

**ROLE OF ANTIBODIES AGAINST ANTIGENS OF *PLASMODIUM*
MEROZOITE AND INFECTED RBC SURFACE IN MALARIA IMMUNITY**

BY

JOHN YAW NUSEDONU

(10507512)

**THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN
PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MPhil
MEDICAL MICROBIOLOGY DEGREE.**

INTEGRI PROCEDAMUS

JULY, 2018

DECLARATION

I hereby declare that except for references to other people's work, which I have duly acknowledged, the experimental work described in this thesis was performed by me, John Yaw Nusedonu at the Department of Medical Microbiology, School of Biomedical and Allied Health Sciences and Immunology Department, Noguchi Memorial Institute of Medical Research, UG, under the supervision of Dr. Patience Borkor Tetteh-Quarcoo and Dr. Kwadwo Asamoah Kusi. This work neither in whole nor in part had been submitted for another degree elsewhere.

Candidate

Sign: _____

Date: _____

JOHN YAW NUSEDONU

(10507512)

Supervisor

Sign: _____

Date: _____

DR. PATIENCE BORKOR TETTEH-QUARCOO

(Department of Medical Microbiology, SBAHS, UG)

Supervisor

Sign: _____

Date: _____

DR. KWADWO ASAMOAH KUSI

(Department of Immunology, NMIMR)

DEDICATION

This thesis is dedicated to God Almighty, the Alpha and the Omega, the beginning and the end for bringing me this far. I also dedicate it to my parents, Jane Kennedy and Richard Nusedonu, my sibling Grace Nusedonu and my cousin Mrs. Esther Effah-Afari for being my pillars.

ACKNOWLEDGEMENT

All praise and glory be to the Almighty God for seeing me through this course. My profound appreciation goes to my supervisors Dr. Patience Borkor Tetteh-Quarcoo and Dr. Kwadwo Asamoah Kusi for their guidance and support throughout this course.

Special gratitude to Prof Ben Gyan, Head of Immunology Department NMIMR for financial assistance during the early phase of my course and for his encouragements. Appreciation goes to Dr. Kwamena Sagoe, Head of Department of Medical Microbiology Department for his encouragements.

I also express my utmost appreciation to Mr. Kakra Dickson, Eric Kyei Baffour, Kojo Akyea Mensah, Dr. John Tetteh, Festus Acquah, Daniel Addo Gyan, Thomas Addison, Elizabeth Coffie, Belinda Acculey all working with the Immunology department for their indispensable support during the course of my work.

Not forgetting the Secretaries of Department of Medical Microbiology, my profound gratitude to them for their formidable help and their encouragements. A word of appreciation also goes to Emmanuel Afutu and Ernest Akomeah of the Department of Medical Microbiology for their support. My appreciation goes my course mates Aba Hayford, Paulina Otu (Mrs), Nancy Nartey, Farid Boadu, Roland Adukpo, Israel Atipoe, Emmanuel Frimpong and Samuel Blankson for their immense encouragements. My final gratitude goes to my family for keeping me going with their support and encouragements.

This thesis was supported by the Post-Doc graduate scholarship, NMIMR.

TABLE OF CONTENTS

DECLARATION	i
DEDICATION	ii
ACKNOWLEDGEMENT	iii
TABLE OF CONTENTS.....	iv
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS.....	xi
ABSTRACT.....	xiv
CHAPTER ONE.....	1
1.0 INTRODUCTION	1
1.1 Background.....	1
1.2 Problem statement.....	5
1.3 Justification.....	5
1.4 Hypothesis.....	6
1.5 Aim	6
1.6 Specific Objectives	6
CHAPTER TWO	7
2.0 LITERATURE REVIEW	7
2.1 <i>Plasmodium</i> and malaria.....	7
2.1.1 Brief history and distribution of malaria.....	8
2.1.2 Life cycle of malaria parasite.....	9
2.1.3 Clinical manifestation	11

2.1.4 Diagnosis.....	13
2.1.5 Treatment	13
2.1.6 Prevention and control	14
2.2 Merozoite surface antigens	14
2.2.1 Merozoite invasion.....	15
2.3 Variant surface antigens (VSAs)	16
2.4 Immunity to malaria.....	18
2.4.1 Innate and adaptive immunity.....	18
2.4.2 Antibody mediated immunity	19
2.4.3 Cell mediated immunity.....	20
2.4.4 Blood stage parasite antigens in vaccine production	20
2.4.5 Immune evasion	21
2.5 Background on Assays Used	22
CHAPTER THREE	25
3.0 MATERIALS AND METHODS.....	25
3.1 Study Design.....	25
3.2 Study Site and Study Population.....	25
3.3 Sample Size:.....	26
3.4 Laboratory Procedures Used:.....	26
3.4.1 Culture of Schizont Stage <i>P. falciparum</i>	27
3.4.2 Isolation of Late Stage <i>P. falciparum</i> Trophozoites	28

3.4.3 Quantifying Antigen – Specific Antibodies to iRBC Membrane Antigens.....	28
3.4.4 Preparation of Free Merozoites.....	29
3.4.5 Measurement of <i>Plasmodium</i> Merozoite Antigen Protein concentration by Bradford Assay	30
3.4.6 Measurement of Antibodies to Merozoite Surface Antigens.....	30
3.4.7 Purification and concentration of IgG using the Spin Purification Protocol	31
3.4.8 Growth Inhibition Assay (GIA).....	32
3.4.9 Lactate Dehydrogenase (LDH) Assay	32
3.4.10 Binding Inhibition Assay (BIA).....	33
3.5 Statistical Analysis.....	34
3.6 Ethical Clearance	34
CHAPTER FOUR.....	35
4.0 RESULTS	35
4.1 Demography and clinical characteristics of study participants.....	35
4.2 Parasite densities of Study samples	36
4.3 Antibody levels of antibodies to merozoite antigens	37
4.3.1 Concentration merozoite antigens by Bradford Assay	37
4.3.2 Antibody levels of antibodies against merozoite surface antigens in symptomatic and asymptomatic individuals by indirect ELISA.....	37
4.4 Antibody levels of antibodies to variant surface antigens	38
4.5 Antibody Purification and Concentration	40
4.6 RBC invasion inhibitory activities of merozoite surface antigen-specific antibodies	41

4.7 Binding inhibitory activities of iRBC surface antigen-specific antibodies on CD36 endothelial receptors	42
CHAPTER FIVE	43
5.0 DISCUSSION	43
5.1 Demography and clinical characteristic of study participants	43
5.2 Antibody levels against merozoite surface antigens and clinical malaria	44
5.3 Antibody levels against variant surface antigens and clinical malaria	44
5.4 Antibody Purification and Concentration	45
5.5 RBC invasion inhibitory activities of merozoite surface antigen-specific antibodies	45
5.6 Binding inhibitory activities of iRBC surface antigen-specific antibodies on CD36 endothelial receptors	46
CHAPTER SIX	48
6.0 CONCLUSION AND RECOMMENDATION	48
6.1 Conclusion	48
6.2 Recommendation	48
6.3 Limitation	49
REFERENCES	50
APPENDICES	66
APPENDIX I	66
Bradford assay OD and sample concentration determination	66
APPENDIX II	68
Antibody (IgG) Purification and Concentration	68
APPENDIX III	69

Ethical Clearance 69

LIST OF TABLES

Table 1: Some of features of malaria parasites that infect humans 8

Table 2: Age demographics of study population 35

LIST OF FIGURES

Figure 1: Life cycle of <i>Plasmodium</i>	Error! Bookmark not defined.
Figure 2: Merozoite invasion of RBC.....	16
Figure 3: Overview of laboratory procedures.....	27
Figure 4: Parasite densities of study population.....	36
Figure 5: Antibody levels of merozoite surface antigen antibodies.....	37
Figure 6: FACSCAN reading of plasma sample containing ethidium bromide treated antihuman IgG-FITC.	39
Figure 7: A graph showing antibody levels of the two study groups in mean fluorescent intensity (MFI).....	40
Figure 8: A graph showing growth inhibitory activities of merozoite surface-specific antibodies in asymptomatic and symptomatic individuals.....	41
Figure 9: A graph showing binding activities of infected RBC surface antigen-specific antibodies in asymptomatic and symptomatic individuals.....	42

LIST OF ABBREVIATIONS

APAD	3-Acetylpyridine Adenine Dinucleotide
AU	Arbitrary Units
BIA	Binding Inhibition Assay
BSA	Bovine Serum Albumin
CD36	Cluster of Differentiation 36
CDC	Centre for Disease Control and Prevention
CIDR	Cystein-Rich Inter Domain Region
CS-A	Chondroitin Sulphate - A
DBL	Duffy Binding-Like
E64	Epoxy succinyl-L-Leucylamido-4-Guanidino Butane
EBA-175	Erythrocyte Binding Antigen
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium Bromide
FACS	Fluorescence Activated Cell Sorting
FBS	Fecal Bovine Serum
FITC	Fluorescein Isothiocyanate
GIA	Growth Inhibition Assay

H ₂ SO ₄	Sulfuric Acid
HRP	Horseradish Peroxidase
ICAM-1	Inter Cellular Adhesion Molecule-1
IE	Infected Erythrocytes
IgG	Immunoglobulin G
iRBCs	Infected Red Blood Cells
KAHRP	Knob-Associated Histidine-Rich Protein
LDH	Lactate Dehydrogenase Assay
MESA	Mature Parasite-Infected Erythrocyte Surface Antigen
MFI	Mean Fluorescence Intensity
MSP1-1	Merozoite Surface Protein 1-1
OD	Optical Density
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline-Tween 20
PCR	Polymerase Chain Reaction
PfEMP1	<i>Plasmodium falciparum</i> Erythrocyte Membrane Protein 1
RAP 1-3	Rhoptry-Associated Protein 1-3
RBCs	Red Blood Cells

RESA	Ring Infected Erythrocyte Surface Antigen
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
SERA	Serine-Rich Antigen
STEVOR	Subtelomeric Variable Open Reading Frame
TMB	3,3',5,5'-Tetramethylbenzidine
VSA	Variant Surface Antigens
WHO	World Health Organization

ABSTRACT

Background: Malaria infection is caused by parasites belonging to the genus *Plasmodium*. There are five (5) species of *Plasmodium* that infect humans, namely: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium knowlesi* and *Plasmodium malariae*. Malaria ensuing from *Plasmodium falciparum* infection is a principal source of disease and death annually, especially among African children in the Sub-Saharan Regions. The erythrocytic stages of the parasites of *Plasmodium falciparum* are linked to malaria symptoms and its complications. Several *Plasmodium* antigens, expressed on the surface of the parasite during the blood stage have been shown to be relevant for RBC invasion. These antigens include merozoite surface protein (MSP1)175 -1, apical membrane antigen (AMA)-1, serine-rich antigen (SERA), erythrocyte binding antigen (EBA)-175, among others. Although humoral and cellular immunity are believed to be involved in malaria immunity, their relative importance in offering protection against the disease is not well established. Antibodies produced against merozoite surface antigens and those against variant surface antigens, VSAs (especially PfEMP-1) on the infected red blood cell (iRBC) membrane have been studied individually but their relative contributions that could lead to protection against clinical malaria using the same samples have not been documented.

General Aim: The aim of this study was to assess the *plasma levels* and *functional effects* of antibodies to merozoite surface and RBC surface antigens between children with asymptomatic infection and clinical malaria.

Methodology: This was a case-control study which utilized a total of 153 archived plasma samples collected (from 81 children with asymptomatic infection and 72 children with clinical malaria) by a larger study (cross sectional) that was conducted at Bongo in the Northern Region and Navrongo in the Upper East Region of Ghana. The following procedures were then carried out: Culture of Schizont Stage *P. falciparum*, Isolation of late stage *P. falciparum* trophozoites and quantification of antigen (specific antibodies to iRBC membrane antigens and preparation of free merozoites). Bradford Assay was used to measure the concentration of *Plasmodium* merozoite antigen protein. Measurement of antibodies to merozoite surface antigens was also done by enzyme-linked immunosorbent assay (ELISA) and that of antibodies to variant surface antigens (VSAs) was done by Flow cytometry. Spin purification protocol was employed in purification and concentration of IgG. Growth inhibition assay (GIA) as well as ligand binding inhibition assay (BIA) were subsequently done.

Results: In determining the antibody levels to merozoite surface antigens by Bradford Assay, a mean sample OD of 0.4798 measured at 590 – 595nm was observed. Sample concentration determined was 0.0839 mg/ml = 83.9 ug/ml. Asymptomatic individuals had statistically ($p < 0.0001$) higher antibody levels against merozoite surface antigens compared to the symptomatic individuals. Antibody levels to variant surface antigens on infected RBC surface between asymptomatic and symptomatic individuals was not statistically significant ($p = 0.6469$). Meanwhile, there was no statistically significant difference ($p = 0.2487$) between asymptomatic and symptomatic patients in terms of growth inhibition ability of antibodies against antigens of *Plasmodium* merozoite and infected RBC surface. Similarly, there seem to be no difference in the ligand binding inhibitory ability of antibodies against antigens on *Plasmodium* infected RBC surface.

Conclusion: Antibodies against antigens of *Plasmodium* merozoite and infected RBC surface seem to give similar contribution in malaria immunity.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is caused by infection with protozoan parasites of the genus *Plasmodium* through the bite of female Anopheles mosquitoes. Over four hundred (400) different species of Anopheles mosquitoes are known, with about thirty (30) of them considered as vectors of major importance. These major vector species usually bite between dusk and dawn. Mosquito is described as the most dangerous animal in the world (Heyman, 2014) causing malaria which happens to be detrimental to humans. There are about 3,500 species of mosquito and those known to cause malaria belong to the genre called *Anopheles* (Cox, 2010). Nearly forty (40) species of this genus can significantly transmit malaria to cause human illness and death. *A. gambiae* complex is the most prevalent vector in Africa (Cox, 2010). In order for the mosquito to transmit malaria effectively it need to present characteristics such as abundance of the species, longevity after feeding on infected blood, ability to carry enough malaria parasites in the salivary glands and contact with humans (MAP, 2018).

Malaria transmission intensity mainly depends on three factors namely: parasite factors, host factors and the environmental factors (WHO, 2016). There are only five (5) species of *Plasmodium* that infect humans out of over 100 *Plasmodium spp* that naturally exist. They include: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium knowlesi* and *Plasmodium malariae* (Pain *et al.*, 2008).

The main source of death and disease recorded annually amongst African children is as a result of *Plasmodium falciparum* infections (Langhorne *et al.*, 2008; WHO, 2015). An estimated amount of 214 million malaria cases were recorded worldwide in 2015 and out of which 438,000 deaths were attributed to children in the African Region (CDC, 2017).

Malaria infection can be identified as symptomatic or asymptomatic dependent on the response of the host immune system. (Mendonça and Barral-Netto, 2015). Individuals infected with malaria present clinical signs and symptoms such as fever, chills, headache, flu-like illness etc. If malaria infected persons are left untreated, they may develop severe complications including severe anaemia, cerebral malaria, pulmonary edema, acute renal failure etc. which can consequently lead to death (Adukpo *et al.*, 2013).

Malaria symptoms and its intricacy usually occur during the blood stages of the parasite. Sporozoites are introduced into circulation through a blood meal of an infected mosquito. Host infection is initiated when these sporozoites invade hepatocytes. At this stage they grow and multiply. About 90 000 exoerythrocytic stage merozoites can be formed during development within the liver (Vaughan and Kappe, 2017). Once *Plasmodium falciparum* merozoites are unleashed into circulation from the liver, they infect RBCs and undergo replication within the cells to yield new merozoites that burst out of the infected erythrocyte and continue the cycle. The merozoites invade the erythrocytes by first attaching to the surface. The parasite reorients and forms a tight junction. Consequently the merozoites enter the host cell by actin myosin motor movement and form a parasitophorous vacuole which encloses it against the host cell cytoplasm.

Thus creating a conducive environment for its development. (Cowman and Crabb, 2006; Ahouidi *et al.*, 2015).

Several *Plasmodium* antigens, formed on the parasite's surface during the blood stage have been shown to be relevant for RBC invasion. These antigens include MSP-1, AMA-1, EBA-175, MSP-1, Pf155/RESA among others (Ramasamy *et al.*, 2001; Kusi *et al.*, 2009; Boyle *et al.*, 2010).

