GENETIC DIVERSITY OF PLASMODIUM FALCIPARUM AND ANTIBODY RESPONSES TO ERYTHROCYTE SURFACE ANTIGENS AMONG CHILDREN IN MINNA, NIGERIA

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PhD/SLS/2016/905

DEPARTMENT OF ANIMAL BIOLOGY

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THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL, FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGERIA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD) IN ZOOLOGY (APPLIED ENTOMOLOGY AND PARASITOLOGY) IN THE DEPARTMENT OF ANIMAL BIOLOGY

ABSTRACT

Malaria is a major global public health concern especially in countries where transmission occur regularly. A larger proportion of the malaria cases that resulted in mortality occurred among children less than five years old. This cross sectional study was aimed at determining the genetic diversity of *Plasmodium falciparum* and antibody responses to erythrocyte surface antigens among children in Minna, Nigeria. Blood samples were collected from children with no signs of fever (asymptomatic cases) within the residential communities in Minna, and children with symptoms of fever (symptomatic cases) who attended selected healthcare facilities within the period of the study. Thick and thin films of blood samples were prepared using Giemsa staining technique to determine the parasite density and the infective stage. Subsequently, a complete blood count to determine the haematological indices of the blood samples was carried out. Blood samples were exposed to enzyme linked immunosorbent assay (ELISA) for the measurement of IgG antibody levels against *P. falciparum.* Samples were further analyzed for the molecular characterization and gene type using polymerase chain reaction (PCR). A total population of 316 children both symptomatic and asymptomatic were sampled for this study from children between 6 months and 17 years between the months of February to July, 2018. The sampled population comprises 181 (57 %) males and 135 (43 %) females. Of the 316 blood samples screened for *P. falciparum* infection, 238 (75.37 %) were infected with *P. falciparum* which comprises of 39 (16.39 %) from asymptomatic children from the communities and 199 (83.61 %) symptomatic children attending the six healthcare facilities. The male and female categories recorded a *P. falciparum* prevalence of 126 (39.61 %) and 112 (35.44 %), respectively, and a non-significant difference (P>0.05) was observed between sex and *P. falciparum* infection. *P. falciparum* infected cases with anaemia recorded a high prevalence rates as compared to the non-infected cases. However, the multinomial logistic regression model demonstrated that *P. falciparum* was not a significant predictor to anaemia. In addition, the results of ELISA showed *P. falciparum* infected cases had higher specific antibody IgG responses of 64 (68.81 %) as compared to the negative *P. falciparum* samples, though a non-significant difference (P>0.05) between the negative and positive *P. falciparum* infected cases and the production of IgG antibody was observed (χ^2=0.896, *P* =0.979). Determination of gene diversity revealed MAD20 and FC27 as the predominant allele for MSP1 and MSP2, respectively. However, K1 and FC27 were the predominant allele from asymptomatic children and on the other hand, FC27 and MAD20 were predominant for symptomatic cases. A high malaria transmission intensity as a result of mixed infection was observed in Minna with an overall multiplicity of infection (MOI) of 2.22. The MOI for symptomatic cases was lower 2.21 than what was observed from asymptomatic cases with 2.41. Finally, findings from this study revealed a high level of gene diversity within the antigenic markers of MSP1 and MSP2 (0.714 and 0.830, respectively). This study has demonstrated the potential of malarial antibody and gene diversity, as important markers for assessing differences in malaria parasite transmission intensity. Continuous genetic surveillance in *P. falciparum* will therefore serve as an important tool in monitoring changes in gene types and for intervention programmes effectiveness.

