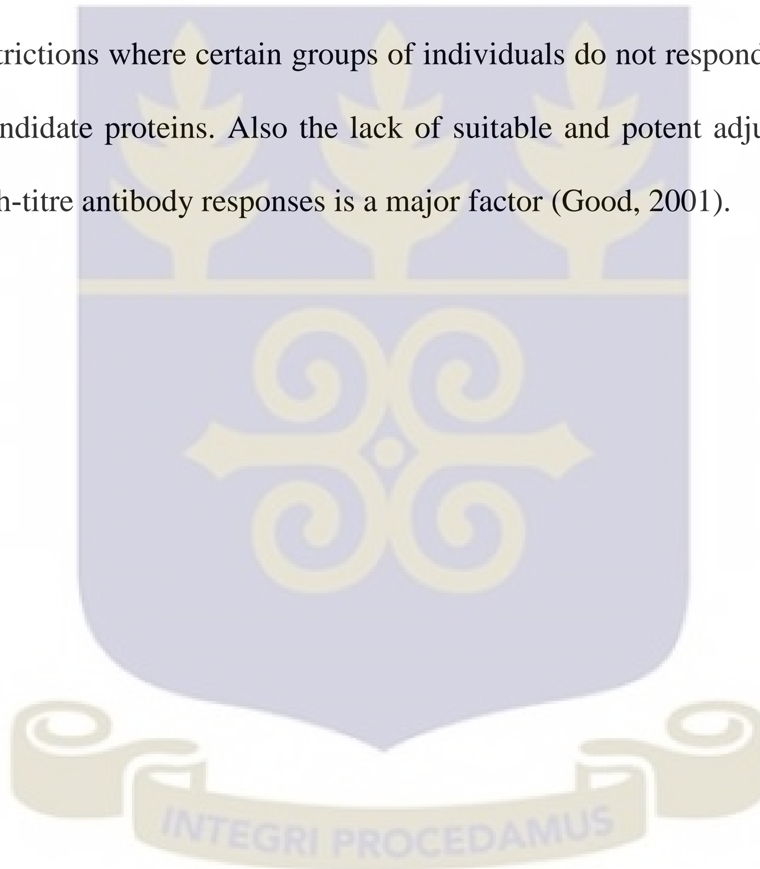
antigens produced by the parasite at every stage of the life cycle which have been identified as potential vaccine candidates.

Though much progress has been made in the recent past to develop vaccines based on these identified candidates, for example the RTS'S vaccine, technical and immune-related difficulties have hindered vaccine development. Some of these difficulties include the cost of producing such vaccines, the complexity of the host parasite relationship, polymorphism and antigenic variation, and Human Leukocyte Antigen (HLA) restrictions where certain groups of individuals do not respond to some of these vaccine candidate proteins. Also the lack of suitable and potent adjuvants that would induce high-titre antibody responses is a major factor (Good, 2001).



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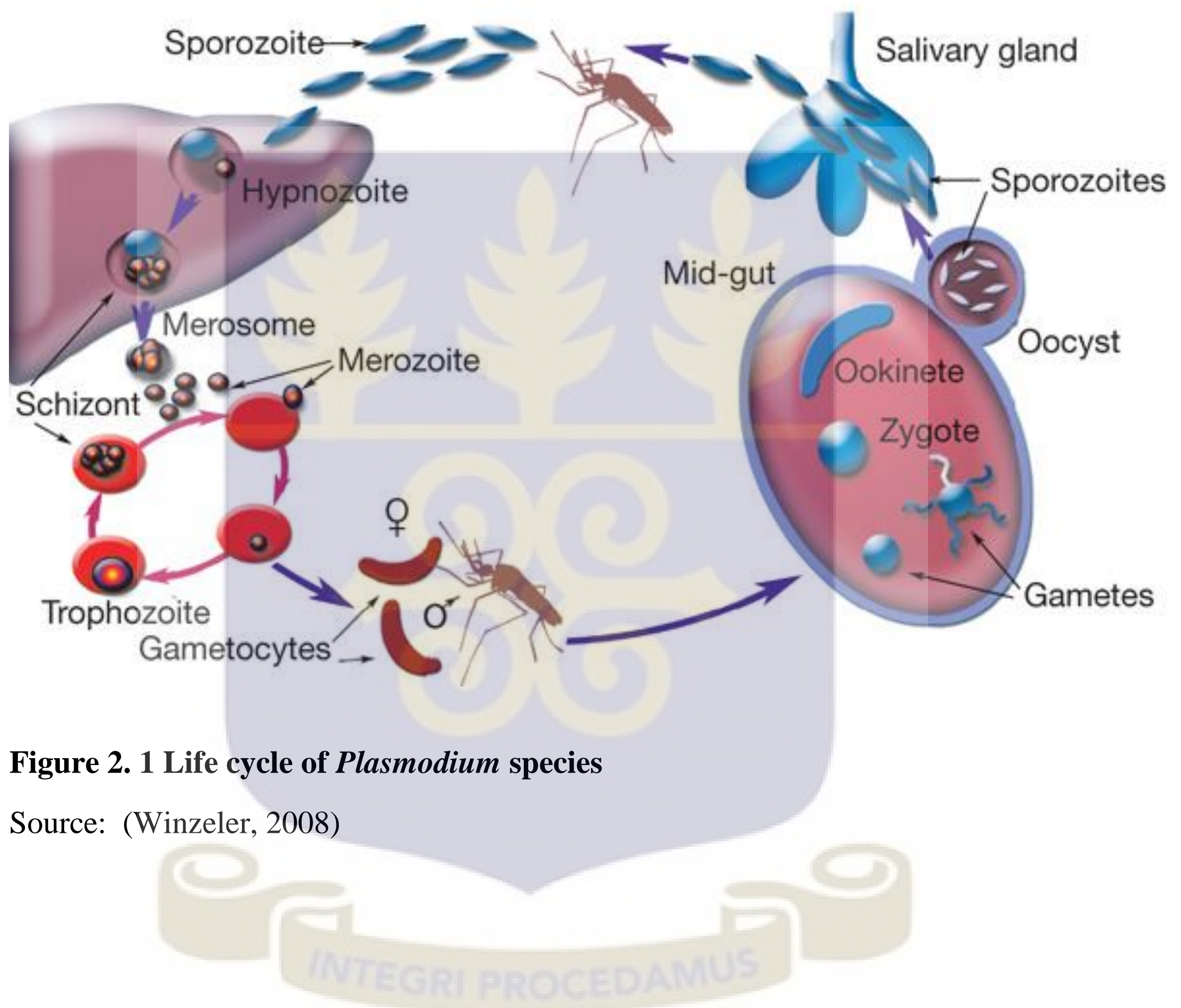

Figure 2. 1 Life cycle of *Plasmodium* species

Source: (Winzeler, 2008)

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2.4 Malaria Situation

2.4.1 Global Malaria Situation

Great efforts have been made over the last 60 years to combat the effect of malaria on human lives. According to a report published by the WHO (1956) malaria was controlled in the 1950s using the most potent tools available then: chloroquine, and dichlorodiphenyltrichloroethane (DDT) with considerable success. By the late 1970s malaria burden had considerably reduced (Wernsdorfer and Kouznetsov, 1980).

However vector and parasite resistance to DDT and chloroquine, respectively, and non-commitment to fight the disease by countries in malarious areas led to a resurgence of the disease until the Global malaria control strategy ministerial conference was held in 1992 (Trape *et al.*, 1998).

Over the past decade, estimated malaria death rates have fallen by 42% in all age groups and 48% in children under 5 years of age (World Health Organization., 2011). In spite of these amazing achievements, about half of the world's population is still at risk of the disease with about 207 million cases, a little over 660000 malaria attributable deaths recorded in 2013. Sub-Saharan Africa still bears considerable burden of the disease with about 80% of the clinical cases and 90% deaths occurring in the region. Most malaria deaths (about 70%) occur in children under 5 years of age and *Plasmodium falciparum* accounts for over 80% of malaria cases worldwide (World Health Organization., 2014).

2.4.2 Malaria in Ghana

Malaria is a major cause of morbidity and mortality in Ghana, particularly among children under 5 years of age, and primigravid pregnant women, and the poor. In a study conducted in 2003 on the economic burden of malaria in Ghana, the disease was

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not only a health problem but a developmental as well as an economic problem. The financial hardships of the disease on households and the economy is enormous and the impact of the disease on real GDP growth is negative and decreases by -0.41% for every increase in malaria morbidity rate (Ankomah Asante and Asenso-Okyere, 2003).

In 2010, malaria accounted for about 34% of all outpatient illnesses and about 37% of all hospital admissions. Malaria attributable deaths increased from 1.22% in 2009 to 1.44% in 2010, representing 19% of all deaths that were recorded. Infection rates in children are very high and peaks at about 80% in those between the ages of 5-9 years. Among pregnant women in 2006, as high as 13.7% of all admissions were as a result of malaria whilst 9% of them died from the disease (Ghana Health Service., 2010).

Malaria is endemic throughout Ghana and varies with season being higher in the wet or rainy season where breeding of mosquitoes is favoured and lower in the dry season where breeding sites are few(Oduro *et al.*, 2007, Owusu-Agyei *et al.*, 2009).

Ahmed (Ahmed, 1989) reported *P. falciparum* as the predominant cause of malaria in Ghana, accounting for over 90% of clinical cases. Another study in the Kassena Nankana District of northern Ghana showed that the disease accounted for about 41% of hospital deaths with parasitaemia around 71% in the high transmission season and 54.3% at the end of the low transmission seasons (Koram *et al.*, 2000). Asante (Asante *et al.*, 2011b) also reported 98.1% of parasites circulating in the middle forest belt of Ghana are *P. falciparum*. In a study conducted in the southern coastal savannah region, *P. falciparum* prevalence among pregnant women was 19.7% compared to the middle forest belt which had a prevalence of 35.1% (Ofori *et al.*, 2009, Glover-Amengor *et al.*, 2005).

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A study by Appawu and his group showed that *Anopheles gambiae s.1* and *Anopheles funestus* constituted 94.3% of all the vectors sampled in the Kassena Nankana District in northern Ghana. They also reported the biting rates of the vectors to be about 36.7% bites per man per night in the irrigated areas and 5.2% in the non-irrigated lowland (Appawu *et al.*, 2004).

2.4.3 Clinical manifestations of Malaria

The clinical outcome of malaria depends on a host of factors including the parasite, host responses, geographic and social factors (Miller *et al.*, 2002). Clinical manifestations of malaria cover a wide spectrum from when one is asymptomatic through mild disease to severe disease and eventually death, especially in young children, if untreated early and properly. An asymptomatic carrier could have the parasite in the blood for a long time without showing any symptom of the disease. Malaria infection can be categorized into two main groups based on the extent of disease severity. These are mild or uncomplicated malaria and severe malaria. Severe malaria includes cerebral malaria and severe malaria anaemia. Other forms of severe malaria include pulmonary edema, impaired consciousness, prostration, seizures, jaundice, haemoglobinuria; respiratory distress, (White, 2003).

Uncomplicated malaria on the other hand present as mild non-specific febrile illness which can be resolved easily if appropriately treated. Some of the common symptoms of uncomplicated malaria are: common cold, chills, dizziness, abdominal discomfort, nausea, vomiting, headaches, cough, general body pains and mild diarrhea which can easily be treated. The other signs which may not be exclusive to malaria include: hepatosplenomegaly (enlargement of liver and spleen), pallor, orthostatic hypotension, tachycardia, jaundice (Murphy and Oldfield, 1996). However in severe malaria cases

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other symptoms are manifested such as: shock, acute renal failure, hypoglycaemia, metabolic acidosis, severe anaemia, multiple convulsions leading to coma. Still births, low birth weight, premature delivery, and miscarriages could also occur in pregnancy associated malaria (PAM) (Wipasa *et al.*, 2002a).

Disease symptoms are as a result of the exponential growth in the number of parasites in erythrocytes and the synchronous bursting of large numbers of these RBCs. These events release waste products of parasite metabolism and other pyrogens, and these are believed to trigger undesirable immune responses (Clark and Schofield, 2000, Good and Doolan, 1999).

2.4.4 Malaria Diagnosis, Prevention, and Treatment

The 'gold standard' for diagnosing malaria is the use of light microscopy to observe Giemsa stained thick and thin blood films on a slide (Moody *et al.*, 2000). Thick smear is used in identification of *Plasmodium* parasite whiles the thin smear is used for speciation. Though light microscopy is the 'gold standard and recommended by WHO, accuracy depends on the quality of smear, and the competency level of the laboratory personnel conducting the test. Other diagnostic methods include the use of the rapid diagnostic test (RDT), florescent microscopy where the nuclei of the parasites are stained with fluorescent dye, and polymerase chain reaction (PCR) assay which is the most sensitive of the methods but expensive (Hanscheid and Grobusch, 2002).

The discovery of the mode of transmission of malaria by Roland Ross and Batista *et al* led to various intervention programs to control and eliminate the disease. Most of these programs were aimed at vector elimination. Large scale vector control and mass drug administration programs were used in controlling the disease because of its success in controlling the disease during the construction of the Panama Canal (Najera *et al.*,

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2011). The lunch of the Global Malaria Eradication Program (GMEP) in the mid-1950s succeeded in eliminating or reducing the burden of malaria in Europe, the Caribbean, North America, South Central America and some parts of Asia, but little success was achieved in Sub-Saharan Africa. The program was however discontinued due to a myriad of problems (Carter and Mendis, 2002, Lopez *et al.*, 2006).

The Roll Back Malaria program RBM launched in 1998 with the Global Malaria Action plan was to reduce malaria deaths to near zero, reduce global malaria cases by 75% and eliminate malaria in 'new' countries by the year 2015. Two main components of the control programme were: Vector control and chemotherapy. Vector control programs include the use of Long Lasting Insecticidal Nets (LLINs) and Indoor Residual Spraying (IRS). These two methods are the core of the vector control programs (World Health Organization., 2014). The second component of the control program is the use of potent drugs to reduce parasite numbers in the blood. The introduction of the intermittent preventive therapy for pregnant women (IPTp), for example, has helped to significantly reduced neonatal deaths resulting from malaria in pregnancy by about 61.3% (Menendez *et al.*, 2010).

Malaria treatment has faced some challenges such as resistance to the available antimalarial drugs. The major causes of resistance are the long term and inconsistent use of antimalarial drugs. WHO now recommends the use of Artemisinin-based combination drugs as the first line drugs to treat *P. falciparum.* However chloroquine is recommended to be used in areas where it is still effective to treat *P. vivax*. (World Health Organization., 2014)

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2.5 The Immune System

The immune system comprises a network of specialised cells and organs that work together to protect the body from attack by 'foreign' substances including disease causing microbes such as viruses, bacteria, fungi, parasites, and other environmental substances. This complex system is highly organized and can recognise and destroy millions of microbes.