For *Plasmodium falciparum* especially, as merozoites develop within the RBC, they export and insert VSAs into the RBC surface. The *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) family of proteins is the most extensively studied variant surface antigen. In each parasite genome, PfEMP1 variants are encoded by 60 *var* genes. The PfEMP-1 protein is made up of different segments, categorized as either duffy binding-like domain (DBL) or cysteine-rich inter domain region (CIDR). The various domains are thought to bind to different host receptors. For instance DBL2b and CIDR1a domains of PfEMP-1 can bind to Cluster of Differentiation 36 (CD36) and Inter Cellular Adhesion Molecule-1(ICAM-1) respectively and mediate cytoadherence to the vascular endothelium (Senczuk *et al.*, 2001). Cytoadherence involves the sequestering of the parasite infected RBCs to the vascular endothelial walls of various organs. When this happens the parasites become non-detectable in host's peripheral blood. They can cause severe and sometimes fatal complications such as pregnancy associated malaria and cerebral malaria when they obstruct blood flow in small vessels coupled with the release of inflammatory cytokines (Coppel *et al.*, 2002; Hviid, 2005).

Immunity to malaria plays a major role in parasite clearance and controlling the infection. Immune response against the parasite include the secretion of parasite-induced inflammatory cytokines, the

actions of effectors such as monocytes, macrophages, complement, and the effector functions of parasite-specific T and B cells (Malaguarnera and Musumeci, 2002; Pawar, 2014).

Antibodies are effector molecules made by B cells. They are glycoproteins found in blood and tissue fluids. Merozoite surface antigen-specific antibodies can protect against disease by binding the parasite to inhibit them from invading RBCs. Thus preparing them for destruction by immune cells (Richards and Beeson, 2009; Stanisic *et al.*, 2015). Antibodies involved in impeding of *Plasmodium falciparum* merozoite invasion into erythrocytes have been shown to play a critical role in anti-malarial immunity (Adamou *et al.*, 2016).

Several methods using Immunoglobulin (Ig) fractions from malaria exposed individuals have been used to study the effect of antibodies on *Plasmodium falciparum in vitro* (Bolad and Berzins, 2000). The functional ability of an antibody to inhibit RBC invasion and parasite development can be measured using Growth Inhibition Assay (GIA).

Studies performed by Courtin *et al.* (2009) and Adamou *et al.* (2016) using GIA showed the inhibitory activities of IgG directed against *Plasmodium falciparum* antigens AMA-1 and MSP-1 involving individuals living in malaria-endemic areas. The establishment of an *P. falciparum in vitro* cultures is very important for studying the parasite's biology, immunology and the development of new drugs and vaccine (Trager and Jensen, 1997; Mata-cantero *et al.*, 2014).

Variant surface protein-specific antibodies have been found to also bind VSAs on iRBCs and prevent cytoadherence in minor blood vessels. Thus leading to increased parasite clearance by the spleen (Giha *et al.*, 2000; Travassos *et al.*, 2013)

Humoral immunity is believed to be actively involved in immunity against malaria (Kinyanjui, 2012), however the relative importance of antibodies against certain *Plasmodium* targets is not

well established. Antibodies produced against merozoite surface antigens and VSAs (especially PfEMP-1) on the iRBC membrane have been studied individually but their relative contributions that could lead to protection against clinical malaria have not been documented.

1.2 Problem statement

The roles played by antibodies against parasitized RBC membrane antigens and merozoite surface antigens in malaria immunity have been studied individually but their relative contributions in asymptomatic and clinical malaria development among infected individuals, as to which one is more important is not known. This situation can be considered among the reasons why malaria vaccine development has been a challenging task for scientists. The purpose of this study is to assess these two sets of antibodies using in vitro assays for their relative contributions to immunity against the parasite.

1.3 Justification

The *Plasmodium* parasite evades protective immunity by employing immune evasion strategies involving sequestration of iRBC in vascular endothelial cells mediated by VSAs especially PfEMP-1 (Bengtsson *et al.*, 2008) and the presentation of highly polymorphic merozoite surface antigens for the invasion of erythrocytes (Wright and Rayner, 2014). This study involving a combination of the two targets will enable better understanding of the immune effects of antibodies in individuals with asymptomatic infection and those with clinical malaria. Thus immune effector mechanisms involving merozoite surface antigens and parasite derived antigens on iRBC surface once understood will contribute to the rational selection of potential vaccine candidates or combinations thereof.

1.4 Hypothesis

The hypothesis of the study is that antibodies against merozoite surface antigens and those against parasitized RBC surface antigens both contribute significantly to curbing *Plasmodium* parasite multiplication and therefore limit the progression of infection to clinical disease.

1.5 Aim

The aim of the study was to assess the plasma levels and the functional effects of antibodies to merozoite surface and RBC surface antigens between children with asymptomatic infection and clinical malaria.

1.6 Specific Objectives

- i.** To determine and compare the plasma levels of merozoite surface-specific antibodies and RBC membrane surface-specific antibodies between individuals with asymptomatic and clinical malaria.
- ii.** To measure and compare the RBC invasion inhibitory activities of merozoite surface antigen-specific antibodies and endothelial adhesion inhibitory activities of iRBC membrane-specific antibodies between the two study groups.
- iii.** To assess the relative contribution of the two antigen-specific antibody fractions to predicting protection from clinical malaria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Plasmodium* and malaria

Malaria is a disease transmitted by the bite of the female anopheles mosquito. Majority of the cases and deaths due to malaria occur in the African sub-regions. Individuals severely affected are mostly children below the age of five and pregnant women (WHO, 2016). The species of *Plasmodium* that cause malaria include *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malaria* and *Plasmodium knowlesi*. Almost 2% of African children with clinical episodes of malaria ensuing in severe disease is attributable to *Plasmodium falciparum* (Gachot and Ringwald, 2014). It is the deadliest among the *Plasmodium* species.

The second major cause of malaria disease among the *Plasmodium* species is *Plasmodium vivax*. Its infection is highly recorded in Asia with about 390 million cases yearly (Price *et al.*, 2009). Considering the global malaria burden, *Plasmodium ovale* and *Plasmodium malaria* have been found to be only accountable to lower proportions (Sutherland *et al.*, 2010). Among the *Plasmodium knowlesi* infection is very rare and usually occurs in South East Asia. It is a zoonotic infection transmitted from macaques to humans through infected mosquitos (Singh and Daneshvar, 2013; Beeson, 2016).

Infected individuals are the main reservoirs of human malaria especially asymptomatics in living in high transmission areas (Heyman, 2014; WHO, 2015). Table 1 shows the various malaria parasites of humans and their characteristics.

Table 1: Some of features of malaria parasites that infect humans

	<i>Plasmodium vivax</i>	<i>Plasmodium malariae</i>	<i>Plasmodium falciparum</i>	<i>Plasmodium ovale</i>
Infected cells	Cell enlarged, Fine stippling (Schüffner's dots). Invades reticulocytes and young RBC.	No enlargement, Absence of stippling. Infects older RBC.	No enlargement, dense stipplings (Maurer's clefts). Infects RBC	Cell enlarged, fine stippling
Maximum parasitemia	30 000/ul of blood	Below 10 000/ul	50 000/ul	Less than 10 000/ul
Trophozoite pigments	Fine, Light brown, Scattered clumps	Coarse, Dark brown, Scattered clumps	Coarse, Black, Few clumps	Coarse, Dark yellow, Scattered
Mature Schizonts	>12 merozoites	<12 merozoites Often large and in rosette.	>12 merozoites	<12 rosetting merozoites

(Adapted from Jawetz *et al.*, 2007)

2.1.1 Brief history and distribution of malaria

Among the ancient diseases thought to have originated from Africa, Malaria is one of the deadliest which cannot be ignored. It comes from the word “mal’aria” which is an Italian word meaning ‘bad air’ (Cox, 2010). The supernatural was blamed for the cause of the disease until Hippocrates, often referred to as “Father of medicine” first described how the disease is manifested in relation to seasons and place of abode.

The Romans were the first to introduce malaria control interventions. Drainage systems were built to curb the disease, as the breeding grounds of the vector, anopheles mosquito was associated to stagnant waters and marshy areas. Parasites residing in the infected individuals was discovered in

1880 by Alphonse Laveran (Cox, 2010). Furthermore, Cyril Garnham and Henry Shortt discovered in 1948 that the parasites enter the bloodstream after development in the liver. Also in 1982, the presence of hypnozoites which happen to be the dormant stages of the parasites which occur in the liver was proved by Wojciech Krotoski (Cox, 2010). The transmission of malaria parasites by mosquitoes was first demonstrated by Ronald Ross. Works by individuals including Ronald Ross, Giovanni Battista Grassi, Robert Koch laid the foundations necessary for malaria control and prevention (CDC, 2017).

Malaria occurs mainly in the tropics especially Africa where highest number of cases are recorded with death toll as high as 90%. Children under 5 years are most infected individuals. The disease is also high in South East Asia and to a less extent in India (WHO, 2018).

Concerning risk factors of the disease, altitudes above 1500m present less risk however the disease can occur up to altitudes of 2900m during favourable conditions (WHO, 2018). Seasonal climatic conditions are among the risk factors. Higher infections are usually recorded during the rainy seasons. Places where risk of the diseases is at a minimum or absent include the Caribbean, Latin America and a few tourist sites in South-East Asia (Hay et al., 2004).

2.1.2 Life cycle of malaria parasite

Sporozoite stage of the parasite is introduced into the bloodstream when a female anopheles mosquito which is infected feeds on a human. The sporozoites then migrate to the liver specifically within hepatocytes for their maturation and multiplication. Inside the liver the stage is collective called exoerythrocytic cycle. The parasites at this stage develop to form numerous merozoites capable of invading erythrocytes (Vaughan and Kappe, 2017) (Figure 1).

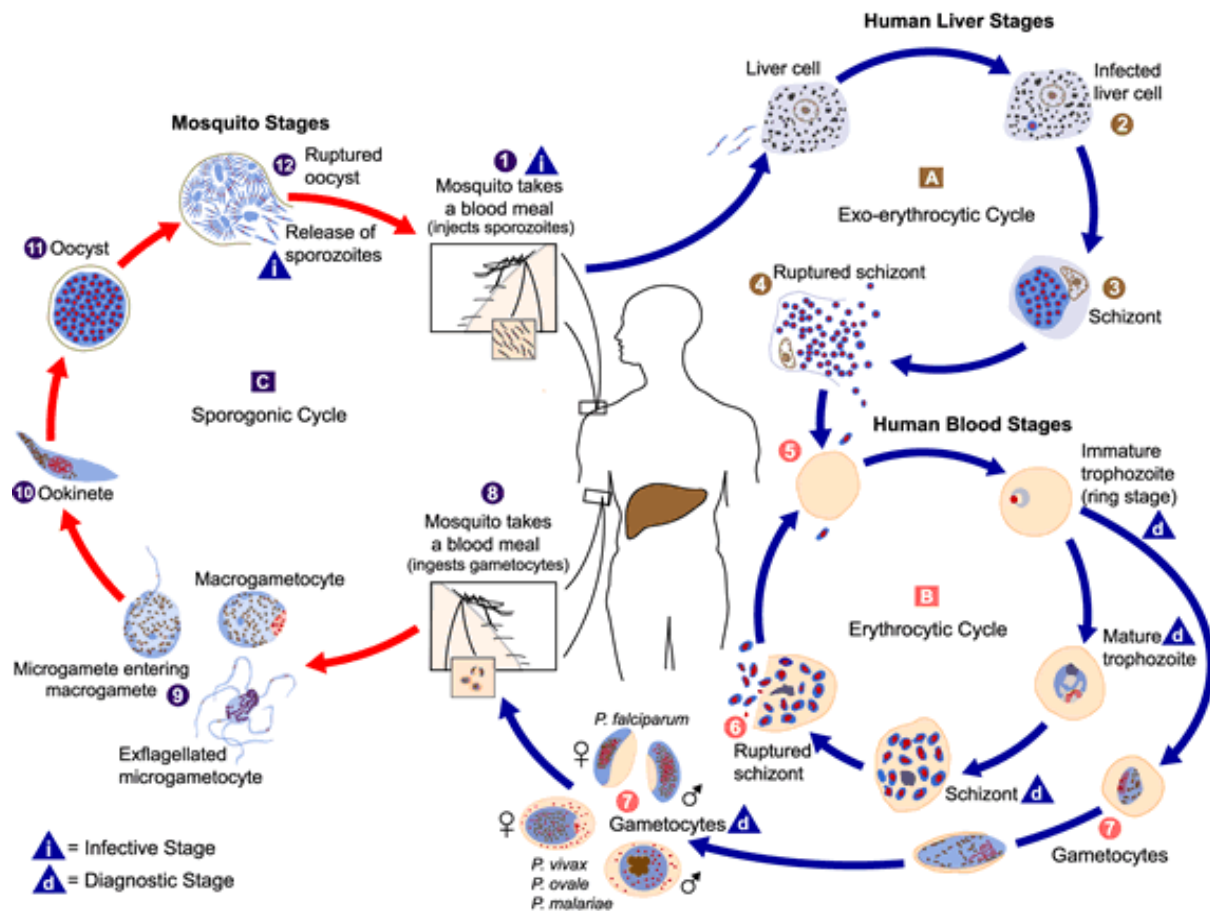


Figure 1: Life cycle of *Plasmodium* species (Adapted from CDC, 2017. Date accessed: 26/06/2018. Accessed from: <https://www.cdc.gov/dpdx/malaria/index.html>)

After multiplication they are released into the bloodstream as merozoites where they pass through the developmental stages to form trophozoites and consequently schizonts. Merozoites develop within mature schizonts and can burst out into the bloodstream to infect other red blood cells. This forms a cycle known as the erythrocytic cycle (Figure 1).

Malaria infections can be asymptomatic or symptomatic depending on the response by host immune system (Adukpo *et al.*, 2013; Mendonça and Barral-Netto, 2015).

Other factors influencing these distinct manifestations include host susceptibility, parasite virulence, the environment and disease tolerance (Andrade and Barral-Netto, 2011; Mendonça and Barral-Netto, 2015).

Individuals presenting asymptomatic plasmodial infection show positive parasitaemia since they contain amount of parasites within them. However these individuals do not show symptoms of the disease over a particular period of time (Lindblade *et al.*, 2013; Mendonça and Barral-Netto 2015). Since asymptomatic individuals can harbour parasites unnoticed within a long period of time, they can impede malaria eradication as they are capable of transmitting the parasites to other persons. (Schneider *et al.* 2007, White 2008; Mendonça and Barral-Netto, 2015)

Individuals infected with malaria present clinical signs and symptoms such as fever, chills, headache, flu-like illness etc. If malaria infected persons are left untreated, they may develop severe complications such as severe anaemia, cerebral malaria, pulmonary edema and acidosis. These complications when unattended can eventually lead to death (WHO, 2015).

2.1.3 Clinical manifestation

Malaria is an acute febrile illness that manifests clinically like other illnesses of bacteria, viruses or parasites in its initial stage (Heyman, 2014). Malaria is characterised by clinical signs which include fever, dizziness, headache, vomiting, jaundice, diarrhoea, cough, anorexia, nausea, lethargy etc. (CDC, 2016). The classical malaria indicators involving severe agitating chills, sweats and high fever which lasts for about 1 – 2 hours seem to be absent in mild or early malaria episodes (PHAC, 2014). Individuals who have lived in malaria endemic areas may present uncharacteristic malaria symptoms as a result of acquisition of partial immunity (Heyman, 2014; Askling *et al.*, 2012). *Plasmodium ovale* and *Plasmodium vivax* are capable of causing relapse

when they persist in the liver. This can occur after taking chemoprophylaxis for more than five years (PHAC, 2014). No clear evidence has been provided for the dormant stage of *Plasmodium falciparum* and *Plasmodium knowlesi* (Askling *et al.*, 2012). Malaria has been shown to increase the death of pregnant mothers and new-borns (PHAC, 2014). Complications in pregnancy malaria which can cause high infant morbidity and mortality include low birth weight and maternal anaemia (Huyuh *et al.*, 2015) Another complication by *Plasmodium falciparum* has to do with severe malaria, this can lead to a case fatality rate as high as 20% (PHAC, 2018). Clinical presentation of severe malaria may include cerebral malaria, severe anaemia etc. (Fairhurst and Wellems, 2015).

Malaria is an acute febrile illness that manifests clinically like other illnesses of bacteria, viruses or parasites in its initial stage (Heyman, 2014). Malaria is characterised by clinical signs which include fever, dizziness, headache, vomiting, jaundice, diarrhoea, cough, anorexia, nausea, lethargy etc. (CDC, 2016). The classical malaria indicators involving severe agitating chills, sweats and high fever which lasts for about 1 – 2 hours seem to be absent in mild or early malaria episodes (PHAC, 2014). Individuals who have lived in malaria endemic areas may present uncharacteristic malaria symptoms as a result of acquisition of partial immunity (Heyman, 2014; Askling *et al.*, 2012). *Plasmodium ovale* and *Plasmodium vivax* are capable of causing relapse when they persist in the liver. This can occur after taking chemoprophylaxis for more than five years (PHAC, 2014). No clear evidence has been provided for the dormant stage of *Plasmodium falciparum* and *Plasmodium knowlesi* (Askling *et al.*, 2012). Malaria has been shown to increase the death of pregnant mothers and new-borns (PHAC, 2014). Complications in pregnancy malaria which can cause high infant morbidity and mortality include low birth weight and maternal

anaemia (Huyuh *et al.*, 2015) Another complication by *Plasmodium falciparum* has to do with severe malaria, this can lead to a case fatality rate as high as 20% (PHAC, 2018) Clinical presentation of severe malaria may include cerebral malaria, severe anaemia etc. (Fairhurst and Wellems, 2015).