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List of Abbreviations

AMA1	Apical Membrane Antigen 1
AR	Attributable Risk
BM	Bone Marrow
bp	Base Pair
CDC	Centre for Disease Control

CSA	chondroitin sulfate
CSP	Circumsporozoite Protein
DNA	Deoxyribonucleic Acid
EBA	Erythrocyte-Binding Antigen
EDTA	Ethylene Diamine Tetra Acetate
ELISA	Enzyme Linked Immuno sorbent Assay
FSP	Family Support Program
Hb	Haemoglobin Concentration
Hc	Haematocrit
He	Heterozygosity index
HPM	Human Population Movements
IgG	Immunoglobulin G
Igs	Immunoglobulin
LSA	Liver Stage Antigen
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MOI	Multiplicity of infection
MSP	Merozoite Surface Protein
Na	Number of Alleles
PCR	Polymerase Chain Reaction
PD	Parasite Density
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1

PHC	Primary Health Care
RBC	Red Blood Cell
RDT	Rapid Diagnostic Test
STEVOR	subtelomeric variant open reading frame
SU	Standard Unit
TAE	Tris Acetate
TMB	Tetramethyl benzidine
VSA	Variant Surface Antigen
WBC	White Blood Cell
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Malaria is a mosquito-borne infectious disease that affects humans which is caused by parasitic protozoans that belong to the genus *Plasmodium* (WHO, 2014). It causes symptoms that normally include fever, fatigue, vomiting, and headaches. In severe cases, it can cause seizures, yellow skin, coma, or death (Caraballo, 2014). There are however, five species of *Plasmodium* that can infect humans and spread by mosquitoes (Caraballo, 2014). These species are *Plasmodium falciparum*, which causes most deaths, while *P. vivax*, *P. ovale*, and *P. malariae* cause a milder form of malaria (Caraballo, 2014; WHO, 2014). Meanwhile, the species *P. knowlesi* rarely causes disease in humans (WHO, 2014). *Plasmodium falciparum* is the most prevalent malaria parasite in sub-Saharan Africa and it accounts for 99 % of the estimated malaria cases in 2016. Outside of Africa, *P. vivax* is the predominant parasite in America, which represent 64 % of malaria cases, and is above 30 % in South- East Asia and 40 % in the Eastern Mediterranean regions (WHO, 2017).

Malaria is a major global public health concern especially in countries where transmission occur regularly. In 2016, an estimated 216 million cases of malaria occurred worldwide, compared with 237 million cases in 2010 and 211 million cases in 2015 (WHO, 2017). About 90 % of most malaria cases in 2016 were found in African, followed by South-East Asia region with 7 % and the Eastern Mediterranean region with 2 %. Out of the 91 countries that reported malaria cases in 2016, 15 countries – all in sub-Saharan Africa – carried 80 % of the global

malaria burden (WHO, 2017). In sub-Saharan Africa, malaria is one of the leading causes of morbidity and mortality, with 191 million cases and over 390 thousand deaths reported in 2016. Nevertheless, with the scaling up of malaria prevention, diagnosis and treatment, the prevalence of *Plasmodium falciparum* infection in many parts of sub-Saharan Africa declined by half, and the frequency of clinical disease fell by 40 % between the year 2000 and 2015 (Bhatt *et al*., 2016). Malaria incidence among populations at risk dropped globally by 21 % between 2010 and 2015. In that same period, malaria mortality rates among populations at risk globally dropped by 29 % among all age groups, and by 35 % among children under the age of five (WHO, 2016). In addition, Sub-Saharan Africa carries an excessively high proportion of the global malaria burden. In 2015, the region recorded 90 % of malaria cases and 92 % of malaria deaths (WHO, 2016). There were an estimated

33,000 malaria cases per 100,000 people in Nigeria, with 110,000 deaths (WHO, 2015a). Malaria prevalence among children in Nigeria has decreased steadily over the years (from 42 % in 2010 to 27 % in 2015), but malaria remains a leading cause of death among them (WHO, 2015a).

A larger proportion of the malaria cases that resulted in mortality probably occurred among children less than 5 years of age. Due to the endemic nature of malaria in Nigeria, partial immunity to malaria is acquired among older children. However, severe forms of anaemia can be seen in children less than 5 years of age who have not yet acquired immunity. According to WHO, in 2015, 69 % of the malaria deaths that occurred worldwide were among children below 5 years (WHO, 2015b).

In 2016, there were an estimated 445,000 deaths from malaria globally, compared to 446,000 estimated deaths in 2015 with a decline of 1000. Meanwhile, the African region accounted for 91 % of all malaria deaths in 2016, followed by South-East

Asia region with 6 %. Fifteen countries from the sub-Saharan Africa recorded 80 % of global malaria deaths in 2016. In 2016, all regions recorded mortality reductions compared to 2010, with the exception of the Eastern Mediterranean region, where mortality rates remained virtually unchanged over the period. The largest decline occurred in the regions of South-East Asia (44 %), Africa (37 %) and the America (27 %) (WHO, 2017).