The immune system is classified into innate and acquired systems. The innate system is made up of all those elements with which one is born including the skin barrier, mucous membranes, phagocytic cells like the neutrophils and macrophages. Other components of the innate immune system are NK cells and inflammatory responses. The body's chemical environment such as pH and secreted fatty acids also forms part of the innate immune system. Acquired immunity is specific and can further be classified into humoral and cell-mediated system. The humoral system defends against infections in most body fluids whiles the cell-mediated arm uses cytotoxic lymphocytes to fight and kill infected cells. There is also the complement system which has components of both the innate and acquired immune systems and has three different pathways of activation to help fight infections. These are the classical, alternative, and lectin pathways. Both vertebrates and invertebrates depend on the innate immune system whilst only vertebrates use the adaptive system to fight against infections. (Coico and Sunshine, 2009).

2.5.1 Immunity to Malaria

Immunity to malaria is the body's ability to resist the disease. Studies have found that adults living in highly endemic areas have partial immunity to malaria though some may carry parasites in their blood (Druilhe and Khusmith, 1987). While some studies

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have found this immunity to be transferred to infants from their mothers from birth to about six months (Brabin *et al.*, 1990, Klein Klouwenberg *et al.*, 2005, Larru *et al.*, 2009), others found no such association (Riley *et al.*, 2000). Individuals living in malaria endemic areas over a period of time acquire immunity to malaria due to their frequent and repeated exposure to the parasites (Snow *et al.*, 1997, Gupta *et al.*, 1999, Marsh and Kinyanjui, 2006). Children living in malaria-endemic areas attain partial immunity against severe malaria as they grow, although they still suffer from uncomplicated malaria. Immunity to clinical disease in adults is believed to be partial and short-lived, and maintenance of this immune status requires the continuous presence of very low levels of parasites (Wipasa *et al.*, 2002a, Doolan *et al.*, 2009), a phenomenon known as premunition.

Though mechanisms underlying protective immunity to parasite infection and clinical malaria are not fully understood in humans, studies in animal models and humans points to the involvement of both cellular and antibody mediated mechanisms(Good and Doolan, 1999, Doolan *et al.*, 2009). In effect both innate and acquired immune mechanisms are mediators of host responses to infection with malaria (Bouharoun-Tayoun *et al.*, 1995).

2.5.2 Innate immunity

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Innate immunity to malaria is the inherent or natural resistance to the disease. This form of immunity is the first line of defense against any infection and can be found in most vertebrates. Studies have found innate responses to malaria are very essential in limiting initial parasite replication in mice while in humans parasite growth can be modulated early in primary infections (Fell and Smith, 1998, Molineaux *et al.*, 2002).

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Innate immunity can be achieved either by genetic, or cell-mediated immunologic mechanisms. Numerous genetic factors play major roles in innate resistance to malaria and this mostly affect the erythrocytes which are critical for parasite development. Some of these genetic factors include Sickle Cell trait (HbS) (Allison, 1954, Friedman, 1978, Aidoo *et al.*, 2002) , Glucose-6-Phosphate Dehydrogenase (G-6PD) deficiency (Smith *et al.*, 2002, Roth *et al.*, 1983), and thalassaemias (Allen *et al.*, 1997, Smith *et al.*, 2002). These genetic factors confer some level of protection against malaria though they are usually seen as inherited genetic disorders of the hemoglobin generally referred to as haemoglobinopathies. These disorders, mostly found in individuals living in malaria endemic regions are usually associated with the development of erythrocytes, thus affecting parasite growth (Smith *et al.*, 2002).

Generally, erythrocytes of genetically protected individuals (individuals with sickle cell, alpha- and beta- thalassemia and G6PD deficiency) are vulnerable to repeated oxidation due to the release of H_2O_2 into the erythrocyte by *P. falciparum* which leads to an irreversible interaction between the oxidized haemoglobin and the red cell membrane. It is suggested that the irreversible oxidation could trigger mechanisms that reduce invasion of RBCs by the *falciparum* parasite, impair parasite survival and development within the cell, and accelerate infected erythrocyte clearance by phagocytosis (Destro Bisol, 1999).

The cell-mediated immunologic mechanisms include phagocytosis of parasitized RBCs and merozoites by macrophages and neutrophils. Some of these immune cells release cytotoxic molecules such as cytokines, chemokines, and anti-parasite molecules such as nitric oxide that kill parasites, and others that help to opsonise parasitized cells. Some parasites are also cleared from the blood stream by the innate immune system (Smith *et*

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al., 2002, McGilvray *et al.*, 2000). It has been demonstrated *in vitro* that components of blood-stage parasites, which include parasite-derived glycosilphosphatidylinositols (GPI), induce macrophages to produce IL-1, IL-6, and TNF-α (Scragg *et al.*, 1999, Tachado *et al.*, 1997). In addition some macrophages have also been shown to have the ability to present malaria antigens to T cells (Quin *et al.*, 2001).

Other innate mechanisms are the activation of the complement system through an alternative or the mannose-binding protein pathway and the cytotoxic activity of natural killer (NK) cells on host infected target cells (Fearon and Locksley, 1996).

2.5.3 Acquired Immunity

2.5.3.1 Cell-mediated immunity

Though it has been establish that both humoral and cellular mechanisms contribute to the control of blood stage parasitaemia, the acquisition and maintenance of protective immunity depends largely on T-cell responses (Troye-Blomberg, 1994).

There are two subpopulations of T-cells: T-helper (TH), and T-cytotoxic (TC). T helper and T cytotoxic cells can be separated from one another by the presence of either CD4+ or CD8+ glycoprotein receptors on their surfaces. T cells displaying the CD4 receptor generally function as helper T cells, whereas those displaying the CD8 receptor are cytotoxic T cells. Responses of both CD4+ and CD8+ T-cells are elicited on encountering parasite antigens in the precise major histocompatibility complex (MHC) context, by antigen presenting cells (APCs) such as dendritic cells, and macrophages. Such responses are very vital in the killing of parasites, and or inhibition of their growth (Chemtai *et al.*, 1984, Good and Doolan, 1999, Frevert and Nardin, 2008).

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There are also different groups of CD4+ T-cells: T-helper 1 (Th1), and T-helper 2 (Th2) and this is based on the type of regulatory functions and the type of cytokines they produce. The Th1 cells activate cells such as macrophages to release inflammatory cytokines like TNF-α, IFN-γ and interleukin 2 (IL2), and are thus responsible for cell-mediated immunity. Th2 cells on the other hand produce IL10, IL4, IL5, IL6, and IL3 which aid the development of humoral immunity by activating B-cells to differentiate, proliferate and produce antibodies (Mosmann and Coffman, 1989, Abbas *et al.*, 1996). In early acute infection with *P. chabaudi*, the prime cytokine produced is IFN-γ, a Th1 cytokine, and this has been shown to decline with decreasing parasitaemia and then replaced with Th2 cytokines such as IL10 and IL 4 in the later stages (Stevenson and Tam, 1993). This suggests that Th1 cells producing IFN-γ and IL-2 are important for controlling infection in its early phases, while Th2 cells, producing IL-4 and IL-10, together with antibodies, are essential for parasite clearance in later phases of infection (Troye-Blomberg *et al.*, 1994). Thus both Th1 and Th2 T-cells play important roles in immunity to malaria at different stages of the disease and the balance between these two subsets is critical for the outcome of an infection (Wipasa *et al.*, 2002b).

Also, cytokines such as TNF-α, and IFN-γ may be produced on antigen stimulation, and may have anti-parasitic activity (Playfair and Taverne, 1987, Mordmuller *et al.*, 1997). CD4+ T-cells play more important role than CD8+ though both carry αβ T cell receptors thus making role of CD8+ T cells in blood stage infections limited since antigen presentation cannot be done as the red blood cells carrying the parasites do not express MHC (Perlmann *et al.*, 1998). Aside the crucial role played by CD4+ T-cells to protect the host by controlling parasitaemia, they can also be detrimental to the host (Amante and Good, 1997, Hermsen *et al.*, 1998).

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Another important group of immune effector cells are the regulatory T cells (Tregs). These cells have been found to regulate cell mediated immunity in rodent malaria (Vigario *et al.*, 2007, Amante and Good, 1997). High numbers of Tregs have been observed in the skin in the study state suggesting that *Plasmodium* specific Tregs are induced during skin stage infection (Guilbride *et al.*, 2010). Inducible T regulatory cells are produced in the periphery and mainly induce the production of IL-10, IL-35, and TGF-β (Collison *et al.*, 2007, Sakaguchi *et al.*, 2009).

Gamma delta T-cells (γδ) are T cells that have been demonstrated to inhibit parasite growth as their numbers increase in the first few days during malaria infection (Farouk *et al.*, 2004), and that mice lacking γδ cells have been found to develop chronic parasitaemia following *P. c. chabaudi* infection (Seixas and Langhorne, 1999). Gamma delta (γδ) T-cells isolated from PBMCs from non-immune individuals inhibit parasite growth in vitro, and the number of γδ T-cells in the culture correlates with their inhibitory activities (Troye-Blomberg *et al.*, 1999).

2.5.3.2 Antibody-mediated immunity

Antibodies, the effectors of humoral immunity, are soluble proteins that are secreted by plasma cells that differentiate when B-cells are stimulated. Antibodies belong to a family of proteins called immunoglobulins (Igs) which have two distinct types of polypeptide chains; light and heavy chains, linked by disulphide bonds. There are five different types of immunoglobulins based on the type of heavy chain they possess, namely IgG, IgA, IgD, IgM, and IgE. IgG is further divided into subclasses: IgG1, IgG 2, IgG3, IgG4.

Studies have shown that antibodies induced by malaria infections are mostly IgM, IgG and IgA (Targett, 1970). IgM is first produced during the primary phase of the immune

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response and is usually short-lived. In secondary responses, class-switching events lead to the production of high affinity IgG, though other antigens such as IgA and IgE are produced in the later stages of the disease (Collins *et al.*, 1971). The main humoral mediators of protection from malaria are IgG.

Anti-malaria antibodies, especially of the IgG isotype, have been shown to be very critical in controlling blood stage infections and symptoms of malaria. These protective antibodies are thought to target mostly merozoite surface antigens, erythrocyte invasion ligands and variant surface antigens expressed by *P. falciparum* infected erythrocytes (Bull *et al.*, 1998, Good and Doolan, 1999). This has been demonstrated by the passive transfer of antibodies from adults who are semi-immune to malaria to treat clinically ill children (Cohen *et al.*, 1961). In another study, pooled IgG from individuals living in various endemic areas in Africa was able to reduce parasitaemia and fever in patients with drug-resistance malaria (Sabchareon *et al.*, 1991).

Antibodies to merozoite antigens are believe to act by inhibition of merozoite invasion into new erythrocytes (Dutta *et al.*, 2003a), blocking cytoadherence of infected RBCs (iRBCs) to endothelial cells (Singh *et al.*, 1988, Taylor *et al.*, 1992), complement-mediated opsonisation of infected erythrocytes (Groux and Gysin, 1990), enhancing phagocytic activity of monocytes and macrophages (Bouharoun-Tayoun *et al.*, 1990), and complement blocking of erythrocyte invasion and erythrocyte stage replication of parasite (Boyle *et al.*, 2015). Antibodies have also been found to neutralize parasite toxins (Schofield *et al.*, 2002). It has also been shown that for individuals living in malaria endemic areas, naturally acquired immunity is largely dependent on the acquisition of a repertoire of specific antibodies directed against *P. falciparum* erythrocyte membrane protein-1(*Pf*EMP-1) that are expressed on the infected RBC

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surface (Bull *et al.*, 1998). The magnitude of protective immunity found in humans (Chizzolini *et al.*, 1988, Piper *et al.*, 1999), and monkeys (Egan *et al.*, 2000) have been shown to correlate with the level of antibodies against asexual blood stage antigens, and are antibody isotype dependent.

The IgG1 and IgG3 subclasses of IgG are cytophilic antibodies that are important in protective immunity against malaria (Bouharoun-Tayoun and Druilhe, 1992). It has also been reported that these cytophilic antibodies, which are the main effectors of Antibody Dependent Cellular Inhibition (ADCI), activates monocytes to secret soluble factors such as nitric oxide and cytokines such as TNF-α to inhibit parasite growth (Bouharoun-Tayoun *et al.*, 1995). While some studies have found IgG2 to be protective (Aucan *et al.*, 2000), others have found IgG2 and IgG4 to inhibit the opsonizing activity of IgG1 and IgG3 (Groux and Gysin, 1990).

It has been reported that high levels of anti-malarial antibodies against pre-erythrocytic antigens are associated with reduced risk of developing malaria in endemic areas (John *et al.*, 2005). Also several studies have associated high levels of anti-malarial antibodies to blood stage antigens and reduced risk of infection (Riley *et al.*, 1992, Theisen *et al.*, 1998, Dodoo *et al.*, 2008, Dodoo *et al.*, 2000). It has also been reported that antibodies whether acquired naturally, or through vaccination, are short lived (Maple *et al.*, 2000). Immunity to malaria is parasite and strain specific, and clonal antigenic variation is common in *P. falciparum (*Hommel *et al., 1983)*. The mechanism behind this antigenic variation is still not clear, but one very likely hypothesis is that there is a frequent on-going switching of variant surface antigens (VSA) in the parasite population, and an outgrowth of subpopulations with newer VSAs would occur when elicited antibodies are mainly against the other VSAs previously presented. The

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importance of VSAs in immunity can be shown by correlating the range of different anti-VSA antibodies to protection (Hviid, 2005). Pregnancy associated malaria (PAM) is caused by accumulation of iRBCs in the placenta. These parasites express a specific VSA that binds to chondroitin sulphate A (CSA), and the immune responses that are induced are parity dependent (Salanti *et al.*, 2004).

2.6 Antigenic targets

Antibodies have been shown to be crucial in acquired protective immunity to malaria. During the developmental stages of *P. falciparum* the parasites express certain proteins or antigens on their surfaces. Antibodies to these antigens confer protection through a variety of mechanisms (Groux and Gysin, 1990, Dutta *et al.*, 2003a, Taylor *et al.*, 1992, Singh *et al.*, 1988, Theisen *et al.*, 1998, Schofield *et al.*, 2002). A number of these target antigens have been identified including:

2.6.1 Apical Membrane Antigen-1 (AMA1)

AMA1 is one of the leading erythrocytic stage malaria vaccine candidates. It plays a crucial role in the invasion of erythrocytes and has also been found in the sporozoite and liver stages of the parasite (Silvie *et al.*, 2004). AMA1 is a merozoite protein located in the micronemes of apicomplexans parasites (Remarque *et al.*, 2008a). It is an integral membrane protein with a large N-terminal cysteine-rich ectodomain, followed by a single transmembrane domain and a short C-terminal cytoplasmic tail. *Plasmodium falciparum* AMA-1 is made of about 622 amino acids and is divided into three domains characterized by eight intra-domain disulphide bonds (Hodder *et al* 1996).