2.1.4 Diagnosis

A patient with malaria infection can be diagnosed through laboratory examination of thin and thick films through microscopy, rapid diagnostic tests or malaria antigen testing and malaria polymerase chain reaction (PCR). For patients under treatment or prophylaxis, parasites may not be detectable in the films (CDC, 2017). Blood smears may test negative for low parasitaemia, however for malaria antigens they can test positive. Malaria PCR is done when microscopy and malaria antigen testing fail with the persistence of clinical presentation. Antibody testing is the last resort when all the above tests are negative. This test however has sub-optimal specificity and results are cautiously interpreted (CDC, 2017).

2.1.5 Treatment

Malaria treatment is specifically dependent on the type of *Plasmodium* species, locality and severity of infection (WHO, 2015). Malaria requires rapid treatment since it is an emergency case. Visiting the hospital is highly recommended. Quinines are the drugs of choice for mild treatment of malaria infections (PHAC, 2014). Combination therapy is the new policy put in place following drug resistance. Various drug combinations were required in treatment of chloroquine resistance (PHAC, 2014). In Ghana Artemisinin based combination therapies are the new policies put in place to deal with drug resistance (WHO, 2015).

2.1.6 Prevention and control

Mosquito control is important in eradicating the disease, however the total destruction of mosquitoes is not necessary as they play very important ecological roles in the ecosystem. Mosquito control measures include personal protection against mosquito bites using insecticide treated bednets and mosquito repellents. Mosquito breeding grounds can also be destroyed using larvicides, preventing waterlogging and draining stagnant waters. Malaria vaccines would be a very good source of prophylaxis. Production of effective malaria has been a challenge as far as malaria eradication is concerned. Not much success has been recorded yet for several malaria vaccine trials done over the years (Genton, 2008). Presently, the only malaria vaccine undergoing Phase III trial is the RTS,S/AS02 vaccine. Current results showed that it has reduced clinical episodes of malaria by 35 - 36% after 12 months and reduced severe malaria to 34.8% in young and older children (RCTP, 2011; Butler, 2011). These initial reports have been considered encouraging and this promising result is very good for the future (Genton, 2008).

2.2 Merozoite surface antigens

Plasmodium antigens such as AMA-1, EBA-175, RAP 1-3, MSP-1, Pf155/RESA etc, are found on the merozoite surface (Egan *et al.*, 1995). These proteins have been shown to be implicated in erythrocyte invasion and are major targets of antibodies that are capable of inhibiting the invasion of merozoites. They proteins are very important vaccine candidates (Richards *et al.*, 2013).

Merozoite surface antigens provide useful information on malaria exposure especially in high transmission seasons. Some of them are capable of showing malaria protective efficacy among individuals in different age groups (Sennang *et al.*, 2014). EBA-140, EBA-175 and EBA-181 are antigens found on the surface of *Plasmodium falciparum* merozoites which are also important targets of naturally acquired inhibitory antibodies. The variation in the expression and function of

all three EBAs plays an essential role in evasion of antibodies by *Plasmodium falciparum* (Persson *et al.*, 2014). Antigenic differences shown by different populations of *Plasmodium falciparum* have implications in the neutralization of the parasite during immune response. These antigenic diversity depict polymorphisms in the allelic gene products. Merozoite surface proteins 1 and 2 (MSP-1 and MSP-2) are the best studied antigens with regard to allelic polymorphisms in which selected regions are used to genotype parasite populations *in vitro* (Bolad and Berzins, 2000). Furthermore MSP-1 antigens are thought to mediate initial attachment of merozoites during erythrocyte invasion by interacting with heparin-like molecules on the RBC surface. Apical membrane antigen 1 (AMA-1) has been associated in merozoite apical reorientation prior to invasion as well as in the formation of a moving junction between the merozoite and the RBCs. Despite the varying functionality of the merozoite surface antigens, there are still a number of them that have unknown functions (Fan *et al.*, 2013). MSP-3 family, MSP-7 family, the 6-cys family among others are a few examples of such antigens.

2.2.1 Merozoite invasion

This happens to be a step by step process (Figure 2). It involves the initial attachment of free merozoites to RBC envelope via their surface antigens. The merozoite then orientates with the apical end coming into contact with the surface of the erythrocyte (Figure 2). This leads to a tight junction formation between the parasite and the RBCs. At this point the merozoite enters the RBC while shedding some of its surface antigens (Beeson and Crabb, 2007; Boyle *et al.*, 2013).

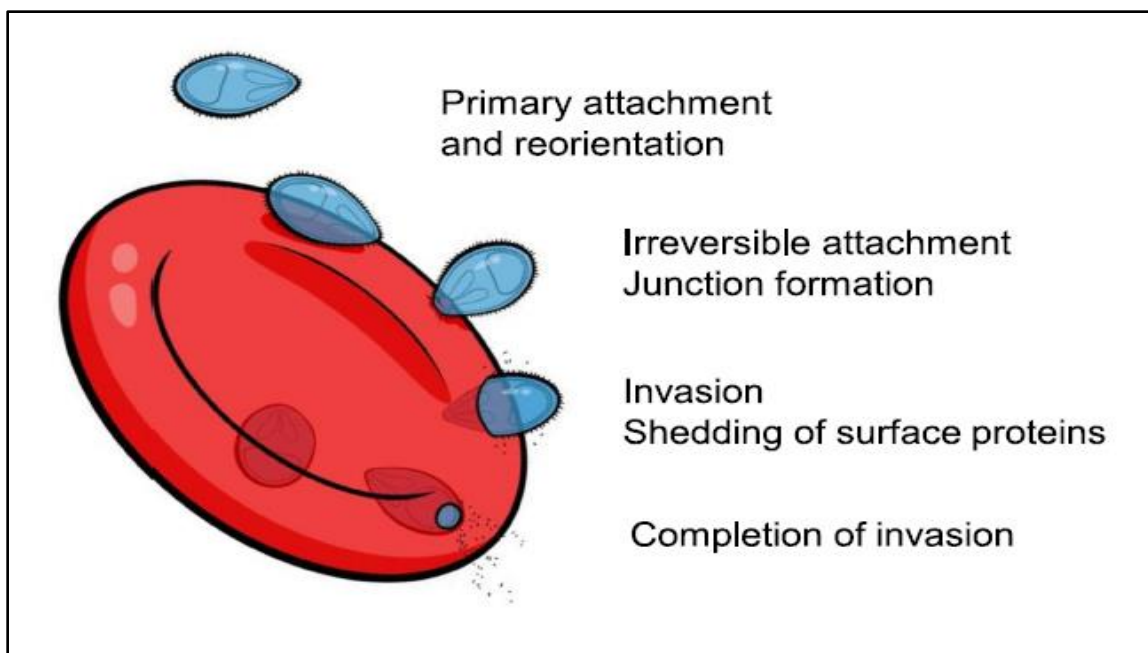


Figure 2: Merozoite invasion of RBC (Adapted from Beeson *et al.*, 2016)

2.3 Variant surface antigens (VSAs)

During intra-erythrocytic development, *Plasmodium falciparum* several proteins usually named variant surface antigens (VSAs) are expressed on the surface of erythrocytes. The accurate role of most of the VSAs is unknown. However it is proposed that they are involved in cellular adhesion (Coppel *et al.*, 2002).

One of the constituents of VSAs that provokes naturally occurring immune responses to infections is the *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1). PfEMP-1 has been shown to facilitate the attachment of infected RBCs to receptors on the human endothelium mediate adhesion of infected red blood cells (iRBC) to endothelial receptors for example CD36 and ICAM-1. CD36 and ICAM-1 are only capable to binding to parasites that express the variable type of PfEMP-1 specific to them (Kaviratne *et al.*, 2002).

Considering various receptor ligand interactions that exist, the interaction of PfEMP-1 with CD36 is known to be involve in major pathophysiological functions (Gamain et al, 2001; Coppel *et al.*, 2002). Examples of Parasite-derived infected erythrocyte VSAs are PfEMP-1, mature parasite-infected erythrocyte surface antigen (MESA), knob-associated histidine-rich protein (KAHRP), surface-associated interspersed gene family proteins (SURFIN) among others (Coppel *et al.*, 2002; Chan *et al.*, 2012).

Unfortunately, insight in malaria immunity and the development of vaccines has not much improvement due to the fact that the significance of the various VSA targets involved in malaria immunity remains obscure. However the ability of iRBCs to attach by way of knob-like protrusion on their surface during normal physiological blood currents has been attributed to KAHRP (Crabb *et al.*, 1997; Coppel *et al.*, 2002). There is no clear understanding of the main function of the other VSAs (MESA, PfEMP3, RESA etc.) as auxiliary molecules in cytoadhesion.

Antigenic assortment and the presence of surface antigen variants primarily leads to frequent infections periodically. PfEMP-1 is the most extensively studied VSA and has been found to be a key target of antibodies acquired through natural immunity (Chan *et al.*, 2012).

A longitudinal study conducted in Kenya by Chan *et al.* (2012) showed how antibodies specific to PfEMP-1 reduced the risk of developing malaria and other surface antigens on the iRBC on the other hand showed no association to protective immunity.

PfEMP-1 has been shown to mediate RBC rosette formation and the attachment of infected erythrocytes to the surface of endothelial cells to prevent splenic clearance of the infected cells. When this happens, it permits the parasite to hide in the host in special organs like the placenta and the brain. This mechanism happens to be an important parasite survival strategy, leading to

parasite pathogenesis (Brown and Beeson, 2002). The hiding of RBCs in the blood vessels causing blockage of blood currents, accompanied by the production of inflammatory cytokines are assumed to lead to severe malaria and other lethal complications like cerebral malaria (Coppel *et al.*, 2002).

2.4 Immunity to malaria

The adaptive and innate immune responses are both important in immune response to malaria. A host requires effective cooperation between these two systems to eliminate malaria infection (Angulo, 2002). Major cause of morbidity and mortality is due to complications of severe anaemia and cerebral malaria (Malaguarnera and Musumeci, 2002). Clinical signs and complications involving malaria are usually triggered by infections in the blood stage. As a result, the bulk of the naturally acquired immune response is directed against these parasites of the blood-stage (Doolan *et al.*, 2009).

2.4.1 Innate and adaptive immunity

Innate immunity involves the first line of defence against the pathogen. The initial encounter against the parasite is followed by the development of specific antibodies secreted by B lymphocytes. These responses are controlled by cytokine and chemokine secretions.

Recurrent and persistent exposure of an individual to malaria parasite over a long period of time can lead to the acquisition of a short-term partial immunity with specificity to the parasite strain and stage. This kind of natural infection to malaria may not induce enough immune protection (Waters, 2006; Chauchan, 2007). It can however result in a symptomatic infections with minor perseverance of the parasites (Chauchan, 2007). Furthermore, in this type of immunity, all the parasites cannot be cleared and for that matter complete protection is not guaranteed for future

infections (Waters, 2006). The complex biology, extensive antigenic diversity and immune evasion strategies of the *Plasmodium* parasite give rise to this kind of partial immune response. These factors make vaccine development very challenging. (Genton, 2008). The dwindling incidence of malaria and its complications observed in people of different age groups, residing in endemic regions following series of repeated infections is an indication of acquired immunity to the disease (Kinyanjui, 2012).

2.4.2 Antibody mediated immunity

Antibodies are mainly made up of glycoproteins and they are present generally in blood and tissue fluids. They are known for their diverse functionality of binding to antigens, neutralization, and preparing foreign materials for destruction by immune cells (Richards and Beeson, 2009).

Antibodies against merozoite antigens may play a role in malaria immunity which may include obstructing parasite growth and blocking merozoites from invading RBCS (Jiang *et al.*, 2011; Richards *et al.*, 2013). Also antibodies can be implicated in opsonisation of merozoites for phagocytosis and antibody dependent cellular inhibition (Richards and Beeson, 2009; Richards *et al.*, 2013). Several methods using Immunoglobulin (Ig) fractions from malaria exposed individuals have been used to study the *in vitro* antibody effects on *P. falciparum* (Bolad and Berzins, 2000). Antibody mediated immunity is prominently present in the erythrocytic stage of the parasite. During the asexual blood stages of the parasite where merozoites are released into the bloodstream, antibodies bind to the surface of these merozoites, and to proteins that are externalized from the apical complex of organelles involved in erythrocyte recognition and invasion. These antibodies could neutralize parasites and lead to Fc-dependent mechanisms of parasite killing by macrophages (Malaguarnera and Musumeci, 2002).

Antibodies produced against the parasites are more strain specific and protection from symptomatic and severe malaria have been connected to antibodies specific to VSAs (Chan *et al.*, 2012).

2.4.3 Cell mediated immunity

This form of immunity largely occurs in the pre-erythrocytic stage of the parasite. During an infection, sporozoites enter into the skin via female anopheles mosquito bites. They are then channeled to the liver, for the invasion of hepatocytes. They can also enter lymph nodes to induce T and B cell priming. After invasion of the hepatocytes, the sporozoites mature into merozoites and enter the bloodstream. Merozoites emerging from the liver into the bloodstream are able to infect RBCs, grow and multiply and reinfect RBCs. The merozoites and iRBCs at this stage can be opsonized and agglutinated by antibodies leading to their phagocytosis by macrophages and other phagocytic cells.

2.4.4 Blood stage parasite antigens in vaccine production

One of the major obstacle to the development of vaccines is primarily the lack of understanding of parasite immune response linked to protection (Greenhouse *et al.*, 2011). However antibodies are central to blood stage immunity (Doolan *et al.*, 2009). Immunity to malaria can be maintained by regular to parasite antigen exposure, and it will take natural exposure to boost an ideal blood stage vaccine to an extent (Doolan *et al.*, 2009). The main target of blood-stage vaccines is to improve and accelerate naturally acquired immunity.

A number of blood stage vaccines have shown inadequate proof of strain effectiveness. Currently, the more potent malaria vaccine is the RTS,S and this vaccine targets the pre-erythrocytic stage of the parasite. Work on this vaccine has reached phase III trials (Thera *et al.*, 2011; Duncan *et al.*, 2012). The effect of varying forms of disease severity transmission is uncertain and the production of a complementary blood stage vaccine will be desirable to deal with epidemic transmission patterns by mopping up leaky pre-erythrocytic immunity (Hill, 2011).

2.4.5 Immune evasion

The malaria parasite, in the course of its interaction with the host immune system receives a lot of selective pressure. As a result of this it has evolved overtime a number of mechanisms of which polymorphism is the commonest, thus enabling it to evade the host immune system (Kinyanjui, 2012). The ability of *Plasmodium falciparum* to elude the immune system permits it to cause recurring and long-lasting infections. It has been suggested that as exposure to the parasite increases, it creates a wide collection of antibodies that are capable of offering protection against a wide variety of parasite strains (Giha *et al.*, 2000). Therefore individuals may ultimately develop immunity which can effectively regulate parasitaemia and serious complications following repetitive exposure to the parasite (Marsh and Kinyanjui, 2006).

Despite developed immunity, the malaria parasites are capable of subsisting in the individual at minimum levels to the point that clinical signs are not evident. The acquisition of this type of immunity may be highly dependent on factors involving genetic background, age, co-infections and host nutritive status (Fortin *et al.*, 2002). Immune response that is protective may be inclined towards the parasite at the pre-erythrocytic stage, free merozoite of the erythrocytic stage or to antigens induced on the iRBC surface (Marsh and Kinyanjui, 2006).

2.5 Background on Assays Used

2.5.1 Parasite Culture & Merozoite Isolation

Plasmodium falciparum Parasite culture involves maintaining the parasites in O+ human blood in RPMI-1640 medium with supplements such as HEPES, gentamycin, L-glutamine etc. This is followed by incubation at 37°C in a gas atmosphere. Slides are prepared and stained to monitor and assess parasitaemia as well as the different stages of the parasite by microscopy.

Considering merozoite isolation, Hill *et al.* (2014) generated free viable merozoites from a highly synchronized *Plasmodium falciparum* culture by treating with E64 protease inhibitor to be used for merozoite opsonin-dependent phagocytosis assay. E64 functions in preventing schizont rupture while allowing merozoites to develop and segregate in within the membrane. The merozoites are released by the filtration of the treated schizonts.