In Africa, malaria was estimated to result in economic loss of US$12 billion a year due to increased healthcare costs, lost ability to work, and negative effects on tourism (Greenwood *et al*., 2005), and by 2016, an estimated US$ 2.7 billion was invested in malaria control and elimination efforts globally by governments of malaria endemic countries and international partners.

In areas of stable malaria transmission, children and pregnant women are the population groups of highest risk for malaria morbidity and mortality (Bourdy *et al.,* 2008) and most children experience their first malaria infections during the first year or two of their life, when they have not yet acquired adequate clinical immunity – which makes these early years particularly dangerous. This rises the burden of deaths due to malaria in young children to about Ninety percent in Africa. Adult women in areas of stable transmission have a high level of immunity, but this is impaired especially in the first pregnancy, with the result that risk of infection increases (WHO, 2003).

During the blood stage of *Plasmodium falciparum* infection, anaemia may be induced as a result of destruction of red blood cells by the parasite and soluble derivatives released by the parasite induce bone marrow dysfunction at the period

of malaria infection. As such, populations most affected by malaria often are at high risk for anaemia (McCuskee *et al*., 2014). Severe malarial anaemia caused by *P. falciparum* is responsible for approximately a third of the deaths associated with the disease (Haldar and Mohandas, 2009). Anaemia is an important contributor to malaria attributable deaths in hospitals, with severe anaemia accounting for between 17 % and 54 % of malaria attributed deaths in children under 5 years of age (Biemba *et al*., 2000).

Plasmodium parasites, expresses many variant antigens on cell surfaces. Variant surface antigens (VSAs) are typically organized into large subtelomeric gene families that play critical roles in virulence and immune evasion (Frech and Chen, 2013). Antigenic variation at the *Plasmodium*-infected erythrocyte surface plays a critical role in malaria disease severity and host immune evasion (Noelle *et al.,* 2010). Severe cases of malaria can occur when parasite invades and then proliferates within red blood cell erythrocytes. The parasite produces many variant antigenic proteins, encoded by multigene families, which are present on the surface of the infected erythrocyte and play important roles in virulence (Chen *et al.,* 2000). The major surface proteins are the Merozoite Surface Protein 1 and 2 (MSP 1 and

2).

Merozoite Surface Protein 1 and 2 of *P. falciparum* are major targets for bloodstage malaria vaccine (Chaitarra *et al.,* 1999) and are also appropriate markers for the identification of genetically distinct *P. falciparum* parasite sub-populations. MSP1 is a major surface protein of approximately 190-kDa size. It plays a major role in erythrocyte invasion (Holder *et al.,* 1992) and is a major

target of immune responses (Apio *et al.,* 2000). MSP1 contains 17 blocks of sequence flanked by conserved regions (Takala *et al.,* 2002) Block 2, which is the most polymorphic part of MSP1, is grouped into three allelic families namely K1, MAD20, and RO33 type (Takala *et al.,* 2006). MSP2 is glycoprotein consisting five blocks where the central block is the most polymorphic (Ferreira and Hart, 2007), and is grouped into two allelic families, FC27 and 3D7/IC1.

Furthermore, individuals living in malaria endemic areas develop naturally acquired immunity to *Plasmodium* spp. infections as they grow, but only slowly and only after repeated exposure (Marsh and Kinyanjui, 2006; Doolan *et al*., 2009). Anti-disease immunity production mechanisms and factors controlling effective protection are still largely unknown (Reddy *et al.,* 2012). However, it was well established that antibodies contribute to protection against clinical malaria due to *P. falciparum*. Antibodies directed against cell surface proteins of either the merozoite form of the parasite or of infected red blood cells have been shown to be important components of acquired protective immunity against malaria (Richards and Beeson, 2009).