With molecular weight of about 83 kDa, AMA1 is cleaved at the N-terminus yielding a mature protein of approximately 66 KDa (Narum and Thomas, 1994, Howell *et al.*,

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2001). This 66 kDa AMA1 translocates to the merozoite surface and is cleaved proteolytically at one of two alternative sites resulting in two soluble fragments of 44 kDa and 48 kDa. The importance of the proteolytic processing is unknown, but it is suggested that it may be required for AMA1 to move to the parasite surface or to play its mediatory role in invasion or both (Howell *et al*., 2001). AMA1 has been shown to be essential for the survival of *Plasmodium* species within their host (Remarque *et al.*, 2008a). AMA1 appears to play a vital role in erythrocyte invasion by participating in the re-orientation and attachment of the merozoite to the host erythrocyte cell surface (Triglia *et al*., 2000, Mitchell *et al*., 2004).

Antibodies against AMA1 have been shown to inhibit merozoite invasion of erythrocytes in both *in vitro* and *in vivo* studies and also confer protection against parasite strains expressing the vaccine AMA1 allele in active immunization studies (Mitchell *et al*., 2004, Dutta *et al*., 2003b). The inhibitory effects are essentially species-specific with a large component of protection being strain-specific (Crewther *et al*., 1996). But to induce parasite-inhibitory activity AMA1 has to be folded correctly (Salvatore *et al*., 2002). Naturally acquired antibodies to AMA1 have been found to be associated with protection from clinical malaria in a population in coastal Kenya (Osier *et al*., 2008).

AMA1 is highly polymorphic (Thomas *et al*., 1990, Remarque *et al*., 2008a) and this polymorphism is not due to repetitive sequence but is mainly as a result of single amino acid substitutions (Chesne-Seck *et al.,* 2005). Animal studies have shown that antibodies to AMA1 obtained from one strain of *Plasmodium* inhibits the growth of a homologous strain effectively but inhibits other strains to various lesser degrees (Kennedy *et al*., 2002, Kocken *et al*., 2002, Kusi *et al*., 2009, Healer *et al*., 2004).

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2.6.2 Merozoite Surface Protein 1-19 ($MSP1_{19}$)

$MSP1_{19}$ is located on the surface of the blood-stage merozoites and is first synthesized as a 200 kDa protein (MSP1) during blood stage schizogony. The various molecular weights that it is processed into include the C-terminal 42 kDa fragment. Majority of the fragments are chopped off before they invade erythrocytes (Holder *et al.*, 1987). A 33kDa protein is processed from the C-terminal 42 kDa, which is further shed into a relatively conserved 19 kDa protein, known as the $MSP1_{19}$ (Holder *et al.*, 1992). $MSP1_{19}$ is anchored to the merozoite membrane by glycosylphosphatidylinositol (GPI) (Blackman *et al.*, 1996). There are 2 epidermal Growth Factor (EGF)-like domains, which may play critical role in merozoite invasion of erythrocytes (Holder *et al.*, 1992). $MSP1_{19}$ has been shown to be an important malaria vaccine candidate and invasion of erythrocytes by parasites have shown to be blocked by this fragment and also inhibit parasite multiplication inside the erythrocyte (Hogh *et al.*, 1995, Holder *et al.*, 1992, O'Donnell *et al.*, 2001). Although MSP1 has been found to be protective, others have found no association of MSP1 with protection from clinical malaria and also control parasite replication (Shi *et al.*, 2007, Dodoo *et al.*, 1999)

2.6.3 Cell-Traversal Protein for Ookinetes and Sporozoites (CelTOS)

CelTOS is a 25kDa protein that is present in both the mosquito and human stages of the parasite and is localized to the micronemes of the sporozoite (Kariu *et al.*, 2006). The protein is required by the parasite for motility and invasion of hepatocytes. CelTOS is a highly conserved protein and anti-*Pf*CelTOS immune response were found to be protective against challenge from *P. berghei* (Bergmann-Leitner *et al.*, 2010). Antibodies to CelTOS in mice have been shown to inhibit sporozoite motility and invasion of hepatocytes *in vitro,* and were also found to induce sterile protection in test animals (Bergmann-Leitner *et al.*, 2011). CelTOS has also been found to induce cell-

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mediated immune responses where CelTOS-specific peptides stimulated high IFN-γ responses in PBMCs (Doolan *et al.*, 2003).

2.6.4 Circumsporozoite Protein (CSP)

CSP is the most abundant protein found on the surface of *Plasmodium* sporozoites and is essential to the survival of *Plasmodium* parasites as it is required for the development of infectious sporozoites in mosquitoes (Menard *et al.*, 1997). The CSP of the 3D7 *P. falciparum* strain has 397 amino acids and is the most advanced and well documented of all the pre-erythrocytic vaccine candidates (Girard *et al.*, 2007). CSP contains two major B cell epitopes consisting of tandem repeats. The most clinically advanced malaria vaccine, RTS, S, is a subunit vaccine consisting of the central repeat and C terminal regions of *P. falciparum* CSP fused to the hepatitis B surface antigen and co-expressed with free hepatitis B surface antigen to form a hepatitis B surface (HBs) Ag-like particle (Gordon *et al.*, 1995). The results from phase III testing of the RTS, S vaccine has reported between 30-55% efficacy (Abdulla *et al.*, 2013, Agnandji *et al.*, 2011, Asante *et al.*, 2011a).

Although antibodies have been thought to be very important in protection from malaria, there is evidence to suggest that not all antibodies are protective. It has been reported that monoclonal antibodies (mAb) against $MSP1_{19}$ which inhibit RBC invasion by merozoites and prevent MSP-1 secondary processing, can be blocked by other mAb to the same antigen (Patino *et al.*, 1997). Also polyclonal antibodies specific to MSP2, but not mAb specific to the same antigen, enhanced invasion of multiple merozoites into RBC (Ramasamy *et al.*, 1999). These findings underscore the importance of finding epitopes that induce protective antibodies when designing a vaccine against malaria.

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2.7 Immune Evasion Mechanisms

2.7.1 Antigenic Variation

Antigenic variation is the change in antigenic phenotype by regulated expression of different genes of a clonal population of parasites over the natural course of an infection (Doolan *et al.*, 2009). Antigenic differences between parasite populations was first detected when mice harbouring chronic infections with *P. berghei* were found to be more susceptible to challenge with relapse parasites than to use the original parasite population (Cox, 1959). Another study with *P. knowlesi* showed a succession of antigenic variants with chronic infection of the erythrocytes (Brown and Brown, 1965).

Plasmodium parasites evade the host immune system by varying the antigenic characters of the infected erythrocytes (Doolan *et al.*, 2009). This evasion is achieved by having a large family of *Plasmodium falciparum* genes called *var* genes which encode a parasite derived protein called *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1) on the surface of infected erythrocytes. *Pf*EMP-1 is a variant protein of approximately 300 kDa, expressed on the surface of RBC infected with late stage parasites and is coded for by approximately 50 genes of the *var* multigene family (Su *et al.*, 1995, Smith *et al.*, 1995).

Antigenic variation of *Pf*EMP1, through switching of the expression of different *var* genes, facilitates evasion of host immune responses, and specific variants of *Pf*EMP1 have been found to mediate adhesion to chondroitin sulphate A (CSA) in pregnant women and to intercellular adhesion molecule 1 (ICAM1) in brain microvasculature (Gamain *et al.*, 2004). *Pf*EMP-1 has been implicated as a key target of naturally acquired immunity to malaria.

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Several studies have reported association between increased episodes of clinical malaria and a transient increase in the level of specific antibodies to variant surface antigens (VSAs) (Marsh and Howard, 1986, Giha *et al.*, 1999). Studies have also found an association between the acquisition of antibodies to VSAs, which is age-dependent, and a decline in parasite density (Piper *et al.*, 1999). In Ghana anti-VSA IgG levels have been correlated with protection from clinical malaria (Dodoo *et al.*, 2001, Ofori *et al.*, 2002). Reports have also been made from other epidemiological settings of correlation between anti-VSA antibody levels and protection from clinical malaria (Kinyanjui *et al.*, 2004, Yone *et al.*, 2005). As a result, clinical immunity probably develops once an individual acquires antibodies against multiple *Pf*EMP1 variants in a natural setting, which may partly explain why natural immunity takes several years to develop (Wipasa *et al.*, 2002a). VSAs have thus been proposed for malaria vaccine development (Chen, 2007).

Other important antigenically variable antigens: are repetitive interspersed family proteins (RIFIN) (Kyes *et al.*, 2001), Subtelomeric variable open reading frame proteins (STEVOR) (Cheng *et al.*, 1998, Kaviratne *et al.*, 2002), and surface-associated interspersed gene family proteins (SURFIN) (Winter *et al.*, 2005).

2.7.2 Allelic Polymorphism

Another immune evasion mechanism employed by *Plasmodium* parasites is allelic polymorphism. Polymorphisms are caused by variations in the sequence of the short tandem repeats, which is a characteristic feature of many malaria antigens and frequently constitute immunodominant regions (Bolad and Berzins, 2000). Studies have discovered that polymorphisms also result from point mutations in several antigens (Bull *et al.*, 1999).

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Polymorphism has resulted in a situation where antibody responses induced against one allelic form of a particular antigen have been shown to be less effective at limiting the multiplication of parasites that express a different allele of the same antigen (Supargiyono *et al.*, 2013, Perraut *et al.*, 2000). For example MSP-1 is a leading malaria vaccine candidate antigen and is highly polymorphic (Qari *et al.*, 1998), thus some antibodies to one MSP-1 allele may not fully recognize the other MSP1 alleles (Burns *et al.*, 1989).

Also, polymorphism severely affects T-cell recognition (Plebanski *et al.*, 1999). T-cell recognition primarily depends on the amino acid sequence rather than protein conformation, which is often recognized by antibodies (Plebanski *et al.*, 1999). The high genetic diversity in the *Plasmodium* parasite, mostly with the expression of variant surface antigens poses a considerable challenge to vaccine formulators. Most of the *P. falciparum* antigens currently under consideration for vaccine development have exhibited extensive polymorphisms in field isolates (Escalante *et al.*, 1998, Barry *et al.*, 2009).

2.7.3 Intracellular Parasitism

Plasmodium parasites have evolved in deploying intracellular parasitism as a mechanism to escape host immune reaction. Anti-malarial antibodies that bind to free sporozoites or merozoites may not be able to access them once the parasites enter the host cells. Moreover, RBCs do not express MHC molecules on their surfaces thus merozoites escape recognition by CD8+ T cells (Hisaeda *et al.*, 2005)

2.8 Strain Specific and Cross Reactive Immunity

Strains results from allelic polymorphisms within a single parasite specie giving rise to different genotypes (McKenzie *et al.*, 2008). Allelic polymorphisms in certain protein

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loci which produce antigenically different forms of the protein in different parasite strains underlie the concept of strain-specific immunity (Day and Marsh, 1991).

Studies with animal models indicated that immunity to malaria is parasite strain-specific (Hommel *et al.*, 1983, Jones *et al.*, 2001). It has been shown in humans that a primary infection by one parasite strain elicited an immune response which was capable of protecting against that strain but not against infection by a different strain (Jeffery, 1966). In *Aotus* monkeys, repeated infections induced increasingly more rapid sterile immunity to homologous challenge (Jones *et al.*, 2000). According to (Doolan *et al.*, 2009), when immunity is induced experimentally using a homologous strain, it is very rapid but in areas of malaria endemicity, immunity is slow due to the challenge by different or heterologous strains.

However in natural populations where malaria is endemic, significant strain-specific and cross-reactive inhibition of parasite multiplication was observed when homologous and heterologous sera from children were assessed for any inhibitory effects on parasite growth (Wilson and Phillips, 1976). In animal model experiments, it was established that immune sera from *Aotus* monkeys contained antibodies that blocked or reversed cytoadherence *in vitro* and were isolate-specific (Udeinya *et al.*, 1983). Also, chronic infections of RBCs with *P. knowlesi* were shown to consist of a succession of antigenically distinct strains (Brown and Brown, 1965), and sera from rhesus macaque monkeys immune to one *P. knowlesi* strain did not react with cells infected with another strain (Brown *et al* 1968). Newbold (Newbold *et al.*, 1992) showed using field isolates and laboratory clones that the predominant agglutinating antibody response in humans was variant-specific and that cross-reactive antibodies to different serotypes

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were rare. It was however demonstrated in a hyperendemic area in India that cross-reactive antibodies against VSA can be found in adults (Chattopadhyay *et al.*, 2003).

Also sera from children living in malaria endemic areas of different geographic regions did not agglutinate cells infected with different strains in agglutination assays. Sera from immune adults contained antibodies that cross-reacted by agglutination majority or all of the different isolates and also reacted with infected cells from most children (Marsh *et al.*, 1986, Forsyth *et al.*, 1989). Also Cortes (Cortes *et al.*, 2005a), found significant allele-specific antibodies in children than in adults while (Terheggen *et al.*, 2014) showed greater antibody cross- reactivity in adults, reflecting a higher level of protective immunity in adults. The passive administration of IgG from immune adult West Africans to children resident in East Africa, or Thailand, demonstrated the importance of cross-reactive antibodies in reducing parasitaemia (Cohen *et al.*, 1961, McGregor, 1963, Sabchareon *et al.*, 1991).

2.9 Multiplicity of Infection and Antibody Responses

Malaria parasites have been found to be highly diverse genetically and that an individual may carry different parasite clones at a given time (Babiker *et al.*, 1991, Branch *et al.*, 2001). When multiple parasite clones simultaneously infect an individual, it is known as multiplicity of infection.

To assess multiplicity of infection, polymorphic genes are genotyped. The merozoite surface proteins 1 and 2 are mostly used for the genotyping. The proteins are encoded by the single-copy genes msp-1 and msp-2 which are polymorphic and a parasite isolate with more than one MSP allele is thus considered multiple infections

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(Viriyakosol *et al.*, 1995). The block two region of MSP1 and the block 3 region of MSP2 are the targeted regions.