2.5.2 Bradford Assay

The Bradford protein assay is a simple method used to determine the total protein concentration of a sample (Bradford, 1976). The method is based on the binding of the Coomassie blue dye to proteins in the sample. The binding of Coomassie dye is proportional to the amount of proteins present. Also the assay is colorimetric; increase in protein concentration leads to deeper coloration of test sample. Absorbance of the dye is measured at 595nm (Bradford, 1976). The protein concentration of the test sample is determined by comparing to the concentration of a protein standard known to reproducibly exhibit a linear absorbance profile. The standard widely used is the Bovine Serum Albumin (BSA) (Bradford, 1976).

2.5.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Significant efforts have been made to identify targets of malaria immunity through antibody titer measurements by ELISA. In one of those studies, antibodies in serum samples were measured

against purified whole merozoites supplemented with protease inhibitors (Osier *et al.*, 2014). The reactivity of IgG1 and IgG3 subclasses were determined in this assay (Osier *et al.*, 2014).

2.5.4 Flow Cytometry

Antibodies to ICAM-1 and CD36-selected parasites present in the plasma of patients were measured by flow cytometry. The ICAM-1 and CD36-binding parasites were obtained from late trophozoite and schizont stage culture and subsequently purified using the magnetic activated cell sorting (MACS) technique. A minimum of 5000 late stage parasite infected erythrocytes were labeled with ethidium bromide and incubated with goat anti-human IgG conjugated to fluorescein isothiocyanate. Anti-VSA antibodies levels were measured using mean fluorescent intensities and analysis was done with CellQuest v3.3 (Adukpo *et al.*, 2013).

2.5.5 IgG Purification

Protein A/G affinity purified IgG are obtained from affinity columns with protein A or G resins using the spin purification protocol or gravity purification protocol. Affinity-purified IgG can be tested for *in vitro* activity in parasite growth inhibition assays (GIAs) (Kusi *et al.*, 2009).

2.5.6 Growth Inhibition Assay

In vitro growth inhibition assays are currently the only functional assays applied to the study of acquired immunity to *Plasmodium falciparum* and candidate blood stage vaccines (Hill *et al.*, 2012). In the assay, parasites are cultured under standard conditions and antibody activity is tested by incubation with the parasites. The growth of the parasite is assessed by measuring parasite lactate dehydrogenase levels in lactate dehydrogenase assay (Kusi *et al.*, 2009).

2.5.7 Lactate Dehydrogenase Assay

This assay assesses parasitaemia by measuring the activity of *Plasmodium falciparum*. It is based on the ability of lactate dehydrogenase (LDH) enzyme of *Plasmodium falciparum* to rapidly use 3-acetyl pyridine NAD (APAD) as a coenzyme in the reaction with lactate to form pyruvate. Human red blood cell LDH can also react with APAD. But this reaction occurs at a very slow rate. Formation of APADH enables the detection of the presence of the parasite from in vitro cultures at paraasitaemia levels of 0.02%. Using this assay with clinical samples, there was a correlation between levels of parasitaemia and the activity of parasite LDH (Makler and Hinrichs, 1993). Parasite LDH activity can be measured in blood hemolysates and in plasma and serum from malaria patients.

2.5.8 Binding Inhibition Assay

Binding inhibition assays tests the inhibitory activity of antibodies against *Plasmodium falciparum*-infected erythrocytes that adhere to endothelia walls of blood vessels. Cytoadherence is mediated by PfEMP-1 and human receptors such as CD 36 and ICAM-1 (Almelli *et al.*, 2014). Study by Beeson *et al.* (1998) used competitive inhibitors of infected erythrocytes against immobilized receptors which in this case were purified chondroitin sulfate A (CS-A). Inhibition of binding to CS-A was found to be dependent on the molecular size of the inhibitors, which were oligosaccharide fragments isolated from CS-A following controlled chondroitin lyase digestion (Beeson *et al.*, 1998).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

The study design was a case-control study, which analyzed archived samples collected from a population at a specific point in time.

3.2 Study Site and Study Population

The study utilized archived plasma samples collected by a larger study that was conducted at Bongo in the Northern Region and Navrongo in the Upper East Region of Ghana. The samples were from children between the ages of 1 to 12 years with asymptomatic infections from community schools and those with clinical malaria from hospitals.

Basic clinical and demographic data of participants were captured by questionnaire to aid data analysis and interpretation, and thick blood smears were prepared for each participant for the detection of blood parasites by light microscopy.

Asymptomatic infection was defined as carriage of malaria parasites without showing clinical symptoms such as fever, headache, chills, myalgia, dizziness, nausea and diarrhea (CDC, 2016).

Clinical malaria was defined as the presence of asexual stage parasites accompanied by any one or more of the following symptoms: fever (temperature of $\geq 37.5^{\circ}\text{C}$ within 24 hours of hospital visit), headache, chills, myalgia, dizziness, nausea, and diarrhea (CDC, 2016).

3.3 Sample Size:

The sample size was estimated using the G Power sample size software with the following parameters:

- i. Effect size (d) = 0.5, meaning that 50% of the children infected with the parasite are expected to have clinical malaria.
- ii. P-value (α) = 0.05
- iii. Power ($1 - \beta$) = 0.8

The above parameters were chosen based on slightly high prevalence of malaria (Koram *et al.*, 2003; Owusu-Agyei *et al.*, 2009). On this basis, the number of samples required was 130 according to the sample size calculation software, G Power. Sample size calculated involved 65 asymptomatic individuals and 65 symptomatic individuals.

Meanwhile, archived samples from a rounded total of 153 participants; 81 from individuals with asymptomatic infection and 72 from individuals with clinical malaria were used.

3.4 Laboratory Procedures Used:

The laboratory procedures used in the current study include: Culture of Schizont Stage *P. falciparum*, isolation of late stage *P. falciparum* trophozoites and quantification of antigen (specific antibodies to iRBC membrane antigens and preparation of free merozoites). Bradford Assay was used to measure the concentration of *Plasmodium* merozoite antigen protein (Figure 3). Measurement of antibodies to merozoite surface antigens was also done by ELISA and that of antibodies to VSAs was done by Flow cytometry (Figure 3). Spin purification protocol was employed in purification and concentration of IgG. Growth inhibition assay (GIA) as well as ligand binding inhibition assay (BIA) were subsequently done (Figure 3).

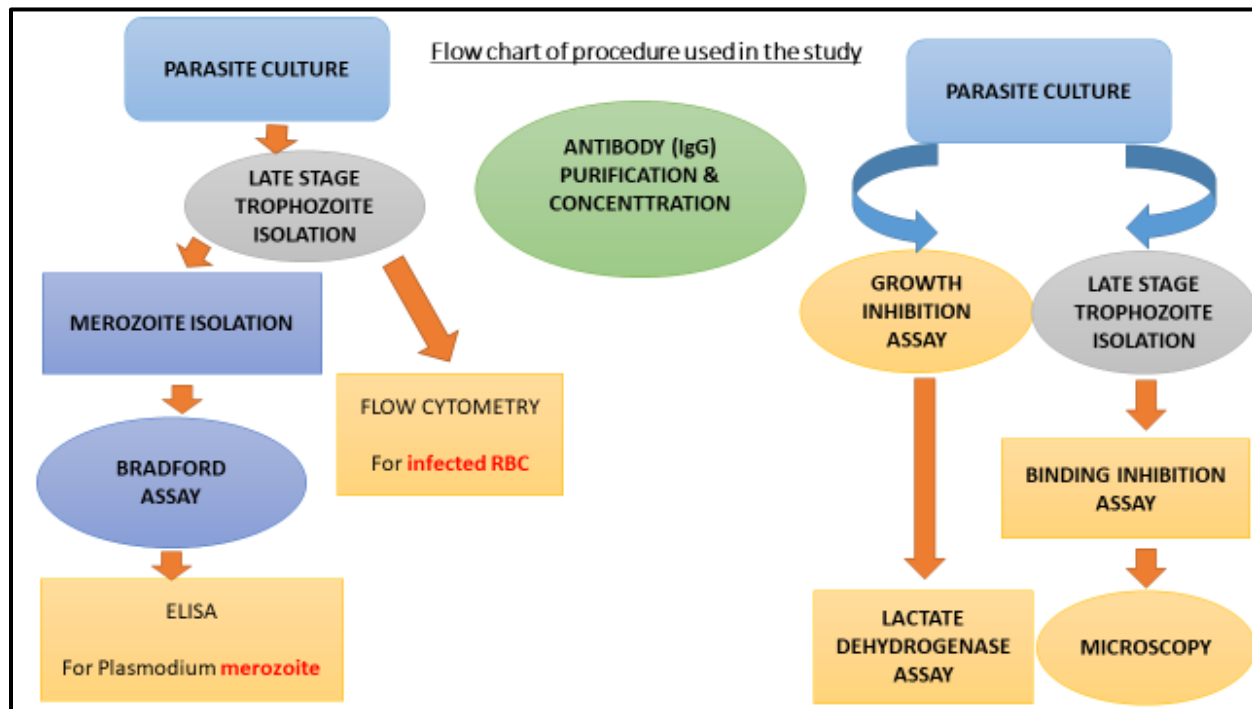


Figure 3: Overview of laboratory procedures

3.4.1 Culture of Schizont Stage *P. falciparum*

All *Plasmodium falciparum* culture procedures were carried out in a level 2 biosafety cabinet under sterile conditions.

3D7 lab strain of *Plasmodium falciparum* was cultured in human RBC using RPMI-1640 medium supplemented with 10% albumax, 20ug/ml gentamicin, and 25.0 mg/ml hepes in a culture flask. The parasites were maintained in a gas atmosphere containing 95% N₂, 4% CO₂ and 1% O₂ and incubated in a 37°C incubator. Thin smears were prepared and fixed with methanol for 10 sec, and consequently stained with 10% Giemsa. The smears were washed after 15min and allowed to dry. Parasitaemia was assessed using 100x oil immersion microscope.

After 24 hours of culture maintenance, the culture is centrifuged and the pellet is treated with sorbitol solution to allow for selection of ring stages. Parasite developmental stages and parasitemia were assessed daily from prepared smears (Hill *et al.*, 2014).

3.4.2 Isolation of Late Stage *P. falciparum* Trophozoites

Twenty-four (24) hours after returning sorbitol treated cultures, cultured cells were centrifuged for 5 min at 300xg. The supernatant was discarded and the pellet put into parasite medium. A magnetic column was connected to a magnet, and equilibrated with parasite medium for 10 min. The culture was transferred to the column and incubated for 15 minutes to allow the trophozoites to be held by the magnet since they contain haemozoin principally containing iron. After incubation the other stages of the parasites were allowed to pass through the column. The trophozoites were retrieved after the column was displaced from the magnet. Parasites were eluted from the column into 50 ml of parasite medium. Thin smear of parasites was prepared, fixed in 100% methanol for 10 sec, and stained with Giemsa. The prepared slide was then be examined under the Microscope.

3.4.3 Quantifying Antigen – Specific Antibodies to iRBC Membrane Antigens

Archived plasma samples were thawed from storage and heated by incubating at 56 °C for 30min. The isolated late stage Trophozoites (iRBCs) with 1 – 5% parasitaemia were washed three times separately in 3ml PBS with 2% Fetal Bovine Serum (FBS).

FACS tubes were precoated with 100µl of 2% FBS in PBS for 30 mins, at room temperature, to block non-specific binding. Fifty microliters (50µl) of iRBCs suspension was added per well containing 20µl test plasma and incubated for 30mins at room temperature (RT) and washed 3 times with 200µl of 2% FBS in PBS. Five microliters (5µl) of secondary antibody, goat anti-human IgG- fluorescein isothiocyanate (FITC) conjugate (Vector Laboratories Inc, USA) diluted

in buffer containing ethidium bromide (10-20 ug/ml) was added to the solution and incubated for 30mins at RT in the dark, then washed 3 times. The cells were re-suspended in 200 µl of FACS buffer. Acquisition was done on a minimum of 5000 iRBCs using FACScan flow cytometer (BD Biosciences, San Jose, CA). The levels of the antibodies bound to antigens on iRBCs and antibodies bound to merozoite antigens were expressed as mean fluorescence intensity (MFI) and determined by a flow cytometry analysis software, Cell Quest version 3.3 (BD Biosciences, San Jose, CA) (Beeson *et al.*, 2004; Adukpo *et al.*, 2013; Hill *et al.* 2014).

3.4.4 Preparation of Free Merozoites

Late stage Trophozoites harvested from previous culture was treated with 10µM E64 (Epoxy succinyl-L-leucylamido (4 guanidino) butane) for 6 - 10 hours to allow for maturation to schizonts without rupture (Osier *et al.*, 2014). After the parasites were incubated with E64, thin smear was prepared. The slides were fixed in methanol, and stained with 10% Giemsa solution. Microscopy was done to assess whether schizonts stages of the parasite have formed membrane with segmented merozoites enclosed. The E-64 schizont cultures was centrifuged at 1,900 x g for 8 min. The pellet was washed with 50ml wash medium containing RPMI-1640 medium with gentamicin, L-glutamine and hepes supplements without albumax. The pellet was resuspended in 2 ml of wash medium and filtered through a 1.2 µm/32 mm syringe filter connected to a syringe into a 15ml tube. The filtrate was passed over a small magnetic column attached to a magnet, and the flow-through was collected. Hemozoin was bound to the column, while merozoites passed through. Analysis of the purified merozoites was assessed by Giemsa-stained smears and flow cytometry (Hill *et al.*, 2014).

3.4.5 Measurement of *Plasmodium* Merozoite Antigen Protein concentration by Bradford Assay

The concentration of crude merozoite surface antigens required for ELISA was measured using Bradford Assay. The aim of this assay was to determine the concentration of free merozoites isolated from E64 treated cultures. The sample was freeze-dried to lyse the merozoites and consequent release the surface antigens in RPMI solution. The volume of the antigen sample was 5ml.

Different bovine serum albumin (BSA) concentrations were prepared from two fold serial dilutions using a BSA stock of 10mg/ml in a 96 well plate (Corning 96 Flat Bottom Transparent Polystyrol). 10 μ l of BSA solution was reacted with 200 μ l Biorad reagent (1X). The same procedure was done to the solution containing merozoite antigens. The Biorad reagent (1X) contains Coomassie blue dye which changes colour upon reaction with the sample from blue to brown. The standard and the sample in the 96 well plate were incubated for 5min. Absorbance was read at 590 – 595nm in a spectrophotometer (infinite 200 Pro, Tecan i-control , 1.7.1.12) and a calibration curve was plotted to serve as a standard curve. Sample concentration was obtained by extrapolation from the standard curve. The concentration of the free merozoite antigens obtained was 83.9 μ g/ml. This concentration was used in ELISA to determine the levels of merozoite surface antigen antibodies.

3.4.6 Measurement of Antibodies to Merozoite Surface Antigens

Whole merozoites (2 μ g/ml) (with crude antigens in solution) were coated in ELISA plates at 100 μ l per well in PBS (pH=7.2) and incubated at 4°C overnight, followed by six washes with PBS or washing buffer (PBST). After incubation the plates was blocked with 200 μ l of 3% milk powder in PBST at room temperature (RT) for 1hr in a humid chamber. This was followed by six washes with PBST and dry padding. 100 μ l of each plasma sample diluted at 1:50 in 1% milk powder in

PBST was added to the in 96 well plates in duplicates and incubated at RT for 1hr, followed by six washes in PBST and dry padding. The plates were further incubated with 100µL per well of secondary antibody (goat anti-human IgG, horseradish peroxidase (HRP)-conjugate) diluted at 1:1000 in 1% milk powder in PBST for 1hr with shaking on a rocker platform at RT followed by six washes and dry padding. 100µl of substrate (3,3',5,5'-tetramethylbenzidine - TMB) was added to each well and incubated for 30 minutes at RT in the dark. Following colour development, 100M 0.2M H₂SO₄ was added to stop the reaction. Colour development was quantified at 450nm and the absorbance was read using the microtitre plate reader. The results was extracted in excel and the optical density (OD) readings were converted to concentrations with the aid of the ADAMSEL FLP b039 software (Osier *et al.*, 2014).

3.4.7 Purification and concentration of IgG using the Spin Purification Protocol

Antibodies required for the functional assays were purified using the spin purification protocol. Archived antibodies were used for the asymptomatic individuals whilst antibodies required from symptomatic individual were purified and concentrated from 300µl of plasma per their availability.

Antibodies (IgG) from plasma were purified using Protein G columns (GE Healthcare, Etten-Leur, The Netherlands). Binding buffer containing PBS at pH 7.2 was used to bind the antibodies to the column. Antibodies were eluted using elution buffer containing mainly glycine at pH 2-3. After the elution of the antibodies, they were concentrated using AmiconUltra-15 tubes filter units (30-kDa cutoff; Millipore, Ireland). Subsequently antibodies were sterile-filtered using 0.22µm filter units (Millipore) and their concentrations were determined with a spectrophotometer (Nanodrop ND2000). The antibodies were then diluted in RPMI to obtain a final concentration of 20 mg/ml and stored at - 20°C until use.