Naturally acquired antibodies to sporozoites can protect against liver-stage infection and antibodies targeting blood-stage antigens can repress high parasite densities and progression to symptomatic disease (Doolan *et al*., 2009). The magnitude of antibody responses are important, with responses to a repertoire of antigens associated with increased disease protection (Richards *et al.,* 2013; Rono *et al.,* 2013), while also acting as biomarkers of past exposure (Elliott *et al.,* 2014).

1.2 Statements of the Research Problem

In malaria-endemic regions, the most vulnerable populations that suffered severe and complicated malaria, including malarial anaemia, are children less than five years of age and pregnant women (Quintero *et al.,* 2011). The vast majority of deaths due to malaria parasite is caused by *Plasmodium falciparum* which is the most virulent species of the malaria parasites (Abdel Hamid *et al*., 2013).The special pathology of *P. falciparum* as compared to other species of *Plasmodium* that infect humans has been attributed to the ability of *P. falciparum*-infected erythrocytes to cytoadhere to receptors expressed on the surface of capillary endothelial cells (Pongponratn *et al.*, 1991; Berendt *et al*., 1994; Turner *et al*., 1994; MacPherson *et al*., 1995), and the parasite mediates adherence of infected erythrocytes to uninfected erythrocytes, which is responsible for the most severe clinical complications of *P. falciparum* malaria. These parasites also have the ability to undergo antigenic switching to evade a developing antibody response (Bull *et al*., 2005). Many important aspects of these variant surface antigen (VSA) function and evolution remain obscure, impeding the understanding of virulence mechanisms and vaccine development (Frech and Chen, 2013).

Severe malaria is one of the most feared complications that is associated with an increased mortality, especially in children and is one of the factors that contributes to the anaemia burden in children. Hence, immunological factors and mechanisms appear to have great relevance in the anaemia pathogenesis during a malaria infection (Quintero *et al.,* 2011). At the period of malaria infection soluble derivatives released by the parasite induce bone marrow dysfunction. These derivatives are therefore implicated in the pathogenesis of malarial anaemia (Silverman *et al*., 1987; Miller *et al.*, 1989; Jootar *et al.,* 1993).

In addition, *Plasmodium* is known to induce a strong inflammatory response with a robust production of immune effectors, including cytokines and antibodies. Therefore, it is possible that the extent of the immune response not only may facilitate the parasite killing but also may provoke severe illness, including anaemia (Castro-Gomes *et al.*, 2014). However, despite enormous health implication of this parasite, the immunological mechanisms involved in malaria-induced anaemia remain incompletely or poorly understood (Castro-Gomes *et al.*, 2014.)

Genetic diversity of malaria parasite creates a great hindrance to the development of vaccine and enhances antimalarial drug resistance (Duah *et al.*, 2016). On the other hand, vaccine designed for *Plasmodium falciparum* was hindered by polymorphisms in certain vaccine candidate loci (Hughes and Verra, 2002; Tongren, 2004). Highly polymorphic regions have been observed in *P. falciparum* antigenic surface proteins, such as the merozoite surface protein 1 (MSP1), circumsporozoite protein (CSP), the apical membrane antigen 1 (AMA-1), the liver stage antigen (LSA-1) and the thrombospondin-related anonymous protein (TRAP) (Escalante *et al.*, 1998).

Therefore, knowledge of the distribution of polymorphic sites on malaria antigens is necessary to obtain a detailed understanding of their significance for vaccine development. Moreover, this study also includes infections occurring in both symptomatic and asymptomatic parasitemia, its relation with anaemia and immune response to the antigen.

1.3 Justification for the Study

Nigeria is an endemic country for malaria, its large population, various weather conditions and beliefs make it a bit difficult implementing the same malaria control measures throughout the country. Genotyping malaria parasites to assess their diversity in different geographic settings have become necessary for the selection of antigenic epitopes for vaccine development and for further implementation of regional intervention programs.