The block 2 region in msp-1 is in the family of three alleles: K1, Mad20 and RO33. The K1 variants and that of Mad20 have been found to differ in tripeptide or hexapeptide repeats, on the other hand the RO33 family does not contain repeats, but exhibits considerable heterogeneity (Miller *et al.*, 1993). For the MSP2 block 3 region, the alleles are grouped into two families, the IC3D7 and FC27 (Smythe *et al.*, 1990). The region 2 of Glutamate rich protein (GLURP) is also used as a molecular marker to detect parasite (Borre *et al.*, 1991).

Multiplicity of infection has been found to be influenced by age and season in central Ghana (Agyeman-Budu *et al.*, 2013). In that MOI decreases with increasing age (Ntoumi *et al.*, 1995). Increased transmission has generally been found to correlate with progressive increase in the average number of malaria parasite clones (Babiker *et al.*, 1997). Multiplicity of infection also varies with malaria transmission season being higher in high transmission season and low in low transmission season.

In areas where malaria is endemic, the average number of malaria parasite strains per person correlates with level of transmission (Arnot, 1998, Babiker *et al.*, 1999). Also high parasite density have been correlated with MOI after malaria transmission (Vafa *et al.*, 2008), and could be an indicator of immune status/premunition (Smith *et al.*, 1999, Arnot, 1998), transmission indicator (Babiker *et al.*, 1999), and an indicator for evaluating control interventions (Mayengue *et al.*, 2009). High parasite diversity have been reported in high transmission season in some cases (Kiwanuka, 2009).

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CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ethics Statement

Ethical and scientific approvals for the study were granted by the Institutional Review Board (IRB) and the Scientific and Technical Committee (STC) of Noguchi Memorial Institute for Medical Research (NMIMR) and the Navrongo Health Research Centre (NHRC) respectively.

The study participants and/or their parents/legal guardians gave written informed consent before enrolment into the study.

3.2 Study Area

Stored plasma and filter paper samples from a previous study conducted in Bongo in the Bongo District of the Upper East Region of Ghana were used in this study. The Bongo District is located in Northern Ghana and shares boundaries with Burkina Faso in the north, Kassena-Nankana District in the west, Bolga Municipal and Talensi-Nabdam to the south, and Bawku West District in the east. Bongo has a size of about 459 sq km. The land is flat with few hills in the south and east. The District has a tropical climate with savanna vegetation and has a rainy season from May to November, with maximum rainfall recorded in August and September, and dry season from December to April the following year. There is one large irrigation dam, the Vea irrigation dam, and other smaller dams scattered across the District (www.geradsn.org) The total population of the district is 84,545. (Ghana Statistical Service., 2010) The study area has marked seasonal malaria transmission that overlaps with rainfall and

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vector distribution patterns and malaria transmission is therefore high from June through November and comparatively lower between January and April.

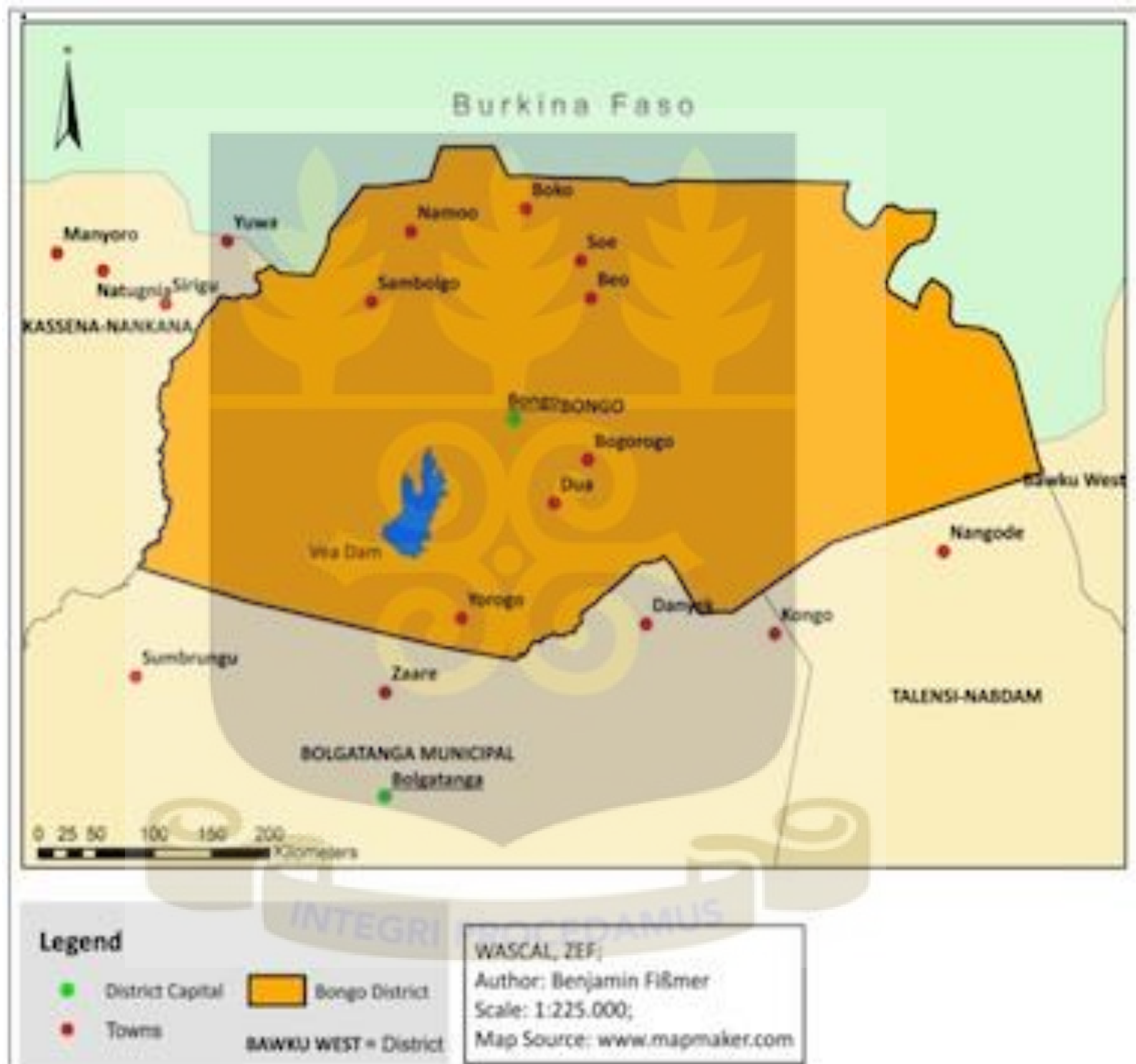

Figure 3. 1 Map of Bongo District

Source: (*http://recas-ghana.com/campus.html*)

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3.3 Study Design and Study Population

The original larger study was a cross-sectional community-based survey which was conducted close to the end of the rainy season in November/December 2012 and a second similar survey conducted at the end of the dry season in April 2013. Participants for the study were selected from two communities in the Bongo district; the Gowrie/Vea community and the Soe community. Gowrie and Vea are located around the Vea irrigation project with a large dam, and the Soe community is located about 20 km away from the irrigation project site. The Bongo district is one of the districts in northern Ghana that is mapped by the demographic surveillance system of the Navrongo Health Research Centre and has a regularly updated database.

Approximately 200 volunteers were recruited from each of the two communities; thus 400 samples were collected per season and about 800 samples in total for both surveys. This current study was curved out of the larger study described, and about 300 stored plasma samples and corresponding filter paper samples per season were used.

3.4 Sample Collection and Processing

About 3ml of venous blood was drawn from each participant into collection tubes that had EDTA as anti-coagulant. Filter paper blood blot, thick and thin film blood slides were obtained from all participants for each survey. Basic clinical and demographic data of participants were also captured by questionnaire. The venous blood was centrifuged using the Harrier 18/80 refrigerated centrifuge (MSE UK Ltd, UK) at 2000rpm for 10 minutes and the plasma taken and stored at -20°C. Thick and thin blood slides were stained with 10% Giemsa for *Plasmodium* parasite examination. Plasma samples were later transported to the Immunology Department, NMIMR in a box containing solid carbon dioxide (dry ice) for immunological analysis.

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3.5 Laboratory Measurements

3.5.1 Parasitological Examination

Light microscopy was used to examine and quantify malaria parasites against 500 WBCs. The parasite density was then calculated using the formula:

Parasite density = (Count x 8000)/500.

Where 'count' is number of asexual blood stage parasites counted and 8000 and 500 are constants representing number of RBCs and WBCs respectively.

3.5.2 Malaria Antigens

Four *P. falciparum* antigens, CSP and CelTOS from the sporozoite stage and the typical blood stage antigens AMA1 and $MSP1_{19}$ were used in this study. For AMA1, four natural alleles (from the 3D7, FVO, CAMP and HB3 parasite strains), and a mixture of three synthetically designed diversity covering (or *DiCo*) AMA1 antigens were used in competition assays (Kusi *et al.*, 2010, Remarque *et al.*, 2008b). The three DiCo antigens were used as a single equimolar mixture termed DiCo mix. All antigens were expressed and purified under GMP conditions.

The CSP protein of the 3D7 strain and containing 19 of the 38 NANP repeats was expressed in an *Escherichia coli* system (Porter *et al.*, 2013). The 3D7 strain CelTOS protein is comprised of 174 amino acids including an N-terminal six-histidine tag within a 16-amino acid linker which was also expressed in *E.coli* (Bergmann-Leitner *et al.*, 2010). The 3D7 strain AMA1 protein is comprised of amino acids 83 to 531 of the AMA1 ectodomain and was also expressed in *E. coli* (Dutta *et al.*, 2002). The full AMA1 ectodomain protein from the FVO strain of *P. falciparum* which is about 75kDa containing 622 amino acid residues was expressed in *P. pastoris* (Kocken et al., 2002). The natural allelic forms from the *P. falciparum* strains HB3, 3D7 and CAMP were

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expressed in *Pichia pastoris* by a similar methodology described by (Faber *et al.*, 2008). The differences in the amino acid sequence of the various AMA1 antigens (Dico1, Dico 2, Dico 3 and AMA1 from the FVO 3D7 HB3 and CAMP parasite strains) are presented in Figure 3.2

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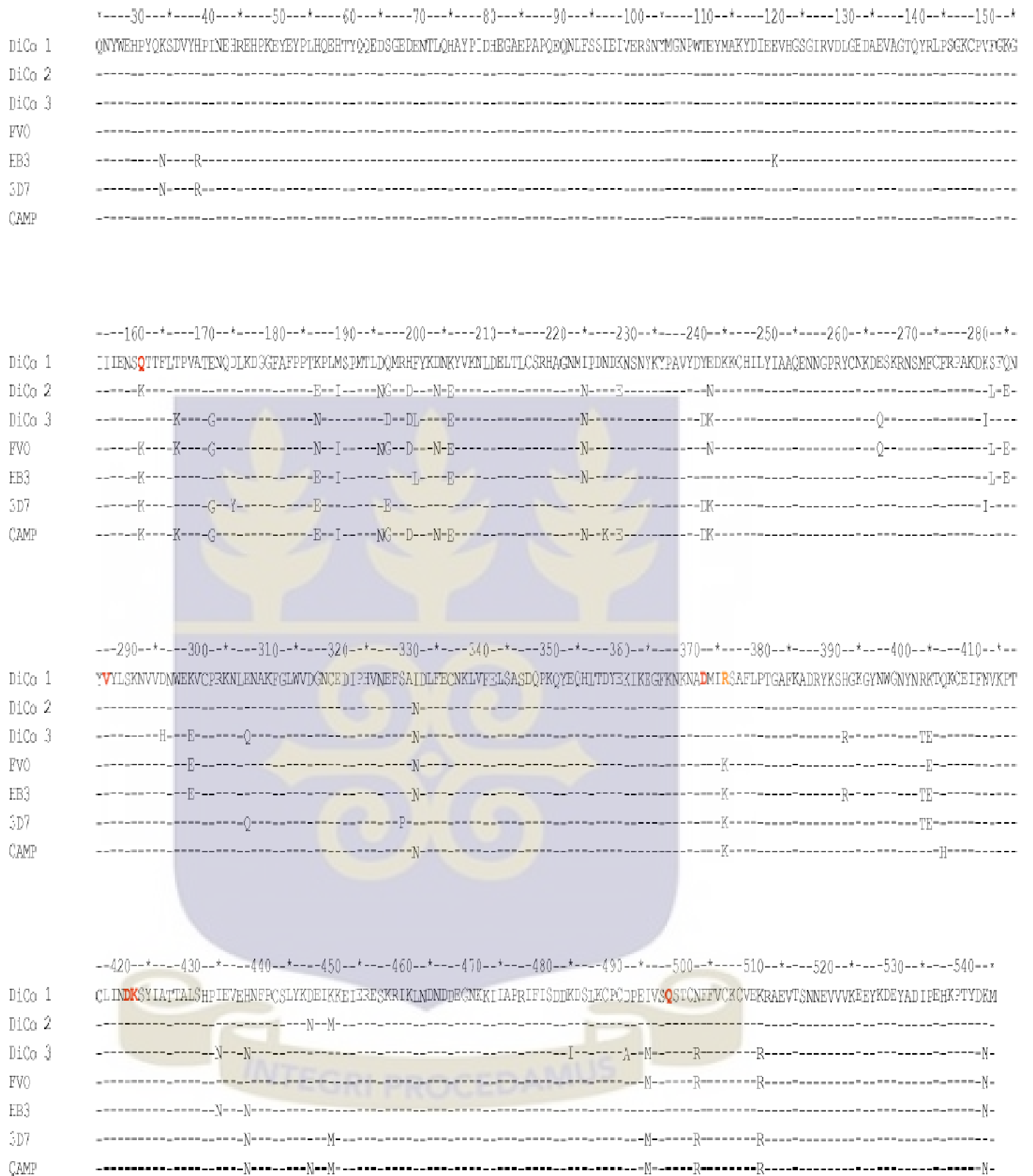

Figure 3. 2 Amino acid sequence alignment for the four AMA1 and three DiCo antigens

Source (Kusi *et al.*, 2010)

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3.5.3 Enzyme-linked Immunosorbent Assay (ELISA)

3.5.3.1 Optimization and Standardization of ELISA

Antigen coating concentration, plasma dilution, and enzyme-conjugate dilution were optimized for the four recombinant antigens, CelTOS, AMA1, CSP and $MSP1_{(19)}$ by ELISA checker-board titrations. In addition, optimization was done to determine the starting dilution of standard pool required for standard curves that convert sample ODs on test plates to concentration in arbitrary units (AU). All antigens tested were optimized and shown to be stable for at least two weeks, when antigen-coated plates were refrigerated (2 to 8°C). Plasma dilutions were also optimized and shown to be stable for 6 weeks when diluted in 1% milk/PBS with 0.02% sodium azide as preservative.