3.4.8 Growth Inhibition Assay (GIA)

3D7 lab strain of *Plasmodium falciparum* were cultured to obtain ring stages at a parasitaemia of about 5%. Sorbitol synchronization was done to obtain a uniform ring stages. The parasites were put back into cultures and maintained till they developed to late trophozoite or early schizont stages within 24 hours. The culture was adjusted to a parasitaemia of 0.3% and a haematocrit of 2% by adding fresh RBCs. Afterward, the parasite was co-cultured with antibodies (IgG) in 96 well plates. Each IgG sample was tested in duplicate at a final concentration of 5mg/ml (Silva *et al.*, 2011). The 96 well assay plates were given a gas atmosphere containing 95% N₂, 4% CO₂ and 1% O₂ within an incubation chamber and placed in a 37°C incubator. After 40 – 44 hours late trophozoites were harvested by adding cold PBS. The harvested plates were stored in a freezer (-20°C) to allow for RBC rupture awaiting lactate dehydrogenase assay. Parasite growth assessment was done based on the activity of parasite lactate dehydrogenase in lactate dehydrogenase assay using lactate/diaphorase/APAD substrate system.

3.4.9 Lactate Dehydrogenase (LDH) Assay

Growth inhibition assay plates were removed from temporary storage (- 20°C) and allowed to thaw at room temperature for at least 30min. Fifty millilitres (50 ml) NBT solution was prepared from 50ml LDH buffer and cover with aluminum foil. Complete lactate dehydrogenase substrate solution was prepared by adding 50µl of APAD stock and 200µl of diaphorase stock to every 10ml of NBT solution. 100µl of complete LDH substrate was added to all the wells of the GIA plates. The reaction was timed for 30min and the plates were covered with aluminum foil and positioned on a flat-bed shaker at room temperature. Plates were read at an absorbance of 650nm in a

microplate reader. Optical densities were converted to percentage growth inhibition and expressed as:

$$\% \text{ Inhibition} = 100 - \left[\left(\frac{\text{OD Sample} - \text{OD RBC only}}{\text{OD Schizont Control} - \text{OD RBC only}} \right) \times 100 \right]$$

3.4.10 Binding Inhibition Assay (BIA)

Recombinant human endothelial receptors (CD36) that bind to PfEMP-1 antigens on iRBCs were diluted in PBS to a concentration of 10ug/ml. About 22 round spots were created on petri dishes and labeled. Twenty microlitres (20µl) of 10mg/ml CD36 receptors were placed on each spot on petri dishes and incubated overnight in a humid container at 4°C. The spots were blocked with 3% BSA for 30min at 37°C in order to avoid non-specific binding.

A culture of *Plasmodium falciparum* was prepared to obtain late trophozoite stage parasites. The parasites purified using magnetic cell sorting columns (MACS, Milteny Biotec) placed within a magnet. Parasites purified were then washed in PBS, centrifuged for 5 min at 800 x g and put in 3% BSA in RPMI 1640 medium with parastaemia and haematocrit adjusted to 20% and 2% respectively. Then 15 µl of parasite suspension and 5 µl of purified total IgGs was added to each spot and incubated for 30min at 37°C in an incubator. After 30 minutes the petri dishes were washed with PBS four times gently with an automated washing system. Unbound RBCs are washed off during the washing process. Bound cell were fixed for 30 min with 1.5% glutaraldehyde in PBS. The plates were subsequently stained in 10% Giemsa in PBS for 15 min and allowed to dry. Microscopy was done to assess the level of binding of infected RBCs. The amount of infected erythrocytes (IEs) bound to the receptors was obtained and expressed as

number of IEs bound/mm² by counting ten fields using the 40× objective. Results obtained was presented as the mean binding capacity of duplicate spots per sample. 1% BSA in PBS was used to assess non-specific binding (Almelli *et al.*, 2014).

3.5 Statistical Analysis

Experimental data was entered in Microsoft Excel (Office 2013) and Statistical analysis was done using Graph Pad Prism version 6. Test of significance was done using t-test and Mann-Whitney U test. P-values less than 0.05 were considered statistically significant.

3.6 Ethical Clearance

Ethical clearance was obtained from the Ethical and Protocol Review Committee of the College of Health Sciences (Protocol Identification Number: CHS-Et/M.7 – P 3.6/2015-2016) (Appendix III). This work also received approval from the Institutional Review Board (IRB) of Noguchi Memorial Institute of Medical Research.

CHAPTER FOUR

4.0 RESULTS

4.1 Demography and clinical characteristics of study participants

A total of 153 individuals (children), out of which 81 were asymptomatic and 72 were individuals presenting with clinical malaria (symptomatic) were involved in the study. The age of the participants in the asymptomatic group ranged from 1 to 12 years while that of the symptomatic group was within the range of 2 – 12 years (Table 2). There was no statistical difference between the age groups of asymptomatic and symptomatic children used for the study (P-value= 0.1434, Mann-Whitney U test).

Table 2: Age demographics of study population

	Asymptomatic	Symptomatic	Total	P-value*
Age				
Median	5.00	5.00		0.1434
Range	(1-12)	(2-12)		
N	81	72	153	

*Mann-Whitney U test

4.2 Parasite densities of Study samples

In terms of parasite density among the study groups, averagely, the level of parasites observed in the symptomatic children was 46 498.60 parasites per microliter of blood whiles that of the asymptomatic children was 644.77 parasites per microliter of blood. Therefore, significantly low level of parasites were observed in the asymptomatic plasma samples compared to the symptomatic individuals ($p < 0.001$, Mann-Whitney U test) (Figure 4).

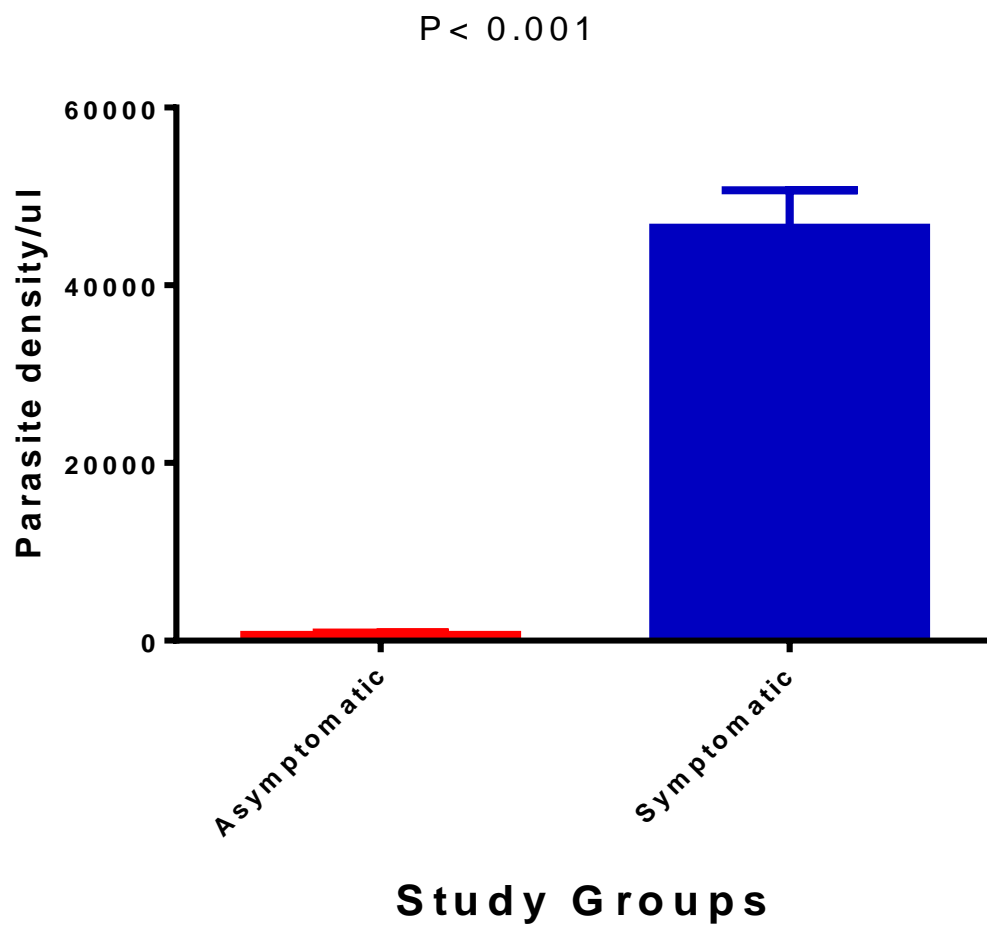


Figure 4: Parasite densities of study population

4.3 Antibody levels of antibodies to merozoite antigens

4.3.1 Concentration merozoite antigens by Bradford Assay

After measuring sample concentration (at 590 – 595nm) following change of colour of Coomassie blue in Biorad Reagent from blue to brown, a mean sample OD of 0.4798 was observed. A sample concentration of 0.0839 mg/ml = 83.9 ug/ml was achieved which represented the concentration of crude merozoites surface antigens required for ELISA (See Appendix I).

4.3.2 Antibody levels of antibodies against merozoite surface antigens in symptomatic and asymptomatic individuals by indirect ELISA

Asymptomatic individuals showed the highest antibody levels with a mean value of 1451.78 AU whilst symptomatic individuals showed a mean value of 380.868 AU ($p < 0.0001$, Mann-Whitney U test) (Fig 5).

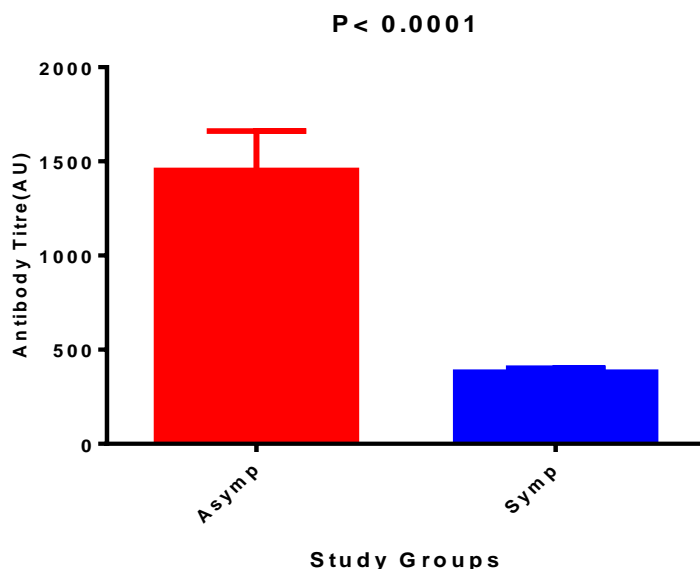


Figure 5: Antibody levels of merozoite surface antigen antibodies

4.4 Antibody levels of antibodies to variant surface antigens

As shown in Figure 6, in measurement of variant surface antigen-antibody levels, a scatter plot showed EtBr stained parasites after flow cytometric analyses. First quadrant of the scatter plot showed EtBr stain and no parasites (Figure 6A). Second quadrant of the scatter plot displayed EtBr staining without parasites (Figure 6A). Third quadrant of the scatter plot showed acquisition for late trophozoite and early schizont stages of the parasite, while fourth quadrant of the scatter plot represents uninfected RBCs and ring stages of the parasite (Figure 6A). Histogram showing IgG-FITC response against the late trophozoite and early schizont stages of the parasite was also achieved where mean fluorescent intensity for the acquisition was shown by the M1 range (Figure 6C). Among samples from asymptomatic children the mean fluorescent intensity (M1) ranged from 20.44 – 3720.96 while that of the symptomatic children was within the range of 82.81 – 1973.97. This corresponded to the quantity of IgG bound to the parasite antigens (Figure 6B). Subsequently, a table showing histogram statistics of acquisition done for more than 5000 events was also obtained (Figure 6C).

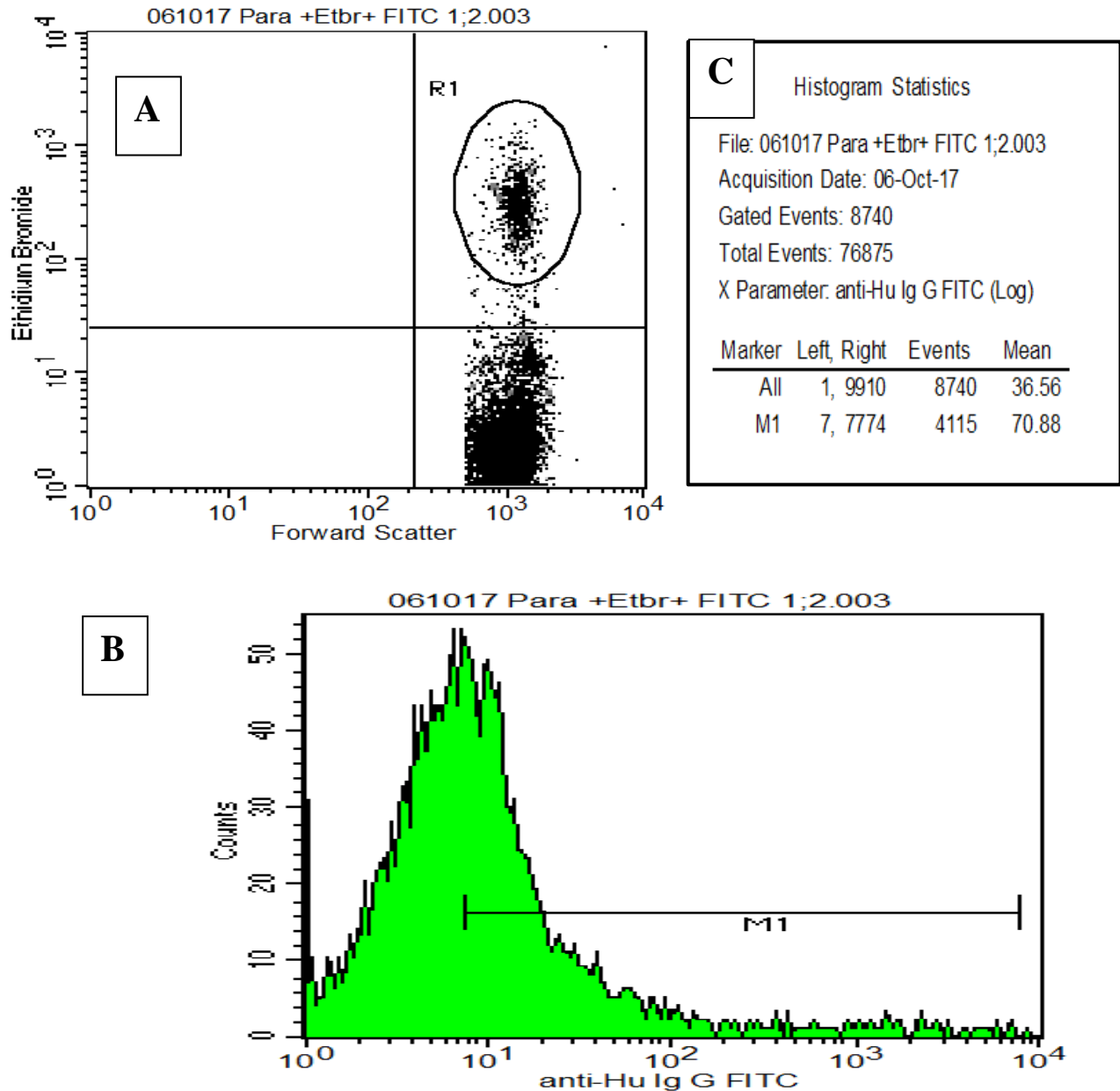


Figure 6: FACSCAN reading of plasma sample containing ethidium bromide treated antihuman IgG-FITC. A. Scatter plot showing EtBr stained parasites. B. Histogram showing IgG-FITC response against the late trophozoite and early schizont stages of the parasite. C. Table showing histogram statistics of acquisition done for more than 5000 events

Total IgG levels between symptomatic and asymptomatic individuals against variant surface antigens showed no statistical significance ($p=0.6469$, Mann-Whitney U test) even though IgG levels for asymptomatic individuals measured (624.366 MFI) was slightly higher than that of the symptomatic individuals (551.643 MFI) (Figure 7).

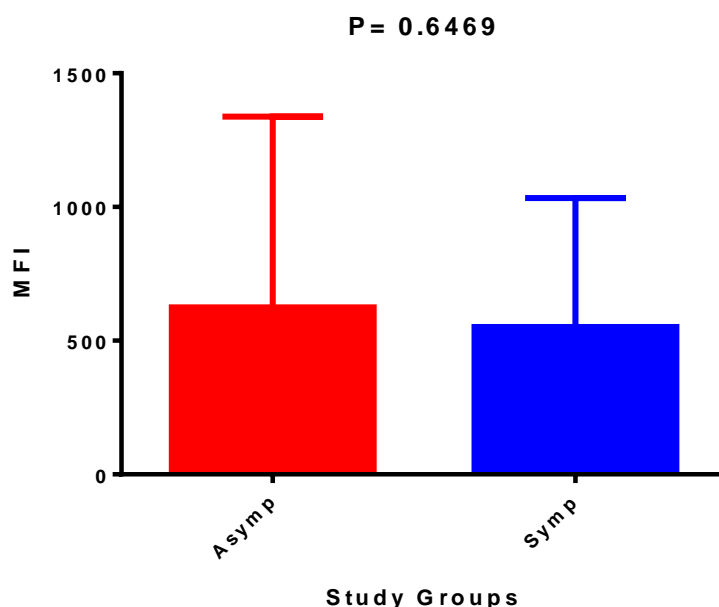


Figure 7: A graph showing antibody levels of the two study groups in mean fluorescent intensity (MFI).

4.5 Antibody Purification and Concentration

A concentration of 19 mg/ml to 21 mg/ml required for the functional assays was obtained after antibodies were purified and concentrated from 24 plasma symptomatic samples. Similar range of concentration (19 mg/ml to 21 mg/ml) was also obtained from archived asymptomatic group. Full results of purified and concentrated antibodies from the symptomatic samples is found in Appendix II.

4.6 RBC invasion inhibitory activities of merozoite surface antigen-specific antibodies

There was a slight difference in percentage growth inhibition of merozoite surface antigen-specific antibodies. Growth inhibitory activities of antibodies in asymptomatic individuals measured was 38.25% and that of symptomatic individuals was 50.15%. However the difference recorded was not statistically significant ($p=0.2487$, Mann-Whitney U test) between the two groups (Fig 8).

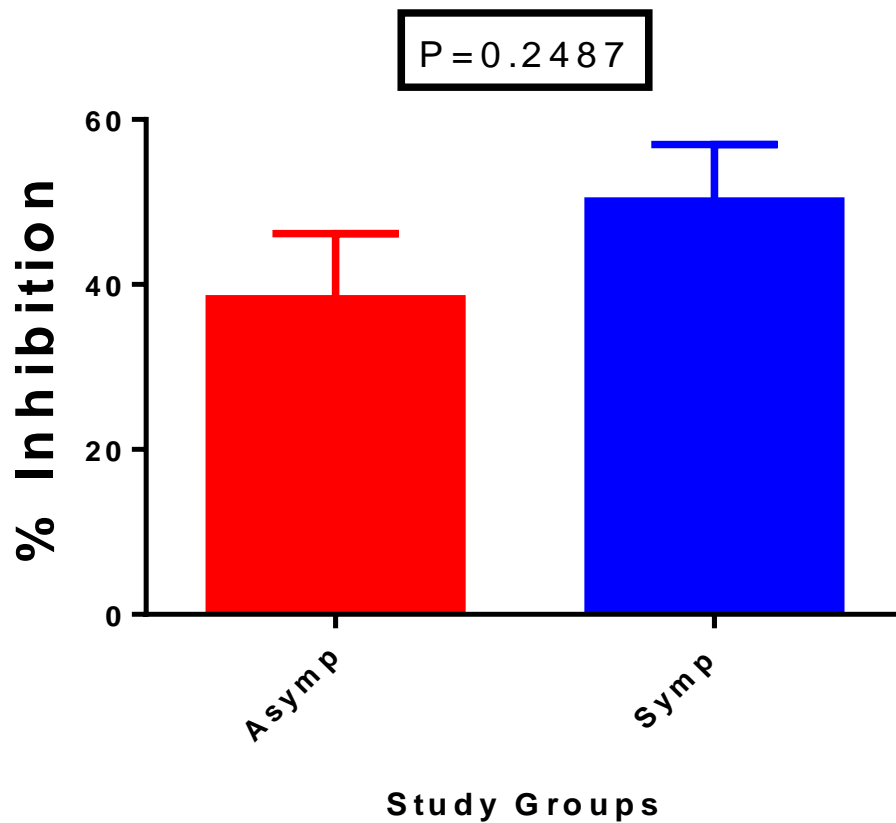


Figure 8: A graph showing growth inhibitory activities of merozoite surface-specific antibodies in asymptomatic and symptomatic individuals

4.7 Binding inhibitory activities of iRBC surface antigen-specific antibodies on CD36 endothelial receptors

Mean binding of infected RBCs observed in asymptomatic individuals was 0.165 iRBC bound per mm^2 and that of symptomatic individuals was 0.106 iRBC bound per mm^2 . There was statistical difference between the two groups ($p= 0.0260$, Mann-Whitney U test) with the binding for the asymptomatic individuals being slightly higher than the symptomatic individuals (Fig 9). Lower binding indicates high inhibitory capacity of the antibodies. However this does not give the true representation of the inhibitory ability of the antibodies as mean iRBC bound per mm^2 may not be equal to percentage inhibition which was not calculated due to absence of standard controls.

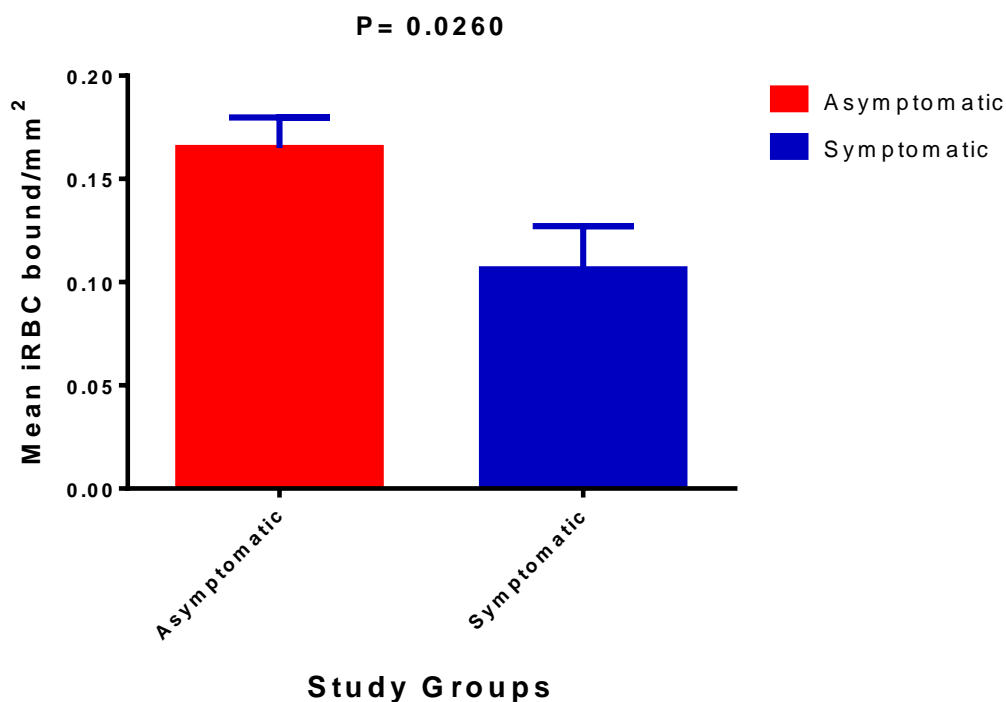


Figure 9: A graph showing binding activities of infected RBC surface antigen-specific antibodies in asymptomatic and symptomatic individuals

CHAPTER FIVE

5.0 DISCUSSION

Levels of antibodies to merozoite surface antigens and antibodies to infected RBC surface found in the study is important in assessing the relative importance of the two sets of antibodies between the two groups by comparing with their functional effects. Findings from *in vitro* assays showed the functional activities of the two sets of antibodies.

5.1 Demography and clinical characteristic of study participants

The study was centered on children between the ages of 1 to 12 years since these individuals have been shown to be more susceptible to the disease and as a result, higher number of deaths recorded are attributed to children. (Langhorne *et al.*, 2008; WHO, 2015)

It was observed in this study that symptomatic individuals had more parasites in their blood than asymptomatic individuals which supports the general assertion that symptomatic individuals have high parasite levels which enables easy detection for treatment (Greenhouse *et al.*, 2011). A study by Andrade *et al.*, 2009 and Barbosa *et al.*, 2014 showed that individuals who have had *Plasmodium* infection repetitively could become asymptomatic carriers with time.

Asymptomatic individuals harbor the parasite without developing any symptoms and this could be a hindrance to malaria eradication as well as a health care canker, because they act as parasite storage, which may allow malaria infection to be preserved within a population. Overtime the *Plasmodium* infection can be transmitted to uninfected people (Gouagna *et al.*, 2004; White, 2008). Furthermore, carriers of asymptomatic malaria infection can pose a risk to the safety of the blood

banks as they can transmit malaria through blood transfusions (Fugikaha *et al.*, 2007; Anthony *et al.*, 2013).

5.2 Antibody levels against merozoite surface antigens and clinical malaria

The presence of antibody response against merozoite surface antigens in the two groups of individuals may be linked to the exposure to the *Plasmodium* parasite. Higher antibody levels in asymptomatic individuals could mean that the antibodies may be associated with the absence of presentation of clinical symptoms even though these individuals carried malaria parasite and this could be linked to clinical protection (Nebie *et al.*, 2008). Also the asymptomatic individuals might have had long term previous exposure that also accounted for the higher antibody titres. Lower antibody levels in symptomatic individuals may explain why these individuals showed clinical signs. These individuals may have used up all the antibodies in fighting the infection. Therefore the antibodies may have degenerated and for that matter their titres were reduced. These findings supports other studies where antibodies have been shown to be potential markers of exposure and protection (Sennang *et al.*, 2014; Greenhouse *et al.* 2011).

5.3 Antibody levels against variant surface antigens and clinical malaria

Statistically insignificant difference between antibody levels of antibodies against variant surface antigens of both symptomatic and asymptomatic individuals obtained from the results gives an indication that, infection from the parasite could be an acute one and not severe. These antibodies appear to have protected the host probably from future clinical episodes. This assumption has been highlighted in binding inhibition assays in studies from Hasler *et al.*, 1993 and Vestergaard *et al.*, 2008. Recent body of evidence shows that VSAs on the surface of parasitized RBCs are the main targets of protective IgGs (Hviid 2005). These antibodies have been shown to be associated with

protection from cytoadhesion and for that matter preventing the parasitized RBCs from sequestering in the blood vessels (Chan *et al.*, 2012). Another possible explanation could be that of inactive parasitaemia or low magnitude of exposure leading to an uncomplicated malaria may have been the reason behind lack of significant difference between the antibodies of the two groups of individuals.

5.4 Antibody Purification and Concentration

Antibodies purified and concentrated (to an optimal concentration of 19 to 21 mg/ml) using protein G sepharose columns provided the required concentration needed for the functional assays, which was in agreement with what was described by Kusi *et al.* (2009). Kusi and colleagues purified serum IgG over protein A sepharose columns and also did affinity fractionation which was subsequently used for competition assays (Kusi *et al.*, 2009).

5.5 RBC invasion inhibitory activities of merozoite surface antigen-specific antibodies

Statistically insignificant levels of Inhibitory activities of merozoite surface antigen-specific antibodies between asymptomatic and symptomatic individuals, indicates that same quantities of antibodies may function similar. This is because in performing the growth inhibition assay, equal quantities (5mg/ml optimal concentration) of antibodies were used for the two groups. However their function might differ *in vivo*, due the higher concentration of anti-merozoite surface antibodies found in asymptomatic individuals. The inhibition activity may involve inhibition of merozoite invasion and intra-erythrocyte parasite development. High antibody levels to antigens have been shown to be often associated with protection (Nebie *et al.*, 2008), even though others have also argued that antibody quantity does not necessarily mean antibody quality as far as protection is concerned (Marsh *et al.*, 1989).

5.6 Binding inhibitory activities of iRBC surface antigen-specific antibodies on CD36 endothelial receptors

Similar to what has been reported by Kyes *et al.*, (2001) and Sherman *et al.*, (2003), in the current study asymptomatic individuals had more of infected RBC binding to the endothelial receptor CD36 via VSA ligands (PfEMP-1) than symptomatic individuals, however less binding of the infected RBCs was observed in symptomatic individuals. 3D7 strain of *Plasmodium falciparum* has been shown in studies that they have affinity to bind to CD36 or ICAM-1 (Kyes *et al.*, 2001; Sherman *et al.*, 2003). The presence of the antibodies may have resulted in the quality of binding observed in the current. Parasites from symptomatic children have been shown to be strongly recognized than parasites from asymptomatic children (Vestergaard *et al.*, 2008). This means that they would have higher inhibition in symptomatic children than asymptomatic children. Calculating percentage binding inhibition which was not addressed in the binding inhibition assay due to few limitations, meanwhile, this could have given a clearer picture of the binding inhibitory activity of the antibodies against the VSAs on the surface of iRBCs. Difference in binding levels was significant ($p=0.0260$, Mann-Whitney U Test), nonetheless this may not be the case for the inhibitory activities of the antibodies of the two groups of individuals. The difference in antibody titres of the two groups measured was not statistically significant ($P=0.6469$). Therefore this may also give an implication of how the inhibitory activity of the antibodies could be. Their inhibitory activities seem not to be too different from each other. This could mean that antibodies in asymptomatic and symptomatic individuals in parallel are capable of preventing cytoadhesion, thus preventing the parasite from sequestering in the blood vessels that may lead to complicated or severe malaria. Several studies have shown that these antibodies specific to VSAs are very

essential for acquired immunity by inhibiting cytoadhesion (Chan *et al.*, 2012; Vestergaard *et al.*, 2008).

Major findings from the study implies that the quantity of antibodies in plasma of an individual may be able to indicate the quality of the antibodies in terms of their functionality. Same concentration of antibodies used gave no much difference in their functionalities. Thus the understanding of antibody levels and functionality adds to basic knowledge required in the search for blood stage vaccine candidates. Antibodies to merozoite surface antigen targets and those against infected RBC surface antigen targets were found to be both important. Hence, it gives an indication that research in the humoral activities concerning these two targets of protective immunity should be extended. Furthermore focus on the merozoite surface antigen targets will enable parasite clearance and a suitable vaccine development whilst focus on infected RBC VSA targets will enhance intercepting malaria from aggravating from acute to severe and complicated malaria.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Antibodies against antigens of *Plasmodium* merozoite and infected RBC surface seem to provide similar contribution in malaria immunity. This work has therefore verified our hypothesis, as the two sets of antibodies have shown similar importance. *Plasmodium falciparum* virulence is connected to the aptitude of the parasite to elude the immune system of the host through antigenic variation of merozoite proteins and adhesion of infected erythrocytes (IEs) to surface receptors of endothelial tissues. Therefore a more critical look at the role of antibodies and their targets as well as the understanding of their immune mechanism and effector functions will contribute enormously in the development of blood stage vaccines.

6.2 Recommendation

Following this study, below are some recommendations made:

- Comparison of various individual merozoite surface antigens to total merozoite surface antigens as well as individual variant surface antigens among symptomatic and asymptomatic individuals is another research area which could be looked at.
- IgG subclasses: IgG1, IgG2, IgG3, IgG4 in comparison with total IgGs against the merozoite surface antigens and variant surface antigens among symptomatic and asymptomatic individuals could be another important research

6.3 Limitation

The limitation of the study has to do with the inability to calculate the percentage binding inhibition of the anti-VSA antibodies due to the absence of standardized controls. As a result, their binding levels which may give an indication of their inhibition abilities were compared. Also the assay could not be repeated due to shortage of the endothelial receptors (CD36) and the shortage of samples.