3.5.3.2 Determination of Total IgG levels

Total IgG levels to the malarial recombinant antigens CSP, celTOS, $MSP1_{19}$, AMA1-FVO, and AMA1-3D7 were measured by indirect ELISA. Briefly, 100µl/well of a 1.0µg/ml antigen solution in coating buffer (plain PBS, pH 7.4) was pipetted into wells of a 96-well microtitre ELISA plate (Maxisorp Nunc, Denmark). Coated plates were kept in a refrigerator at 4°C overnight.

Plates were then washed four times in washing buffer (PBS with 0.1% Tween-20) with 30 seconds incubation between each wash using the Biotek ELx 405 automated ELISA plate washer (Biotek Instruments, Winooski, VT; USA). The washed plates were padded dry on a tissue paper and blocked with 200µl blocking buffer (PBS with 5% milk powder, 0.1% Tween-20) and incubated at room temperature for 1 hour.

Plates were then washed four times in washing buffer and plasma samples diluted at 1:1000 in sample dilution buffer (PBS with 1% milk powder, 0.1% Tween-20 and

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0.02% Na-azide) was added at 100µl/well in duplicates. To control for inter-assay and day-to-day variations in the standardized ELISA procedure, each assay (ELISA plate) included a calibration curve obtained by a 3-fold serial dilution of pooled hyper immune plasma (Standard pool) known to be positive for total IgG to the specific malaria antigens tested (CSP, CelTOS, $MSP1_{19}$, AMA1-FVO, AMA1-3D7). For CSP and CelTOS, the pool was diluted at 1:50, 1:100 for $MSP1_{19}$, and 1:10000 for AMA1-FVO and AMA1-3D7. A buffer blank (plasma dilution buffer) served as control for detection of background responses. The plates with the samples and standard pool plasma were then incubated at room temperature for 1 hour in a humidified chamber after which they were washed four times in washing buffer.

Secondary antibody, goat anti-human IgG (H+L), HRPO conjugated (Invitrogen Corporation, Camarillo, CA; USA), diluted at 1:10,000 in conjugate dilution buffer (PBS with 1% milk powder, 0.1% Tween-20), was added at 100µl/well. The plates with the conjugates were incubated for 1 hour at room temperature after which they were washed four times in wash buffer and padded dry. Bound secondary antibody was quantified by incubation with ready to use TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate (Kem-En-Tec Diagnosis A/S, Taastrup, Denmark) for 5 minutes in the dark. The enzymatic reaction was stopped with 100µl/well of 0.2 M H_2SO_4.

Optical density (OD) was read at 450 nm with a reference wavelength of 630 nm using a Biotek EL 808 ELISA plate reader (Biotek Instruments, Winooski, VT; USA). Optical density (OD) values for the test samples were converted into antibody units (AU) with the standard reference curves generated for each ELISA plate using a Microsoft Excel-based four parameter logistic curve-fitting application (ADAMSEL b040, Ed Remarque© 2009).

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Samples were re-tested if the coefficient of variation between duplicate optical densities were higher than 30% and plates were also re-tested if the R-square value of the standard curve was less than 95%.

3.5.4 Competition ELISA

3.5.4.1 Determination of dilutions for Competition assay

Competition ELISA was done to determine relative specificities of antibodies against 2 AMA1 antigens (AMA1-FVO, AMA1-3D7) using a modified version of the protocol described by (Kusi *et al.*, 2009)

Competition assay was performed first by estimating dilution that yields approximately 2AU based on the ELISA procedure described above and these dilutions were used for the competition assay.

The concentration of antigens to use in the competition assay was determined by serially diluting the competing antigens and it was realised that at 30µg/ml almost all antigens are depleted after competing with same antigen as the coating antigen. The assay was also used to determine the concentration at which much of the other competing antigens would be depleted by using two plasma samples, one from a 6 year old child, and the other from a 45 year old adult. AMA1-3D7 and AMA1-FVO were used as coating antigens. The competition antigens were serially diluted three (3) fold from a high concentration of 30µg/ml to 0.04µg/ml; the last well had no competing antigen. Competing antigens were added first followed by the diluted samples at 50µl/well. Plates were incubated for 1 hour at room temperature in a humidified chamber and developed as describe above. ODs for the wells with competing antigens were expressed as a percentage of OD values from wells without competing antigens as percentage antibody depletion by the competing antigens.

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The percentage depletion was then plotted with a predicted percentage curve with a least square approximation using a four-parameter logistic function:

$$Y = \frac{(100\text{-}Y\ min)}{1 + e^{(Xmid-X)sc}} + Y\ min$$

Results were reported as percentage depletion/Residual (minimum) binding value.

3.5.4.2 Competition assay

Microtitre plates (96 well) were coated with each antigen (AMA1-FVO and AMA1-3D7) at 1µg/ml with 100µl/well coating buffer, incubated overnight and blocked as described above. 50µl/well of competing (alleles) antigens (AMA1-3D7, AMA1-FVO, AMA1-CAMP, AMA1-HB3, and AMA1 –DICO MIX) were diluted at 30µg/ml and first added to the wells in duplicates. All five competing antigens were added on one plate with a standard plasma pool titrated 3 fold, with a highest dilution of 1:10,000. Appropriately diluted plasma sample each was added at 50ul/well to the competitor antigens to co-incubate. For any competing antigen added per sample, another 50µl/well of the same sample was also added in duplicate wells without competing antigens to be used to calculate percentage inhibition by the competing antigens. Plates were incubated at room temperature for 1 hour and developed as described above. OD values were converted to arbitrary units and expressed as a percentage of AU values from wells without soluble antigens using the formula below:

$$\text{Percentage antibody depletion} = \frac{\text{Average AU with competing antigen}}{\text{Average AU without competing antigen}} \times 100\%$$

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3.5.5 DNA Extraction

DNA was extracted from stored filter paper (Watman 3MM, Watman Int. Ltd, England) using a modified version of the Chelex extraction protocol described by Wooden (Wooden *et al.*, 1993).

A 3 mm square piece of a blood blot on filter paper was cut out with a sterile punch into labeled 1.5ml microfuge tubes. About 1ml of autoclaved 1X PBS was added followed by 50µl of 10% saponin solution. The tube was vortexed (Stuart Scientific, UK) gently, and incubated at 4°C overnight. The tubes were then centrifuged at 14000 rpm for 30 seconds and the supernatant discarded. One milliliter (1ml) 1X PBS without saponin was added to the tubes and inverted several times and incubated at 4°C for 30 minutes. The tubes were again centrifuged at 14000 rpm for 30 seconds and the supernatant discarded leaving pellets.

The pellets were re-suspended in 100µl of sterile distilled water and centrifuged at 14000rpm for 30 seconds and the supernatants discarded. The pellets were again re-suspended in 100µl of sterile distilled water followed 50µl of 20 % w/v Chelex-100 resin suspension in deionized water. The tubes were vortexed and incubated at 95°C using a Techne heating block, (Bibby Scientific Ltd, UK) for 10minutes with vortexing at 2minutes intervals. After incubation, the tubes were centrifuged at 14,000 rpm for 5mins and then the supernatant (DNA) was transferred into pre-labeled 0.5 ml microfuge tubes for use as template in PCR applications.

The concentration of the genomic DNA was determined using Nanodrop 2000C spectrophotometer (Labteck International, UK) and stored at -20°C until ready for use.

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3.5.6 PCR Amplification

The oligonucleotide primers used in this study were picked from already published sequences by (Smythe *et al.*, 1990), to amplify the polymorphic block 3 region of the MSP-2 gene. Polymerase Chain Reaction (PCR) method was adapted from (Snounou and Singh, 2002, Snounou, 2002) with slight modifications.

Block 3 region of the MSP2 gene was amplified using the published sequence specific primers by (Smythe *et al.*, 1990): forward primer, (5'-ATGAAGGTAATTAAAACATTGTCTATTATA-3'), and the reverse primer (5'-CTTTGTTACCATCGGTACATTCTT-3'). Template DNA was amplified using nested PCR, with primers 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 A-3').

Separate reactions were performed for each pair of primary and nested primers. For the primary reaction, the following cycling conditions were used: denaturation at 94°C for 2 minutes preceded 30 amplification cycles: denaturation for 30 seconds at 94°C, annealing for 1 min 30 seconds at 55°C, and extension for 1 min at 72°C. The last extension was carried out for 5 minutes.

The family specific (IC3D7 family) nested reaction was performed with the following conditions: denaturation at 94°C for 2 minutes preceded 30 amplification cycles: denaturation for 30 seconds at 94°C, annealing for 1 min 30 seconds at 58°C, and extension for 1 min at 72°C. The last extension was also carried out for 5 minutes. Both primary and nested reactions were done in a 25ul final volume, containing 2.5ul 10X reaction buffer, 2uM $MgCl_2$, a 200uM concentration of a mixture of the four dNTPs, a 300nM concentration of each of the two appropriate primers, and 1U of *Taq* DNA

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polymerase (Sigma-Aldrich, St. Louis, MO, USA). In the first reaction, 5ul of Chelex-extracted DNA was added as a template, and 1ul of the first PCR product was added in the second reaction. All reactions had a genomic DNA from 3D7 laboratory strain which was used as positive control, and molecular grade water was used as negative control.

The PCR products were analysed by electrophoresis on 2% agarose with ethidium bromide as the stain with a standard 100pb ladder. The DNA (bands) was visualized using UV trans-illumination, and fragments obtained were compared by size with a ladder loaded onto the gel.

Positive samples were recorded with their band size and the number of bands per sample which refers to the clones in a sample.

3.5.7 Data Analysis

Optical density OD values were converted to antibody units using a four parameter logistic fit, ADAMSEL (developed by Remarque). Mann-Whitney (Non parametric) test was used to determine differences in antibody levels between the sites and seasons. Percentage antibody depletion/residual binding (or minimum) values in competition assay, and their matching confidence intervals were generated by a 4-parameter logistic fit with least squares approximation using the R statistical package (R Development Core Team, 2008). Multiplicity of infection was calculated by adding all the clones per individual and finding their mean in each cohort. Chi-square test was used to compare multiplicity of infection between the two sites and across seasons.

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CHAPTER FOUR

4.0 RESULTS

A total of 560 stored plasma samples from volunteers with ages ranging from 1-70 years were used in this study with 257 being males, and 303 females. During the rainy/wet season, 232 samples were obtained and 328 samples from the dry season. Forty-five percent of the samples obtained for the wet season were from males and 46% of dry season samples were from males. Mean age between the wet (17.1±0.9 years) and dry (16.8±0.7 years) seasons was not significantly different (*p = 0.937*, Mann-Whitney test). Stored filter paper samples were also used to determine parasitaemia by polymerase chain reaction (PCR). In the wet season 154 filter paper samples were used and in the dry season 134 filter paper samples were used. The overall median parasite density by microscopy among the subjects between the two seasons was also not significantly different (*p=0.971,* Mann-Whitney test). At enrolment during the rainy season, 79 subjects (34.1%) were carrying malaria parasites by microscopy, and 51 (15.5%) had parasites during the dry season (**Table 4.1).**

Table 4. 1 Characteristics of study subjects for the Wet and Dry Seasons

	Season		***P value***
Characteristics	**Wet Season**	**Dry Season**	
No. of Subjects	232	328	
Sex			
Males (%)	105 (45)	152 (46)	
Females (%)	127 (55)	176 (54)	
Age (SE)	17.1±0.9	16.8±0.7	*0.937*
Parasite Density (SE)	2703.2±1001.8	1872.1±474.6	*0.529*
Parasite Carriage (%)	79 (34.1)	51 (15.5)	

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4.1 Parasite Density and Proportions at the Sites

Parasite detection was done by microscopy (459 samples) and PCR in a subset of participants (290) whose filter paper samples were available. Samples were grouped into two based on proximity to the dam site and proportion of parasites compared between the sites. For both microscopy and PCR there was no difference in the proportions that were parasite positive at the two sites during the wet season. However the proportion that was parasite positive at the dam site was significantly greater than that at the non-dam site in the dry season (Tables 4.2 and 4.3). For samples that were parasite positive there was no significant difference in median parasite density between the dam sites and non-dam sites at any of the two seasons (p>0.05 in both cases).

Table 4. 2 Proportion of individuals carrying parasites by microscopy

Season	Dam	Total No. of Subjects	No. parasitaemic	No. Non-parasitaemic	P value*
Wet	Yes	92	27	65	0.21
	No	139	52	87	
Dry	Yes	155	26	129	0.0061
	No	73	24	49	

* P value obtained after Chi-square test for differences in proportion

Table 4. 3 Proportion of individuals carrying parasites by PCR

Season	Dam	Total No. of Subjects	No. parasitaemic	No. Non-parasitaemic	P value
Wet	Yes	67	40	27	0.4520
	No	89	48	41	
Dry	Yes	69	66	3	<0.0001
	No	65	28	37	

* *P-value* obtained after Chi-square test for differences in proportion. Filter paper blood samples were not available for all participants, hence differences in the number of subjects tested by microscopy and PCR.

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4.2 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 wet and dry seasons were 1.76 and 1.46 (*p=0.001*) respectively (Table 4.4). When samples were categorized based on proximity to the dam, there was no difference in the MOI between the wet season and the dry season in individuals close to the dam and those who live far away. However significant differences were observed in the MOI values in the dry season between the sites, and also the dry season had lower MOI compared to the wet season. Also 60.2% of samples in the wet season had multiple infections compared to 40.3% in the dry season. Figure 4.1 shows a gel photograph with DNA bands that indicate whether a sample has single or multiple parasite clones.

Table 4. 4 Multiplicity of *Plasmodium falciparum* infection as assessed with MSP-2 marker

Number of PCR Fragments	Wet Season (%)	Dry Season (%)	*p value*
1	43 (39.8)	70 (58.8)	
2	49 (45.4)	43 36.1)	
3	15 (13.9)	5(4.2)	
4	1 (0.9)	1 (0.8)	
Multiplicity of infection	1.76	1.46	*0.001**

Table 4. 5 Mean multiplicity of *Plasmodium falciparum* infection between sites

	Wet Season	Dry Season	**P value*
Dam site	1.8	1.6	*0.242*
Non-Dam Sites	1.7	1.0	*<0.001*
#*P value*	*0.599*	*<0.001*	

P values obtained following comparison between season (*) or between sites (#) by the student t test

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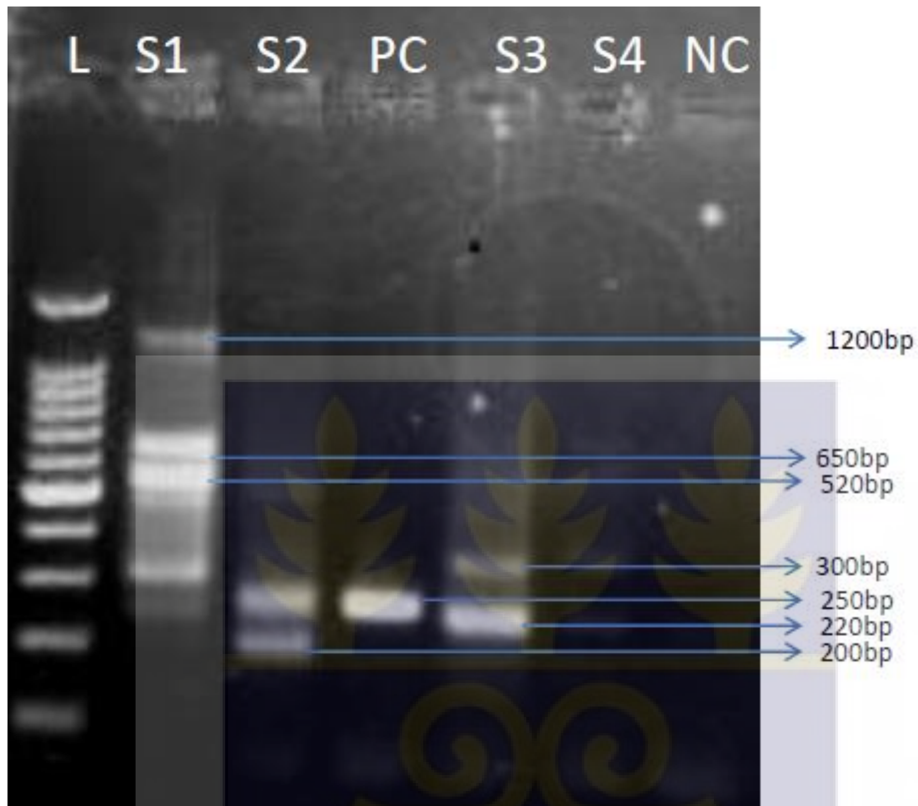

Figure 4. 1 Agarose gel photograph with a 100bp molecular weight DNA marker indicating multiple infection

L	100bp molecular weight marker
NC	Negative control
S1, S2 &S3	Samples with single infections
PC	Positive control (single infection)

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4.3 Total IgG Levels in Plasma Samples between Sites

Levels of anti-malarial antibodies were compared between the two study sites for each season. The levels of antibodies to the two blood stage antigens, AMA1 and, $MSP1_{19}$, were not significantly different between the sites for both the wet and dry seasons. However the levels of antibodies to the two sporozoite antigens, CSP and CelTOS, were significantly different between the two sites for both seasons (Figure 4.2).

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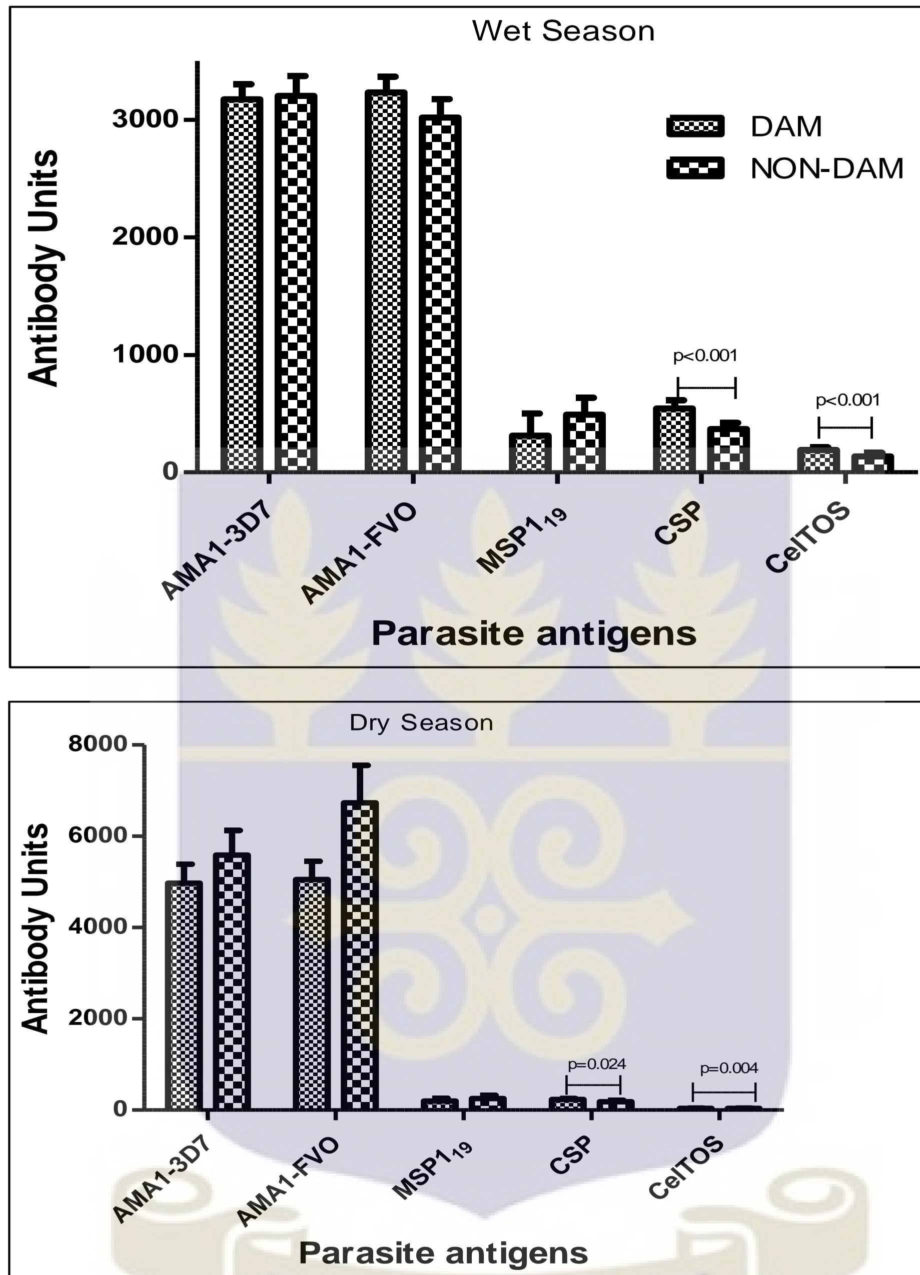

Figure 4. 2 Comparison of anti-malarial IgG responses between the two sites.

Error bars are standard error of the mean (SEM).

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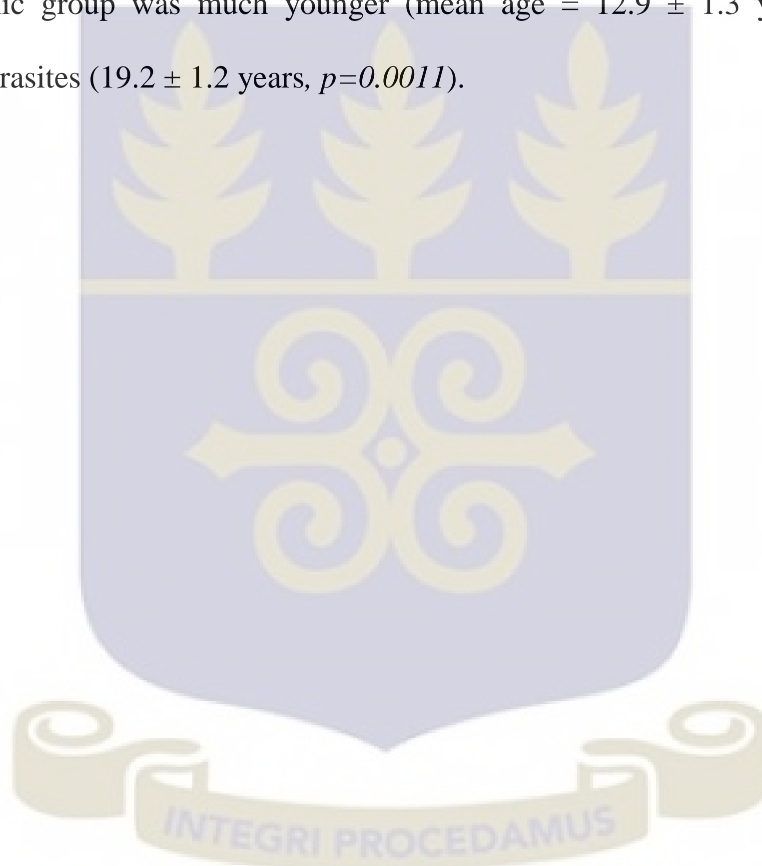
4.4 Parasite Carriage and IgG Responses

Total IgG responses were categorized according to season, proximity to the dam, and parasitaemia (Figure 4.3). The wet season the data showed no significant differences in the IgG levels to all the parasite antigens in samples with and without parasites across dam sites. A similar result was obtained during the dry season where differences were not found between the parasitaemic and non-parasitaemic groups. However the parasitaemic group was much younger (mean age = 12.9 ± 1.3 years) than those without parasites (19.2 ± 1.2 years, $p=0.0011$).



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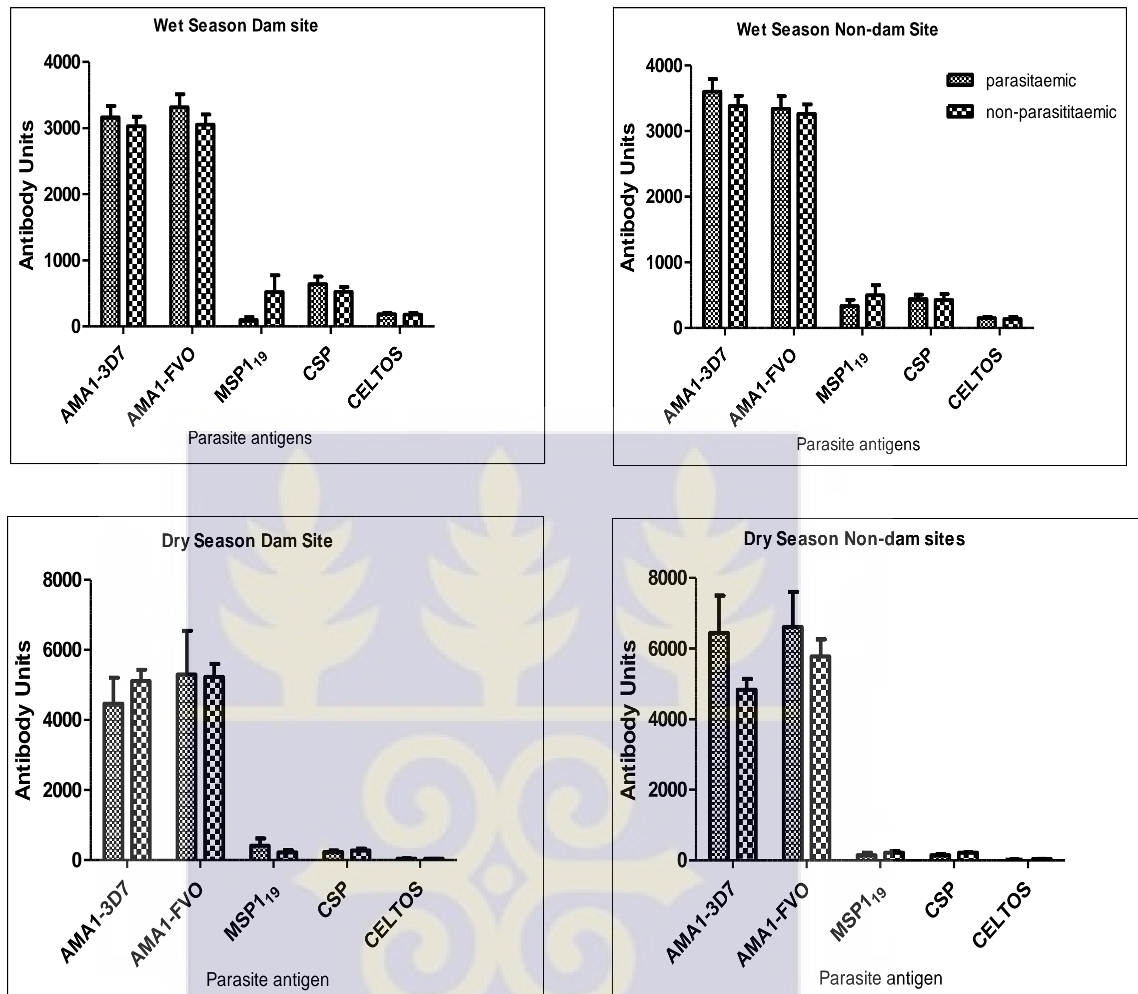

Figure 4. 3 Total IgG levels and parasitaemia between sites in the two seasons

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4.5 Cross-reactive and Strain-specific Antibodies

Competition ELISA was used to determine whether naturally acquired anti-AMA1 antibodies in the different age groups were more strain-specific or cross-reactive. Four natural alleles of AMA1 (3D7, FVO, HB3, and CAMP), expressed as recombinant antigens, and three *in-silico* designed AMA1 proteins known as *Di*versity-*Co*vering (or DiCo) proteins, were used in the assay. An equimolar mixture of the three DiCo proteins, referred to as DiCo-mix, was used as a positive control in all assays.

Initially, cross-reactive and strain-specific antibodies in the plasma of two study participants (a 6 year old child and a 45 year old adult) against the AMA1-3D7 and AMA1-FVO allelic antigens were determined. This was to prove the concept that antibody responses in children are most likely to be skewed towards strain specificity since they would have had limited exposure to diverse parasite populations. Adult antibody responses, in contrast, may be more focused on the common antigenic components of the numerous parasite variants they are likely to have been exposed to, and would hence be more cross-reactive in nature.

Cross reactive antibodies are identified in competition ELISA on the basis of their depletion from an allelic antigen-coated plate by a soluble competitor allele that is co-incubated with the diluted plasma sample. Strain-specific antibodies (against the coated allele) cannot be depleted by the competitor allele in a similar system and therefore bind to the coated allele. Thus if the same allelic antigen is used for plate coating and as a competitor antigen, it is expected that there will be near complete depletion of antibodies. If two different allelic forms of the antigen are used for coating and competition respectively, only the fraction of antibodies that is cross reactive between these allelic forms will be depleted by the competitor antigen. Antibody depletion by

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competitor antigens was expressed as residual binding (minimal) value. Thus for example a 20 residual binding value means 20% antibodies are bound to the coated antigens and are therefore specific to the coating antigen relative to the competitor antigen while the rest 80% in solution are cross reactive and share common epitopes.