REFERENCES

- Adamou, R., Chénou, F., Sadissou, I., Sonon, P., Dechavanne, C., Djilali-saïah, A., ... Courtin, D.** (2016). Acta Tropica *Plasmodium falciparum* infection and age influence parasite growth inhibition mediated by IgG in Beninese infants. *Acta Tropica*, 159, 111–119. <https://doi.org/10.1016/j.actatropica.2016.03.020>.
- Adukpo, S., Kusi, K. A., Ofori, M. F., Tetteh, J. K. A., Amoako-sakyi, D., Goka, B. Q., ... Dodoo, D.** (2013). High Plasma Levels of Soluble Intercellular Adhesion Molecule (ICAM) -1 Are Associated with Cerebral Malaria. *PLoS ONE* 8(12): e84181. <https://doi.org/10.1371/journal.pone.0084181>.
- Ahouidi, A. D., Amambua-Ngwa, A., Awandare, G. A., Bei, A. K., Conway, D. J., Diakite, M., ... Zenonos, Z. A.** (2015). Malaria Vaccine Development: Focusing Field Erythrocyte Invasion Studies on Phenotypic Diversity. *Trends in Parasitology*, 1453, 1–10. <http://doi.org/10.1016/j.pt.2015.11.009>.
- Almelli, T., Ndam, N. T., Ezimegnon, S., Alao, M. J., Ahouansou, C., Sagbo, G., ... Tahar, R.** (2014). Cytoadherence phenotype of *Plasmodium falciparum* - infected erythrocytes is associated with specific pfemp-1 expression in parasites from children with cerebral malaria. *Malaria Journal*, 13(333), 1–9.
- Andrade, B. B. and Barral-Netto, M.** (2011). Biomarkers for susceptibility to infection and disease severity in human malaria. *Mem Inst Oswaldo Cruz* 106 (Suppl. I), 70-78.
- Andrade, B. B., Rocha, B. C., Reis-Filho, A., Camargo, L. M. A., Tadei, W. P., Moreira, L. A., Barral, A., Barral-Netto, M.** (2009). Anti-Anopheles darlingi saliva antibodies as marker

of *Plasmodium vivax* infection and clinical immunity in the Brazilian Amazon. *Malar J* 8, 121.

Angulo, I. F. M. (2002). Cytokines in the pathogenesis of and protection against malaria. *Clin Diagn Lab Immunol*, 9, 1145–52.

Anthony, C. N., Lau, Y-L., Sum, J-S., Fong, M-Y., Ariffin, H., Zaw, W-L., Jeya- jothi. I., Mahmud, R. (2013). Malaysian child infected with *Plasmodium vivax* via blood transfusion: a case report. *Malar J* 12: 308.

Askling, H. H., Bruneel, F., Burchard, G. et al. (2012). Management of imported malaria in Europe. *Malaria Journal*; 11:328.

Barbosa, S., Gozze, A. B., Lima, N. F., Batista, C.L., Bastos, M. da S., Nico-lete, V. C., Fontoura, P. S., Gonçalves, R. M., Viana, S. A. S., Menezes, M. J., Scopel, K. K. G., Cavasini, C. E., Malafrente, R. dos S., da Silva-Nunes, M., Vinetz, J. M., Castro, M. C., Ferreira, M. U. (2014). Epidemiology of disappearing *Plasmodium vivax* malaria: a case study in rural Amazonia. *PLoS Negl Trop Dis* 8: e3109.

Beeson, J. G. and Crabb, B. S. (2007). Towards a vaccine against *Plasmodium vivax* malaria. *PLoS Med*, 4, e350.

Beeson, J. G., Brown, G. V., Molyneux, M. E., Mhango, C., Dzinjalama, F., and Rogerson, S. J. (1999). *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. *Journal of Infectious Diseases*, 180(2), 464–472. <https://doi.org/10.1086/314899>.

Beeson, J. G., Chai, W., Rogerson, S. J., Lawson, A. M., & Brown, G. V. (1998). Inhibition of Binding of Malaria-Infected Erythrocytes by a Tetradecasaccharide Fraction from

Chondroitin Sulfate A. *Infect Immun*, 66(7), 3397–3402.

- Beeson, J. G., Drew, D. R., Boyle, M. J., Feng, G., Fowkes, F. J., & Richards, J. S.** (2016). Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. *FEMS microbiology reviews*, 40(3), 343-72.
- Beeson, J. G., Mann, E. M., Elliott, S. R., Lema, V. M., Tadesse, E., Molyneux, M. E., Brown, G. V., and Rogerson, S. J.** (2004). Antibodies to variant surface antigens of *Plasmodium falciparum* infected erythrocytes and adhesion inhibitory antibodies are associated with placental malaria and have overlapping and distinct targets. *J Infect Dis* 189, 540-551.
- Bengtsson, D., Sowa, K. M., Salanti, A., Jensen, A. T., Joergensen, L., Turner, L., ... Arnot, D. E.** (2008). A method for visualizing surface-exposed and internal PfEMP1 adhesion antigens in *Plasmodium falciparum* infected erythrocytes. *Malaria Journal*, 7, 101. <http://doi.org/10.1186/1475-2875-7-101>.
- Bolad, A., and Berzins, K.** (2000). Antigenic Diversity of *Plasmodium falciparum* and Antibody-Mediated Parasite Neutralization. *Scand J Immunol*, 52(3), 233–239.
- Boyle, M. J., Wilson, D. W., and Beeson, J. G.** (2013). New approaches to studying *Plasmodium falciparum* merozoite invasion and insights into invasion biology. *International Journal for Parasitology*, 43(1), 1–10. <https://doi.org/10.1016/j.ijpara.2012.11.002>.

- Boyle, M. J., Wilson, D. W., Richards, J. S., Riglar, D. T., & Tetteh, K. K. A. (2010).** Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development. *PNAS*, *107*(32), 14378–14383. <http://doi.org/10.1073/pnas.1009198107//DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1009198107>.
- Bradford, M. M. (1976).** A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248-254.
- Brown, Jg. and Beeson, Gv. (2002).** Pathogenesis of *Plasmodium falciparum* malaria : the roles of parasite adhesion and antigenic variation. *Cell Mol Life Sci.*, *59*(2), 258–271.
- Butler, D. (2011).** Malaria vaccine results face scrutiny: Experts question early release of incomplete trial data. *Nature*, *478*, 439-440. Doi: 10.1038/478439a.
- Centers for Disease Control and Prevention. (2016).** Malaria Surveillance–United States, 2013. *Morbidity and Mortality Weekly Report*; *65*(2), 1-22.
- Centers for Disease Control and Prevention. (2017).** <https://www.cdc.gov/malaria/>. [Accessed 15/03/17].
- Chan, J. A., Howell, K. B., Reiling, L., Ataide, R., Mackintosh, C. L., Fowkes, F. J. I., ... Beeson, J. G. (2012).** Targets of antibodies against *Plasmodium falciparum* -infected erythrocytes in malaria immunity. *Journal of Clinical Investigation*, *122*(9), 3227–3238. <https://doi.org/10.1172/JCI62182>.

- Chauhan, V. S.** (2007). Vaccines for malaria – prospects and promise. *Curr Sci.*;92(11), 1525-1524.
- Coppel, R., Cooke, B. M., Glenister, F. K., Mohandas, N., and Coppel, R. L.** (2002). Assignment of functional roles to parasite proteins in malaria-infected red blood cells by competitive flow-based adhesion assay . Assignment of functional roles to parasite proteins in malaria-infected red blood cells by competitive flow-based adhesion. *British Journal of Haematology*, 117(MAY), 203–211. <https://doi.org/10.1046/j.1365-2141.2002.03404.x>.
- Courtin, D., Oesterholt, M., Huismans, H., Kusi, K., Milet, J., Badaut, C., Gaye, O., Roeffen, W., Remarque, E. J., Sauerwein, R., Garcia, A., Luty, A. J. F.** (2009). The quantity and quality of African children’s IgG responses to merozoite surface antigens reflect protection against *Plasmodium falciparum* malaria. *PLoS One*, 4, e7590, <http://dx.doi.org/10.1371/journal.pone.0007590>.
- Cowman, A. F., and Crabb, B. S.** (2006). Review Invasion of Red Blood Cells by Malaria Parasites. *Cell*, 124, 755–766. <http://doi.org/10.1016/j.cell.2006.02.006>.
- Cox, F. E. G.** (2010). History of the discovery of the malaria parasites and their vectors. *Parasites and Vectors*, 3(5), 1–9.
- Crabb, B. S., Triglia, T., Waterkeyn, J. G., and Cowman, A. F.** (1997). Stable transgene expression in Plasmodium falciparum. *Mol Biochem Parasitol*, 90(1), 131-144.
- Duncan, C. J. A., Hill A. V. S., and Ellis, R. D.** (2012). Can growth inhibition assays (GIA) predict blood-stage malaria vaccine efficacy? *Hum Vaccin Immunother.* Jun 1; 8(6): 706–714. doi: 10.4161/hv.19712.

- Doolan**, D. L., Doban, C., and Baird, J. K. (2009). Acquired Immunity to Malaria. *Clinical Microbiology Reviews*, 22(1), 13–36. <https://doi.org/10.1128/CMR.00025-08>
- Egan**, A. F., Chappel, J. A., Burghaus, P. A., Morris, J. S., Bride, J. S. M. C., Holder, A. A., ... Riley, E. M. (1995). Serum Antibodies from Malaria-Exposed People Recognize Conserved Epitopes Formed by the Two Epidermal Growth Factor Motifs of MSP1 19 , the Carboxy-Terminal Fragment of the Major Merozoite Surface Protein of *Plasmodium falciparum*. *Infection and Immunity*, 63(2), 456–466.
- Fairhurst**, R. M., and Wellems, T. E. (2015). Malaria (plasmodium species). In *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)*, 3070-3090.
- Fan**, Y. T., Wang, Y., Ju, C., Zhang, T., Xu, B., Hu, W., & Chen, J. H. (2013). Systematic analysis of natural antibody responses to *P. falciparum* merozoite antigens by protein arrays. *J Proteomics*, 78, 148–158. <https://doi.org/10.1016/j.jprot.2012.11.020>.
- Fugikaha**, E., Fornazari, P. A., Penhalbel, R. de S. R., Lorenzetti, A., Maroso, R. D., Amoras, J.T., Saraiva, A. S., Silva, R. U da., Bonini-Domingos, C. R., Mattos, L. C de., Rossit, A. R. B., Cavasini, C. E., Machado, R. L. D. (2007). Molecular screening of *Plasmodium* sp. asymptomatic carriers among transfusion centers from Brazilian Amazon Region. *Rev Inst Med Trop Sao Paulo*, 49, 1-4.
- Fortin**, A., Stevenson, M. M. and Gros, P. (2002) Susceptibility to malaria as a complex trait: Big pressure from a tiny creature. *Hum Mol Genet*, 11(20):2469-2478.
- Gachot**, B. and Ringwald, P. (2014) ‘Severe malaria’. *Tropical Medicine and International Health*, 19, pp. 7–131. doi: 10.1111/tmi.12313.

- Gamain, B., Miller, L.H. and Baruch, D.I.** (2001). The surface variant antigens of *Plasmodium falciparum* contain cross- reactive epi- topes. Proceedings of the National Academy of Sciences of the United States of America, 98, 2664–2669.
- Genton, B.** (2008). Malaria vaccines: A toy for travelers or a tool for eradication? *Expert Review Vacc*; 7(5), 597-611.
- Giha, H. A., Staalsoe, T., Dodoo, D., Roper, C., Satti, G. M., Arnot, D. E., ... Theander, T. G.** (2000). Antibodies to variable *Plasmodium falciparum* -infected erythrocyte surface antigens are associated with protection from novel malaria infections. *Immunology Letters*, 71(2), 117–26. [http://doi.org/10.1016/S0165-2478\(99\)00173-X](http://doi.org/10.1016/S0165-2478(99)00173-X).
- Greenhouse, B., Ho, B., Hubbard, A., Njama-meya, D., Narum, D. L., Lanar, D. E., ... John, C. C.** (2011). Antibodies to *Plasmodium falciparum* Antigens Predict a Higher Risk of Malaria But Protection From Symptoms Once Parasitemic. *Journal of Infectious Diseases* 1899(204), 19–26. <https://doi.org/10.1093/infdis/jir223>.
- Gouagna, L. C., Ferguson, H. M., Okech, B. A., Killeen, G. F., Kabiru, E. W., Beier, J. C., Githure, J. I., Yan, G.** (2004). *Plasmodium falciparum* malaria disease manifestations in humans and transmission to *Anopheles gambiae*: a field study in Western Kenya. *Parasitology* 128, 235-243.
- Hasler, T., Albrecht, G. R., Schravendijk, Van, M. R., Aguiar, J. C., Morehead, K. E., Pasloske, B. L., Ma, C., Barnwell, J. W., Greenwood, B., and Howard, R. J.** (1993). An improved microassay for *Plasmodium falciparum* cytoadherence using stable transformants of Chinese hamster ovary cells expressing CD36 or intercellular adhesion molecule-1. *Am J Trop Med Hyg*, 48:332–347.

- Hay, S. I., Guerra, C. A., Tatem, A. J., Noor, A. M., and Snow, R. W. (2004).** Europe PMC Funders Group The global distribution and population at risk of malaria : past , present , and future.*The Lancet Infectious Disease*, 4(6), 327–336. [https://doi.org/10.1016/S1473-3099\(04\)01043-6](https://doi.org/10.1016/S1473-3099(04)01043-6).
- Heyman, D. L. (2014).** Malaria. In: *Control of Communicable Diseases Manuel 20th ed*, American Public Health Association, Washington, 372-389.
- Hill, A.V.** Vaccines against malaria. *Philos Trans R Soc Lond B Biol Sci.* (2011);366:2806–14. doi: 10.1098/rstb.2011.0091.
- Hill, D. L., Eriksson, E. M., Carmagnac, A. B., Wilson, D. W., Cowman, A. F., Hansen, D. S., & Schofield, L. (2012).** Efficient Measurement of Opsonising Antibodies to *Plasmodium falciparum* Merozoites. *PLoS ONE*, 7(12), 1–10. <https://doi.org/10.1371/journal.pone.0051692>.
- Hill, D. L., Eriksson, E. M., & Schofield, L. (2014).** High Yield Purification of *Plasmodium falciparum* Merozoites For Use in Opsonizing Antibody Assays. *J Vis Exp*, e51590 (89), 1–11. <http://doi.org/10.3791/51590>.
- Hviid, L. (2005).** Naturally acquired immunity to *Plasmodium falciparum* malaria in Africa. *Acta Tropica*, 95(3), 270–275. <http://doi.org/10.1016/j.actatropica.2005.06.012>.
- Huyuh, B., Cottrell, G., Cot, M. and Briand, V. (2015).** Burden of Malaria in Early Pregnancy: A Neglected Problem? *Clinical Infectious Diseases*; 60(4):598-604.

- Jawetz**, Melnick and Adelberg. (2007). *Medical Microbiology* 24th Edition, McGraw-Hill Medical. 0-07-151054-0.
- Jiang**, L., Gaur, D., Mu, J., Zhou, H., Long, C. A., Miller, L. H. (2011). Evidence for erythrocyte-binding antigen 175 as a component of a ligand-blocking blood-stage malaria vaccine. *Proc. Natl. Acad. Sci. U. S. A.* 108, 7553–7558.
- Kaviratne**, M, Khan, S.M., Jarra, W., Preiser, P.R. (2002). Small variant STEVOR antigen is uniquely located within Maurer’s clefts in *Plasmodium falciparum* -infected red blood cells. *Eukaryotic Cell.* 1(6), 926–935.
- Kinyanjui**, S. M. (2012). The Immunology of Malaria. *Malaria Parasites*, Dr. Omolade Okwa (Ed.), ISBN: 978-953-51-0326-4. <http://www.intechopen.com/books/malaria-parasites/immunity-tomalaria>.
- Koram**, K. A., Owusu-agyei, S., Fryauff, D. J., Anto, F., Atuguba, F., Hodgson, A., ... Nkrumah, F. K. (2003). Seasonal profiles of malaria infection , anaemia , and bednet use among age groups and communities in northern Ghana. *Tropical Medicine and International Health*, 8(9), 793–802.
- Kusi**, K. A., Faber, B. W., Thomas, A. W., and Remarque, E. J. (2009). Humoral Immune Response to Mixed PfAMA1 Alleles ; Multivalent PfAMA1 Vaccines Induce Broad Specificity. *PLoS ONE*, 4(12), 19–21. <https://doi.org/10.1371/journal.pone.0008110>
- Kyes**, S., P. Horrocks, and C. Newbold. (2001). Antigenic variation at the infected red cell surface in malaria. *Annu. Rev. Microbiol.* 55:673–707.

- Langhorne, J., Ndungu, F. M., Sponaas, A., and Marsh, K. (2008).** Immunity to malaria : more questions than answers. *Nature Immunology*, 9(7), 725–732. <https://doi.org/10.1038/ni.f.205>
- Lindblade, K. A., Steinhardt, L., Samuels, A., Kachur, S. P., Slutsker, L. (2013).** The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Rev Anti Infect Ther* 11, 623-639.
- Makler, M. T., Hinrichs, D. J. (1993).** Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *Am J Trop Med Hyg*, 48(2), 205-210.
- Malaguarnera, L., and Musumeci, S. (2002).** The immune response to *Plasmodium falciparum* malaria. *The Lancet Infectious Diseases*, 2(8), 472–478. [https://doi.org/10.1016/S1473-3099\(02\)00344-4](https://doi.org/10.1016/S1473-3099(02)00344-4)
- Malaria Atlas Project. (2018).** University of Oxford. <https://map.ox.ac.uk/mosquito-malaria-vectors/> [Accessed 20/03/2018]
- Marsh, K. and Kinyanjui, S. (2006)** Immune effector mechanisms in malaria. *Parasite Immunol*, Jan, 28 (1-2):51-60.
- Marsh, K., Otoo, L., Hayes, R.J., Carson, D.C. and Greenwood, B.M. (1989).** Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. *Trans. R. Soc. Trop. Med. Hyg.* 83, 293-303.