On the basis of this principle, assays were performed with either AMA1-3D7 or AMA1-FVO as the coating allele and for each coated plate; AMA1-3D7, AMA1-FVO, AMA1-HB3, AMA1-CAMP and DiCo mix were used as competitor antigens. The depletion patterns show that the child plasma sample does have anti-AMA1 antibodies that may be 3D7 strain-specific, especially relative to the FVO, HB3 and CAMP AMA1 alleles (Figure 4.4 A & C, Table 4.6) whilst the adult plasma sample has a higher proportion of cross-reactive antibodies as all AMA1 alleles depleted statistically similar proportions of anti-AMA1 antibodies (Figure 4.4 B & D, Table 4.6). The DiCo mix control in all cases depleted similar proportions of antibodies as the homologous 3D7 antigen (over 95% since the highest residual binding observed was 4.4% for the adult sample assayed on the 3D7 AMA1-coated plate (Figure 4.4, Table 4.6).

Based on the above interpretation, 60 samples with high responses to both AMA1-3D7 and AMA1- FVO antigens were selected from each season and the assay was run with only the highest competing antigen concentration of 30µg/ml. The data was categorised into single and multiple clones and the mean residual (minimal) binding values plotted

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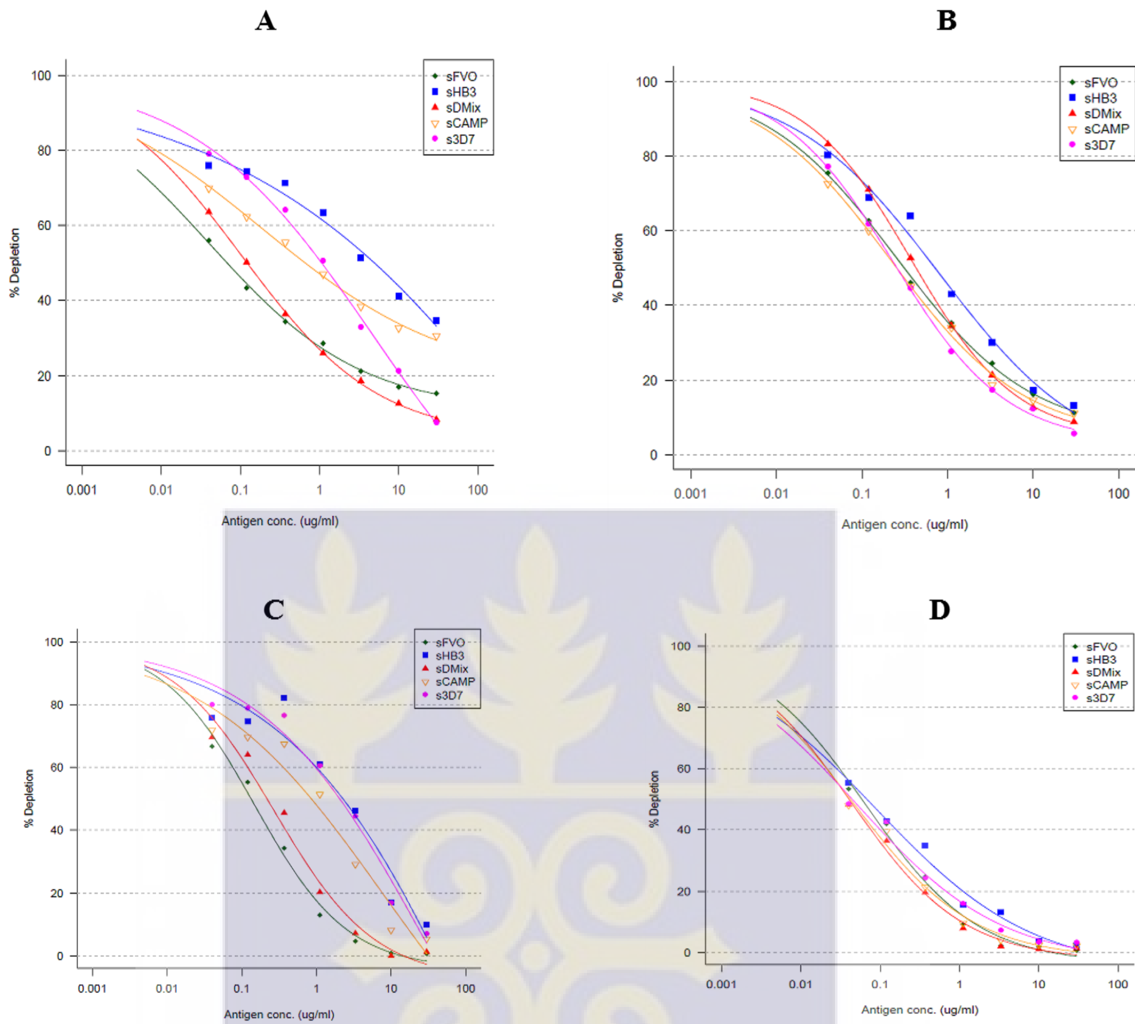

Figure 4. 4 Competition ELISA with plasma from a child and an adult

Assay on AMA1-3D7 coated plate. The experimentally determined data (points) were fitted to a 4-parameter logistic (lines). Plot A is the curve for the plasma from a child and plot B is the curve for the plasma for an adult. For the AMA1-FVO coated plates, plot C is for a child and plot D for adult.

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Table 4. 6 Residual anti-AMA1 IgG binding (minimum values) estimates for Adult and child plasma samples.

Coating Antigen	Plasma Sample Source	Competitor Antigens				
		3D7^	**FVO**	**CAMP**	**HB3**	**DICO MIX**
3D7	Child	-39.0# (-81.0-2.9)	10.8* (6.4-15.1)	18.3* (7.7-28.9)	-182.7# (-1686.8-1.3)	3.6 (1.5-5.7)
	Adult	3.0 (-0.5-6.5)	1.9 (1.9-9.7)	4.3 (-2.9-11.5)	-3.1# (-27.0-20.8)	4.4 (3.0-5.8)
		3D7	**FVO^**	**CAMP**	**HB3**	**DICO MIX**
FVO	Child	-91.5# (-403.9-221.0)	-4.1* (-12.8-4.5)	-61.1# (-278.2-156.1)	-187.3# (-1769.1-1.4)	-7.6# (-24.3-9.1)
	Adult	-3.4# (-15.1-8.3)	3.5 (-11.6-4.5)	-2.1# (-10.7-6.5)	-6.5# (-22.6-9.7	-2.5# (-8.6-3.6)

Residual binding (minimum) value with 95% confidence intervals
Residual binding values were generated with a four parameter logistic fit with least square approximation. These values are predicted minimum values based on measured values for competitor antigens.
^homologous competitor antigen, ie the same competitor antigen was used for coating the ELISA plate for that assay.
* Antibody depletion levels for these competitor antigens are significantly different from that of the homologous competitor antigen for the same plasma sample.
#Negative values indicates that the curves (minimum values) have not yet reached a plateau, but the confidence interval includes zero and hence the minimum values are not significantly different from zero (Fig 9 C).

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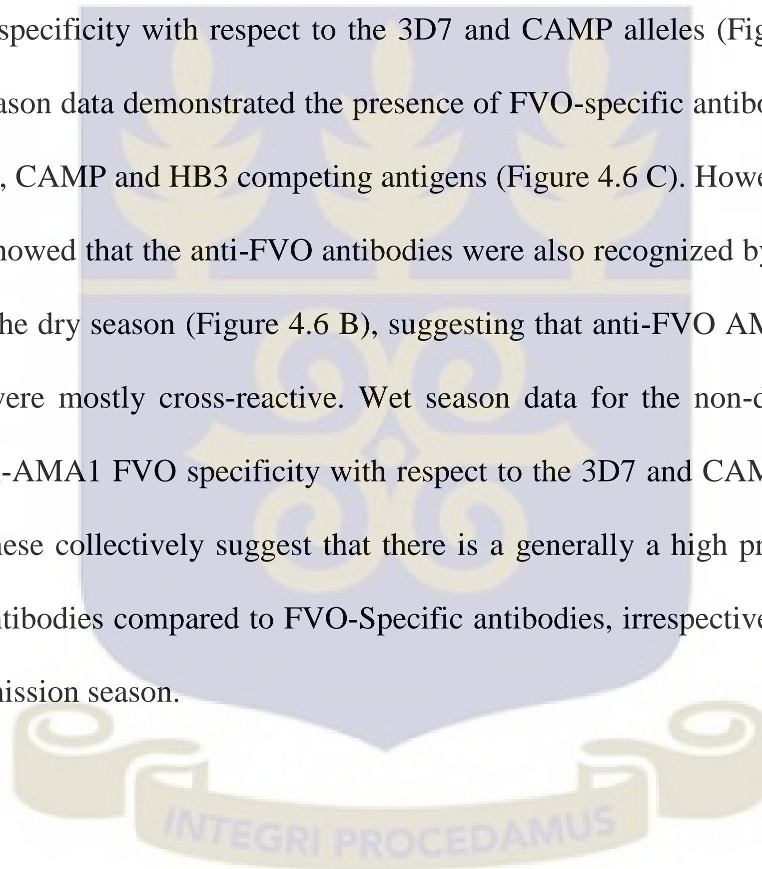
4.6 Cross Reactive and Strain Specific Responses at the Sites

On the basis of the explanation of the preliminary competition ELISA data above, the complete data for each season was compared between the two sites. For both the wet and dry seasons, assays on the 3D7-coated plate showed the presence of 3D7-specific since the other 3 competing antigens (FVO, CAMP and HB3) depleted significantly less of the antibodies recognized by the 3D7 antigen at both sites (Figure 4.5). For assays done on FVO coated plates, the dry season data for the dam site only showed anti-FVO specificity with respect to the 3D7 and CAMP alleles (Figure 4.6 A) while the wet season data demonstrated the presence of FVO-specific antibodies with respect to the 3D7, CAMP and HB3 competing antigens (Figure 4.6 C). However, the non-dam site data showed that the anti-FVO antibodies were also recognized by all other AMA1 alleles in the dry season (Figure 4.6 B), suggesting that anti-FVO AMA1 antibodies at that site were mostly cross-reactive. Wet season data for the non-dam site however shows anti-AMA1 FVO specificity with respect to the 3D7 and CAMP alleles (Figure 4.6 D). These collectively suggest that there is a generally a high proportion of 3D7-specific antibodies compared to FVO-Specific antibodies, irrespective of the study site and transmission season.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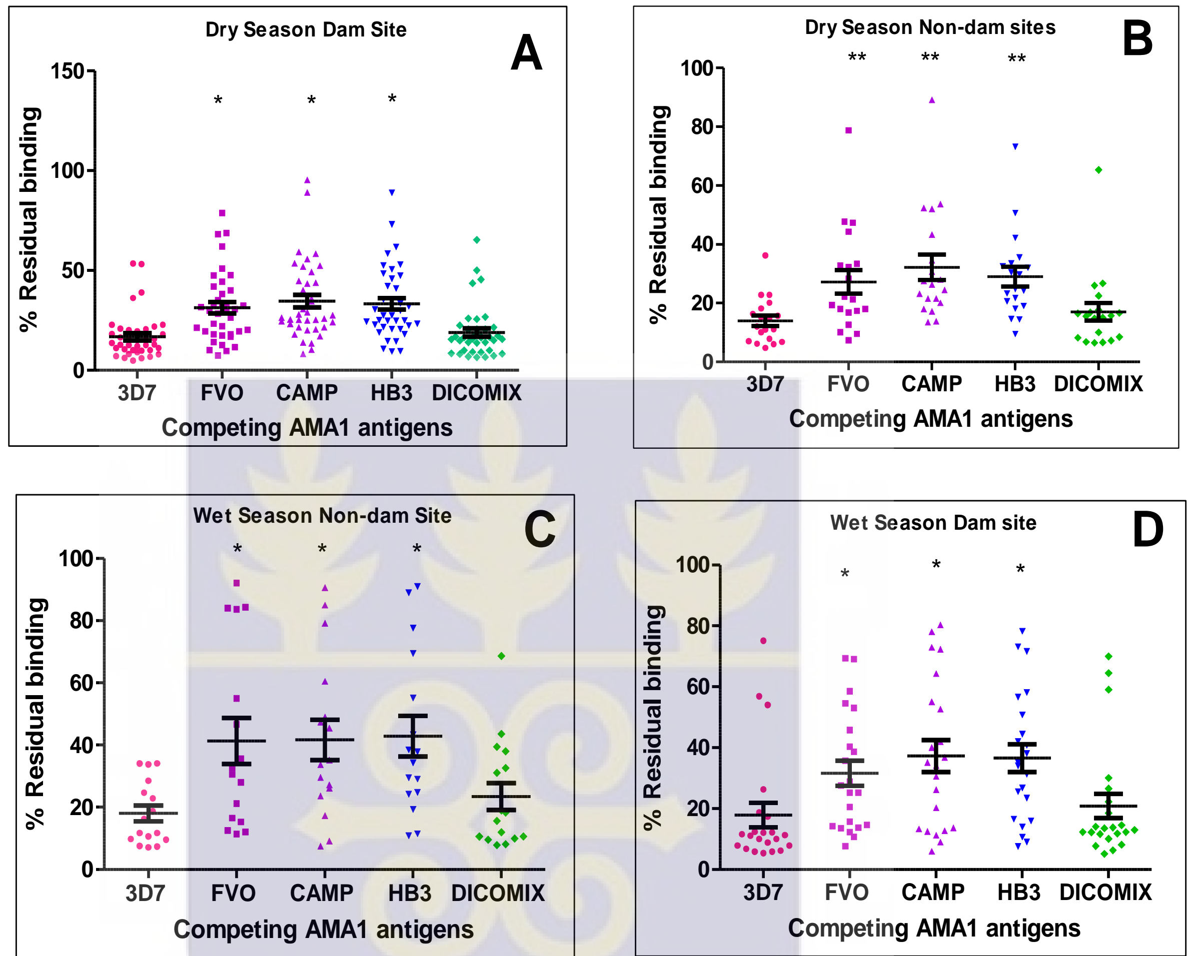

Figure 4. 5 Residual binding estimate for all competing antigens on 3D7 coated plates.

The 3D7 competing antigen is expected to have the least residual binding since it will deplete the most antibodies from the coated plate.

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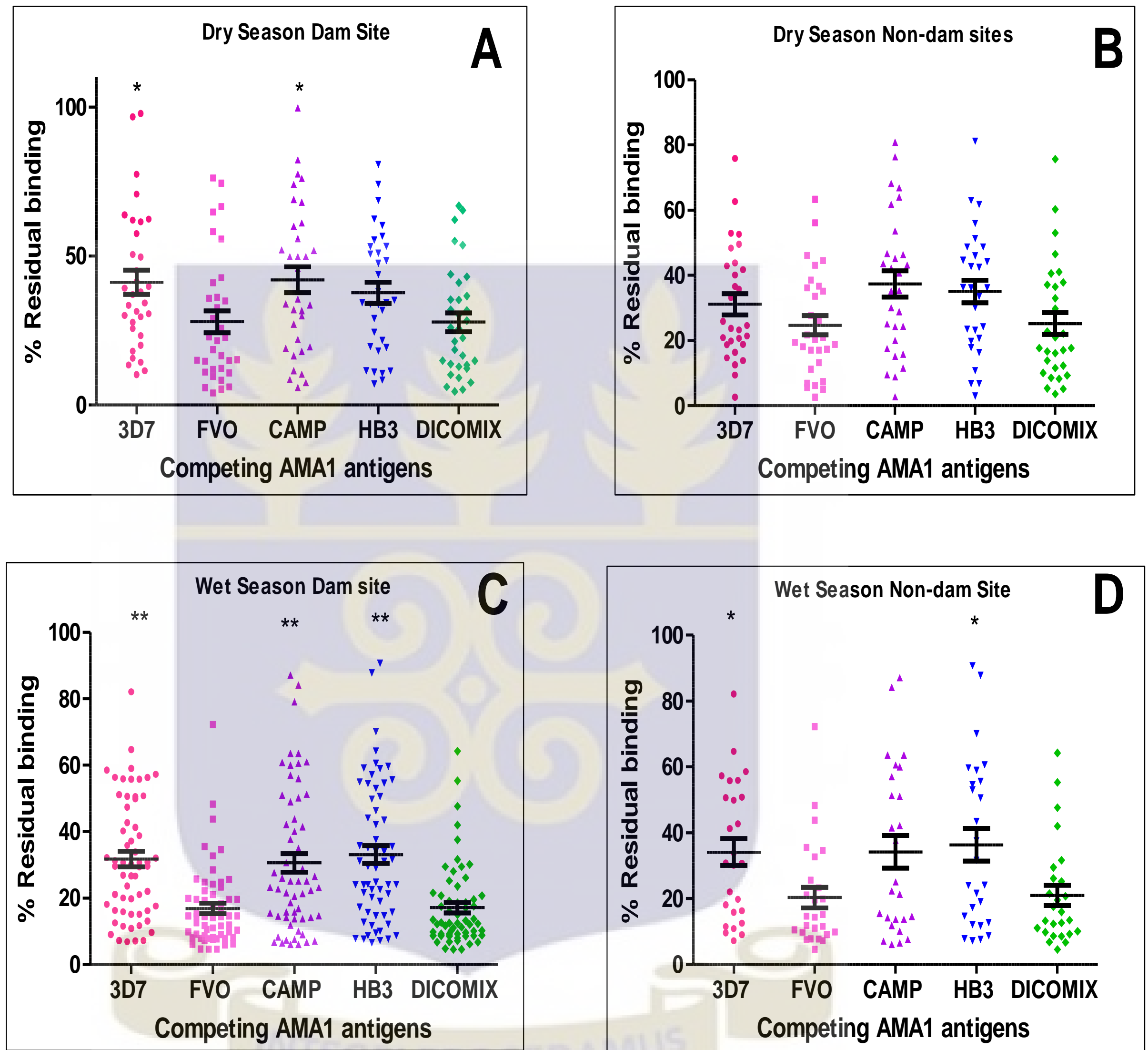

Figure 4. 6 Residual binding estimate for all competing antigens on FVO coated plates.

The FVO competing antigen is expected to have the least residual binding since it will deplete the most antibodies from the coated plate.

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CHAPTER FIVE

5.0 DISCUSSION

Anti-malarial antibodies are an important component of immune response to *P falciparum* infection in humans and the induction of the right quality of antibodies is important for protection against infection. In malaria endemic areas, adults beyond a certain age are partially protected from clinical disease and this protection is mediated in part by antibodies following repeated infection. These antibodies in adults have been proposed to be an accumulation of different strain specificities following infection with diverse parasites or are more cross-reactive in nature as the immune response is focused on epitopes that are common to the diverse infecting parasites (Doolan *et al.*, 2009). Irrespective of which of these proposed mechanisms is true the quality of antibodies induced within a given population would depend on the transmission dynamics and the parasite diversity within that population.

This study was aimed at investigating the effect of parasite diversity and disease transmission pattern on the levels of antibody responses to *P. falciparum* in individuals living in an area of seasonal malaria transmission. Two blood stage antigens ($MSP1_{19}$ and seven variants of AMA1) and two liver stage antigens (CSP and CelTOS), were used for antibody analysis in this study since they are amongst the leading vaccine candidate antigens currently in pre-clinical or clinical testing. These antigens were tested against stored plasma samples from individuals living in two sites; one in close proximity to an irrigation dam and the other at least 20 kilometres away from the dam.

Parasites were detected by microscopy and PCR and for both methods, the proportions of study participants who were parasite-positive were significantly greater at the dam site compared with those at the site away from the dam (non-dam site) during the dry

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but not the wet season (Tables 4.2 and 4.3). This may be a direct consequence of the presence of the dam, which serves as possible breeding grounds for disease vectors and results in higher levels of disease transmission at this site, relative to the non-dam site. Thus although the area has been generally described as one with seasonal transmission (Appawu *et al.*, 2004) malaria transmission in communities around the dam may not be as seasonal.

Diversity of infecting parasites was investigated by molecular typing of the block 3 region of the *MSP 2* gene and the results showed the number of infections per person ranged from 0 to 4 in both seasons and the mean multiplicity of infection (MOI) was generally higher in the wet season compared to the dry season (1.76, and 1.46, $p=0.001$, Table 4.4). Comparing MOI between study sites there was a statistically significant difference between sites during the dry but not the wet season. (Table 4.5). It has been shown that in malaria endemic regions parasite diversity in high malaria transmission areas are high and that individuals could carry multiple genotypes (clones) but the opposite pertains in low endemic areas with most infections being monoclonal (Peyerl-Hoffmann *et al.*, 2001, Babiker *et al.*, 1997, Haddad *et al.*, 1999). This therefore supports the conclusion that transmission at the dam site may be higher than at the non-dam sites during the dry season.

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Comparison of antibody levels between study sites showed that there were statistically significant difference in anti-sporozoite (CSP and CelTOS) antibody levels between the two sites for both transmission seasons whilst no such differences were observed for the two blood stage antigens (AMA1 and $MSP1_{19}$) (Figure 4.2). This observation may be explained by the fact that antibodies to AMA1 and MSP1, due to the cyclic nature of the blood stage infection, may persist for much longer periods while antibodies to the

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sporozoite antigens are short lived and are boosted only when there are new infectious bites (Campo *et al.*, 2011, Torres *et al.*, 2008, Kusi *et al.*, 2014). This finding therefore supports the use of sporozoites antigens for monitoring malaria transmission

Though differences in the proportions of infected individuals between the two study sites were observed, the median parasite densities for infected individuals were not significantly different by study site or season.

Comparison of antigen specific antibody levels between parasitaemic and non-parasitaemic individuals within the different study sites did not show any significant differences (Figure 4.3). Thus antibody levels were not dependent on the presence of parasites at the sampling time point. It is possible that individuals who were non-parasitaemic may have recently recovered from an infection

Apical membrane antigen, AMA1 has been found to be a promising blood stage vaccine candidate antigen because of the critical role it plays in erythrocyte invasion (Remarque *et al.*, 2008a). But this potential has been dampen due to extensive polymorphism (Marshall *et al.*, 1996).

In a study by (Kusi *et al.*, 2009), rabbits immunized with alleles of AMA1: 3D7, FVO HB3, and a mixture of these three antigens elicited high levels of cross-reactive antibodies to the antigen mixture compared the single allele immunization and concluded that cross-reactive antibodies were to epitopes that are shared by the three antigens. Thus the competition ELISA assay could be used to distinguish between responses to a single antigen and responses to simultaneous multiple antigens.

Application of this to human infection is however limited by the fact that the infecting parasite strain history may not be known. Nevertheless this concept was tested in two naturally exposed humans and the data showed the possibility of detecting strain

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specific antibodies in the child and a more cross-reactive antibodies in the adult (Figure 4.4) While this data is based on just two samples two main conclusions can be drawn; i) that adults with numerous exposures to parasites may have a more cross-reactive repertoire of antibodies compared to children who have had only a few parasite exposures, corroborating previous findings (Cortes *et al.*, 2005a, Kusi *et al.*, 2012), and ii) the competition as described for the rabbit data can be applied to human samples for the detection of strain specific and cross-reactive antibodies.

Applying the assay to samples from the two sites using 3D7 coated plates the 3D7 competing antigen always depleted significantly more antibodies compared to the FVO, CAMP and HB3 alleles irrespective of the study site or season (Figure 4.5), suggesting that there were 3D7 specific antibodies (relative to the other competing antigens) at both study sites. Assays done on the FVO coated plates however showed high proportions of cross-reactive antibodies in the dry season at the non-dam sites since the FVO competing antigen depleted similar quantities of antibodies as the other competing antigens (4.5 B). This therefore suggest a relationship between antibody specificity and MOI; antibody specificities to the both 3D7 and FVO AMA1 alleles were observed where the MOI was greater than 1 but specificity to the only the 3D7 allele was observed where the MOI was 1. This observation can be explained by the fact that individuals with multiple infections may be better protected because of the production of cross-reactive responses whiles individuals with single infection are not because of the production of antibodies specific to only the single clone hence introduction of a new clone could easily cause disease. This could explain why in high transmission settings some individuals are better protected compared to low transmission areas (Rogier and Trape, 1993, Doolan *et al.*, 2009).

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CHAPTER SIX

6.0 Conclusions and Recommendations

6.1 Conclusions

Data from the current study shows the multiplicity of infection was greater than one (MOI > 1) at the dam site irrespective of the season, and at the non-dam site only during the wet season. This suggests that MOI is high in areas of moderate to high transmission and similar findings have been described in other areas. This observation is possibly linked with the availability of water, most likely as possible breeding sites for the mosquito vectors. Thus despite the label of the larger study area being one with seasonal malaria transmission, disease transmission in communities around the dam, on the basis of the MOI estimated in this study may have transmission levels as high as occurs during the wet season. Communities around such large water bodies could therefore experience year round malaria transmission with high diversity of parasites. If these findings are confirmed, it will provide a basis for targeted control of malaria.

It was also observed that when transmission intensity was relatively higher (as determined by the estimated MOI > 1), antibodies that were specific to either the 3D7 or FVO allele of AMA1 were elicited, while mostly 3D7 AMA1-specific antibodies were elicited (MOI = 1) under periods of low transmission. If this observation is confirmed for other polymorphic antigens, it could partly explain the difference in acquired malaria immunity between moderate to high transmission areas and areas with very low transmission. In addition, levels of antibodies to sporozoite antigens, but not those to blood stage antigens, were different between the wet and dry seasons, reflecting possible exposure to a greater number of infectious bites during the wet

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season. This finding supports the use of anti-sporozoite antibodies as markers for monitoring malaria transmission in endemic areas.

6.2 Recommendations

1. Future studies should include an entomological component to directly assess the vector population and possible differences in inoculation rates between communities around such large water bodies and those that are a good distance from it.

2. 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.

3. The use of other polymorphic antigens to assess antibody responses is important for confirmation for conclusions drawn on the possible effect of AMA1 diversity on immune responses

4. *In vitro* functional studies such as growth inhibition assays involving purified IgG must be conducted in future to determine the functional quality of antibodies and the relative contributions of strain-specific and cross-reactive antibodies to parasite invasion of red cells.

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Appendixes

7.1 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 PCRs solutions were obtained commercially.

Blocking Buffer (PBS with 5 % milk powder, 0.1% Tween-20)

To prepare 500ml, 1 tablet of PBS was added to a beaker containing 500ml deionised water and mixed using a magnetic stirrer. 0.5ml of tween-20 was added and then 25.0g of skimmed milk was then added and stirred until all were in solution.

Plasma Dilution Buffer (PBS with 1 % milk powder, 0.1% Tween-20 and 0.02% Na-azide)

To prepare 500.0ml of the plasma dilution buffer, 1 PBS tablet was added to a beaker containing 500.0ml deionised water and mixed on a magnetic stirrer. After the tablet was dissolved, 5.0 g of skimmed milk, 0.5 ml of Tween-20 and 1.0 ml of 10 % Na-azide solution were added and the solution stirred until a homogeneous mixture was obtained.

The 10 % Na-azide solution was prepared by adding 40.0ml of deionised water to 4.0g of Na-azide.

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Conjugate Dilution Buffer (PBS with 1% milk powder and 0.1% Tween-20)

500.0ml of conjugate dilution buffer was prepared by adding 1 tablet of PBS a beaker containing 500.0ml deionised water on a magnetic stirrer to mix. 5.0 g of skimmed milk and 0.5ml of Tween-20 was added.

Washing Buffer (PBS with 0.1% Tween-20)

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.

Colour Solution [TMB (3, 3', 5, 5'-Tetramethylbenzidine)]

Ready to use TMB plus2 (3, 3', 5, 5'-Tetramethylbenzidine) solution was obtained from commercially from the manufacturer (Kem-En-Tec Diagnosis A/S, Taastrup, Denmark).

Stop Solution (0.2M H2SO4)

500.0ml of stop solution, 10.0ml of 10.0M H_2SO_4 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

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7.2 PCR Materials

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

Gel loading buffer

Commercially obtained gel loading buffer (GelPilot DNA loading dye 5X, Qiagen,) was obtained and stored at 4°C until required.

DNA Ladder

DNA molecular weight size markers Direct Load 100bp DNA molecular weight marker was obtained commercially and stored at -20°C until required.

Agarose gel

To prepare a 2%w/v agarose gel, 4.0g of agarose powder (SeaKem®GTG® Agarose, Lonza, Rockland, ME, USA) was put into flask and X1 TAE was added to make a volume of 200ml. It was heated in microwave oven for 4 minutes to dissolve. 3ul of Ethidium Bromide (AppliChem, Damstadt, Germany) was added. The gel was then cast to set in a chamber with comb to make the wells.