- Mata-cantero**, L., Lafuente, M. J., Sanz, L., & Rodriguez, M. S. (2014). Magnetic isolation of *Plasmodium falciparum* schizonts iRBCs to generate a high parasitaemia and synchronized in vitro culture. *Malaria Journal*, 13 (112),1–9.
- Medzhitov**, R., Schneider, D. S., Soares, M. P. (2012). Disease tolerance as a defense strategy. *Science* 335, 936-941.
- Mendonça**, V. R. de, and Barral-Netto, M. (2015). Immunoregulation in human malaria: the challenge of understanding asymptomatic infection. *Memórias Do Instituto Oswaldo Cruz*, 110(8), 945–955. <https://doi.org/10.1590/0074-02760150241>
- Najem**, G. R., Sulzer, A. J. (2003). Transfusion-induced malaria from an asymptomatic carrier. *Transfusion* 16: 473-476
- Nebie**, I., Diarra, A., Ouedraogo, A., Soulama, I., Bougouma, E.C., Tiono, A.B., Konate, A.T., Chilengi, R., Theisen, M., Dodoo, D., Remarque, E., Bosomprah, S., Milligan, P. and Sirima, S.B. (2008). Humoral responses to *Plasmodium falciparum* blood-stage antigens and association with incidence of clinical malaria in children living in an area of seasonal malaria transmission in Burkina Faso. *West Africa. Infect. Immun.* 76, 759-766.
- Osier**, F. H. A., Feng, G., Boyle, M. J., Langer, C., Zhou, J., Richards, J. S., ... Beeson, J. G. (2014). Opsonic phagocytosis of *Plasmodium falciparum* merozoites: mechanism in human immunity and a correlate of protection against malaria. *BMC medicine*, 12(1), 1–15. <https://doi.org/10.1186/1741-7015-12-108>.
- Owusu-Agyei**, S., Asante, K. P., Adjuik, M., Adjei, G., Awini, E., Adams, M., Newton, S., Dosoo, D., Dery, D., Agyeman-Budu, A., Gyapong, J., Greenwood, B., & Chandramohan, D.

- (2009). Epidemiology of malaria in the forest-savanna transitional zone of Ghana. *Malaria Journal*, 8, 220. doi:10.1186/1475-2875-8-220.
- Pain, A.**, Bohme, U., Berry, A. E., Mungall, K., Finn, R. D., Jackson, A. P., Mourier, T., Mistry, J., Pasini, E. M., Aslett, M. A., and Berriman, M. (2008). The genome of the simian and human malaria parasite *Plasmodium knowlesi*. *Nature*, 455, 799-803.
- Pawar, A.** (2014). Immune mechanisms involved in malaria : A review. *Int J Cur Res Rev*6(15), 10–14.
- Persson, K. E. M.**, Fowkes, F. J. I., Mccallum, F. J., & Gicheru, N. (2014). Europe PMC Funders Group Erythrocyte-binding antigens of *Plasmodium falciparum* are targets of human inhibitory antibodies and function to evade naturally acquired immunity. *J Immunol*, 191(2), 785–794. <https://doi.org/10.4049/jimmunol.1300444>.Erythrocyte-binding.
- Price, R. N.**, Tjitra, E., Guerra, C. A., Yeung, S., White, N. J., and Anstey, N. M. (2009). Europe PMC Funders Group Vivax malaria : neglected and not benign. *PMC*, 77(61), 79–87.
- Public Health Agency of Canada.** (2014). Canadian Recommendations for the Prevention and Treatment of Malaria: An Advisory Committee Statement (ACS) Committee to Advise on Tropical Medicine and Travel (CATMAT). http://publications.gc.ca/collections/collection_2014/aspc-rctp/HP40-102-2014-eng.pdf. [Accessed: 20/03/2014].
- Public Health Agency of Canada.** (2018). Medical Access to Artesunate or Quinine for Malaria Treatment Streamlined in Canada through the Canadian Malaria Network (CMN). <http://www.phac-aspc.gc.ca/tmp-pmv/quinine/index-eng.php>. [Accessed 20/03/2018]

- Ramasamy, R., Ramasamy, M., & Yasawardena, S. (2001).** Antibodies and *Plasmodium falciparum* merozoites. *Trends in Parasitology*, 17(4), 194–197. [http://doi.org/10.1016/S1471-4922\(00\)01946-2](http://doi.org/10.1016/S1471-4922(00)01946-2).
- Richards, J. S., Arumugam, T. U., Reiling, L., Healer, J., Hodder, A. N., Fowkes, F. J. I., ... Beeson, J. G. (2013).** Identification and prioritization of merozoite antigens as targets of protective human immunity to *Plasmodium falciparum* malaria for vaccine and biomarker development. *Journal of Immunology (Baltimore, Md. : 1950)*, 191(2), 795–809. <https://doi.org/10.4049/jimmunol.1300778>
- Richards, J.S., Beeson, J.G. (2009).** The future for blood-stage vaccines against malaria. *Immunol. Cell Biol.* 87, 377–390
- Schneider, P., Bousema, J. T., Gouagna, L. C., Otieno, S., Vegte-Bolmer, M van de., Omar, S. A., Sauerwein, R. W. (2007).** Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am J Trop Med Hyg*, 76, 470-474.
- Senczuk, a M., Reeder, J. C., Kosmala, M. M., & Ho, M. (2001).** *Plasmodium falciparum* erythrocyte membrane protein 1 functions as a ligand for P-selectin. *Blood*, 98(10), 3132–5. <http://doi.org/10.1182/blood.V98.10.3132>
- Sennang, N., Rogerson, S., Wahyuni, S., Yusuf, I., and Syafruddin, D. (2014).** Antibody response against three *Plasmodium falciparum* merozoite antigens in Mamuju District , West Sulawesi Province , Indonesia. *Malaria Journal*, 13(381), 1–7.
- Sherman, I. W., Eda S., and Winograd, E. (2003).** Cytoadherence and sequestration in *Plasmodium falciparum* : defining the ties that bind. *Microbes Infect.* 5:897–909.

- Silva, H. D. De, Saleh, S., Kovacevic, S., Wang, L., Black, C. G., Plebanski, M., & Coppel, R. L.** (2011). The antibody response to *Plasmodium falciparum* Merozoite Surface Protein 4 : comparative assessment of specificity and growth inhibitory antibody activity to infection-acquired and immunization-induced epitopes. *Malaria Journal*, *10*(1), 266. <https://doi.org/10.1186/1475-2875-10-266>
- Singh, B. and Daneshvar, C.** (2013). Human infections and detection of *Plasmodium knowlesi*. *Clinical Microbiology Reviews*, *26*, 165–184.
- Stanisic, D. I., Fowkes, F. J. I., Koinari, M., Javati, S., Lin, E., Kiniboro, B., & Richards, J. S.** (2015). Acquisition of Antibodies against *Plasmodium falciparum* Merozoites and Malaria Immunity in Young Children and the Influence of Age , Force of Infection , and Magnitude of Response, *83*(2), 646–660. <http://doi.org/10.1128/IAI.02398-14>.
- Sutherland, C. J., Tanomsing, N., Nolder, D., Oguike, M., Jennison, C., Pukrittayakamee, S., ... Polley, S. D.** (2010). Two Nonrecombining Sympatric Forms of the Human Malaria Parasite *Plasmodium ovale* Occur Globally. *The Journal of Infectious Diseases*, *201*(10), 1544–1550. <https://doi.org/10.1086/652240>.
- The RTS,S Clinical Trials Partnership.** (2011). First Results of Phase 3 Trial of RTS,S/AS01 Malaria Vaccine in African Children. *New Eng J Med*. doi: 10.1056/NEJMoa11002287.
- Thera, M. A., Doumbo, O. K., Coulibaly, D., Laurens, M. B., Ouattara, A., Kone, A. K., et al.** (2011). A field trial to assess a blood-stage malaria vaccine. *N Engl J Med.*, *365*:1004–13. doi: 10.1056/NEJMoa1008115.
- Trager, W, and Jensen, J.** (1997). Continuous culture of *Plasmodium falciparum* : its impact on

malaria research. *Int J Parasitol*, 2, 989–1006.

Travassos, M. a., Niangaly, A., Bailey, J. a., Ouattara, A., Coulibaly, D., Laurens, M. B., ... Plowe, C. V. (2013). Seroreactivity to *Plasmodium falciparum* erythrocyte membrane protein 1 intracellular domain in malaria-exposed children and adults. *Journal of Infectious Diseases*, 208(9), 1514–1519. <http://doi.org/10.1093/infdis/jit339>.

Vaughan, A. M. and Kappe, S. H. (2017). Malaria Parasite Liver Infection and Exoerythrocytic Biology. *Cold Spring Harb Perspect Med.* pii: a025486. doi: 10.1101/cshperspect.a025486.

Vestergaard, L. S., Lusingu, J. P., Nielsen, M. A., Mmbando, B. P., Dodoo, D., Akanmori, B. D., ... Theander, T. G. (2008). Differences in human antibody reactivity to *Plasmodium falciparum* variant surface antigens are dependent on age and malaria transmission intensity in Northeastern Tanzania. *Infection and Immunity*, 76(6), 2706–2714. <https://doi.org/10.1128/IAI.01401-06>.

Waters, A. (2006). Malaria: New vaccines for old? *Cell*; 124:689-693. Doi: 10.1016/j.cell.2006.02.011.

White, N. J. (2008). The role of anti-malarial drugs in eliminating malaria. *Malar J* 7 (Suppl.1):S8.

World Health Organization. (2015). Guidelines for the Treatment of Malaria Third Edition 2015. http://apps.who.int/iris/bitstream/10665/162441/1/9789241549127_eng.pdf?ua=1andua=1. [Accessed 21/03/2018].

World Health Organization. (2016). <http://www.who.int/mediacentre/factsheets/fs094/en/> [Accessed 16/03/17].

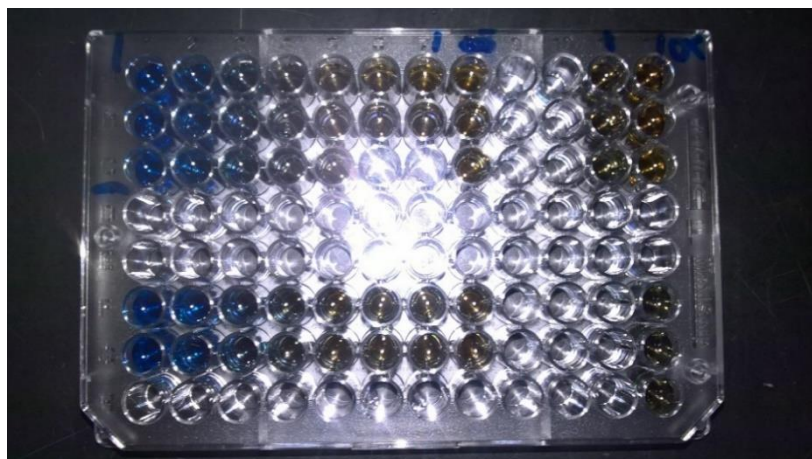
World Health Organization. 2018. <http://www.who.int/ith/diseases/malaria/en/> [Accessed 07/05/18].

Wright, G. J., & Rayner, J. C. (2014). *Plasmodium falciparum* Erythrocyte Invasion : Combining Function with Immune Evasion, *10*(3), 1–7. <http://doi.org/10.1371/journal.ppat.1003943>.

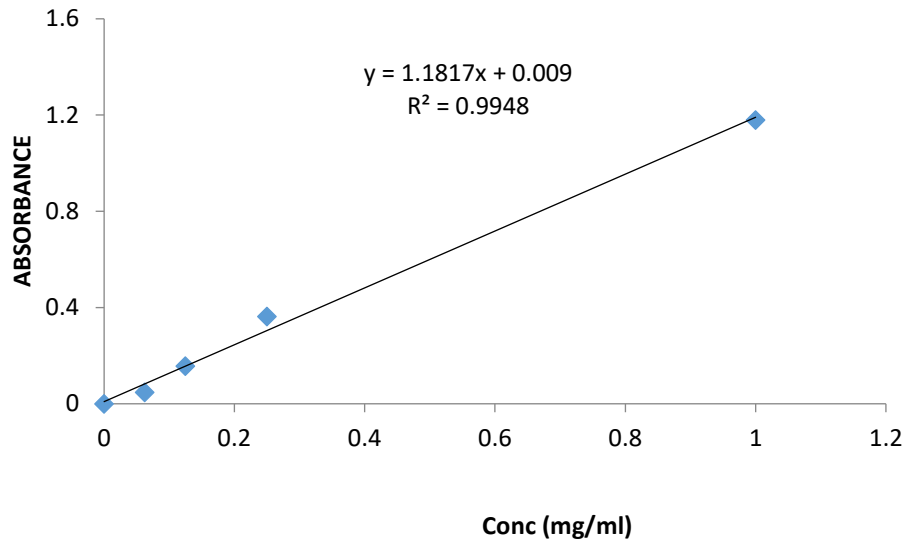
APPENDICES

APPENDIX I

Bradford assay OD and sample concentration determination



	Conc (mg/ml)	A	B	MEAN ABS	MEAN-CTRL
STANDARD	0	0.3745	0.3687	0.3716	0
	0.0625	0.4285	0.4088	0.41865	0.04705
	0.125	0.5334	0.5216	0.5275	0.1559
	0.25	0.7481	0.7194	0.73374999	0.36215
	1	1.532	1.5685	1.55024999	1.17865
SAMPLE	0.4868	0.4763	0.4763	0.4798	0.1082
					0.083947 mg/ml
SAMPLE CONC (mg/ml)		0.0839			
		83.9ug/ml			



APPENDIX II

Antibody (IgG) Purification and Concentration

Sample	Volume Of Plasma (ul)	Purified Antibody Pool Eluted (mg/ml)	Total Volume Of Pool (ml)	Concentrated Antibody (mg/ml)	Volume Of Antibody Concentrated (ul)
EIM-N001	300	0.030	5.00	19.02	171.5
EIM-N013	300	0.212	5.00	20.24	168.6
EIM-N017	300	0.039	5.00	4.123	20
EIM-N018	300	0.188	5.00	9.017	40
EIM-N032	300	0.426	5.00	21.38	100
EIM-N042	300	0.825	5.00	23.92	120
EIM-N054	300	0.880	5.00	21.83	150
EIM-N084	300	0.778	5.00	20.17	120
EIM-N093	300	0.588	5.00	22.73	60
EIM-N143	300	0.946	5.00	23.91	110
EIM-N145	300	0.546	5.00	21.94	200
EIM-N166	500	0.66	5.00	23.66	200
EIM-N167	300	0.507	5.00	23.91	190
EIM-N176	400	0.326	5.00	20.20	100
EIM-N231	400	0.631	5.00	23.86	160
EIM-N232	400	0.518	5.00	19.86	115
EIM-N156	500	1.434	5.00	23.24	250
EIM-N235	250	0.365	5.00	22.06	100
EIM-N243	450	1.445	5.00	22.65	260
EIM-N259	350	0.989	5.00	23.52	170
EIM-N279	350	0.646	5.00	21.01	75
EIM-N245	400	0.171	5.00	16.17	40
EIM-N247	350	0.273	5.00	20.53	55
EIM-N253	350	0.296	5.00	16.32	55

APPENDIX III

Ethical Clearance



UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

My Ref. No.....

28th April, 2016.

Mr. John Yaw Nusedonu
Department of Medical Microbiology
School of Biomedical and Allied Health Sciences
University of Ghana
Korle-Bu

Dear Mr. Nusedonu,

ETHICAL CLEARANCE

Protocol Identification Number: CHS-Et/M.7 – P 3.6/2015-2016

The Ethical and Protocol Review Committee of the College of Health Sciences on the 28th of April, 2016 unanimously approved your research proposal.

TITLE OF PROTOCOL: “Role of Antibodies against antigens of Plasmodium merozoite and infected RBC surface in malaria immunity”

PRINCIPAL INVESTIGATORS: Mr. John Yaw Nusedonu

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

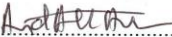
Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till 30th September, 2016.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: 

PROFESSOR ANDREW A. ADJEI
CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE

cc: Provost, CHS
Dean, SBAHS
Head of Department

• P. O. Box 52, Korle-bu, Accra, Ghana • Tel: +233 (0) 302665103/244061270 • Fax: +233 (0) 302660762
• Email: eprc@chs.edu.gh / provost@chs.edu.gh • Website: www.chs.ug.edu.gh