Furthermore, the level of antigenic diversity varies from one malaria endemic region to another and even between countries, in such a way that the variant forms of the parasite exist at different frequencies in different geographic areas presenting different complexities of infection (Raj *et al.,* 2004). Merozoite Surface Protein 1 and 2 of *P. falciparum* are one of the variant forms of the parasite which are the most abundant and a major blood-stage malaria vaccine targets (Chaitarra *et al.*, 1999) and are also suitable markers for the identification of genetically distinct *P. falciparum* parasite sub-populations. Antibodies against several of these merozoite antigens have been found to be associated with protective immunity (Fowkes *et al.,* 2010) and Immunoglobulin IgG which is a type of antibody found in all body fluids was used. The lack of *in vitro* functional assays that correlate with protective immunity *in vivo* has hampered the development of effective blood stage vaccines (Crompton *et al.,* 2010). There have been inconsistencies in the correlations of antibody responses to recombinant antigens and protection from malaria using enzyme-linked immunosorbent assays (ELISAs) (McCallum *et al.*, 2008). *P. falciparum* Parasite genetic diversities has been implicated in evolutionary fitness and consequently populations with high diversity have the

ability to survive against most intervention efforts in the control of malaria thereby frustrating control efforts (Barry *et al.,* 2013).

Therefore, to control and eventually eradicate malaria, an effective vaccine is considered to be needed in addition to the existing strategies such as insecticidetreated bed nets (ITNs) and chemotherapy (Kilama and Ntoumi, 2009). However, the development of an effective vaccine is being hampered by genetic diversity (Healer *et al.,* 2004) even though extensive research on the diversity in the parasite field isolates have been conducted in several studies (Babiker *et al.,* 2000 and Zakeri *et al.,* 2005). This diversity has the potential to alter the conformation of antimalarial drug targets and render the parasites drug resistant (Blasco *et al.,* 2017) which will hinder malaria treatment outcome (Felger *et al.,* 1999 and Raj *et al.,* 2004). Genetic diversity and multiplicity of *P. falciparum* infections are therefore, essential parasite indices that could determine the impact of malaria intervention programs as well as the endemicity of parasite infections in varying transmission settings (Barry *et al.,* 2013, Nabet *et al.,* 2016 and Razak *et al.,* 2016).

Malarial anaemia is acknowledged as an important cause of morbidity and mortality in endemic regions (Quintero *et al.,* 2011). Its importance as a cause of death may well be underestimated because of difficulty in diagnosis, especially where parasitemia may be low and the clinical picture may be confused with other causes of anaemia (Phillips and Pasvol, 1992). Complications of severe anaemia and cerebral malaria were assumed to be the major cause of morbidity and mortality, however, recent evidence indicates that the immunological response of the host could also contribute to the pathophysiology of the disease in human

beings (Malaguarnera and Musumeci, 2002). However, giving that specific haematological changes associated with malaria infection may vary with the level of malaria endemicity (Idro *et al*., 2006), nutritional status (Friedman *et al.*, 2005), demographic factors (Barcus *et al.*, 2007), malaria immunity (Langhorne *et al.*, 2008) and the parasite species. It is therefore, essential to study and characterize the indices involved in malarial anaemia in endemic regions like Minna metropolis.

Furthermore, to elucidate the molecular mechanisms involved in the pathogenesis of malaria-induced anaemia, this study addresses the malarial anaemia immune pathogenesis process.

In view of this, haematological, immunological and molecular studies are essential for better understanding of the parasite diversity, and to generate information on the immunological mechanisms involved in malaria induced anaemia as well as the nature and extent of antibody response to infected *P. falciparum* erythrocyte which is essential in understanding the mechanism underlying the pathology of malaria, the acquisition of immunity, and also the development of vaccine candidate against the parasite. This research, therefore, focuses on hematological indices, genetic diversity of parasite antigens and immune responses to the parasite antigens *in vitro*.

1.4 Aim and Objectives

1.4.1 Aim of the study

This study evaluated the genetic diversity of *P. falciparum* and antibody responses to erythrocyte surface antigens among children in Minna, Nigeria.

1.4.2 Objectives of the study

The objectives of this study are to determine the:

i. prevalence of *P. falciparum* among children in Minna ii. haematological indices for malaria induced anaemia iii. specific immunoglobulin IgG levels among *P. falciparum* infected children in Minna iv. immunological indices involved in malaria-induced anaemia

v. molecular characterization of *P. falciparum* field isolates from Minna vi. estimate of multiplicity of infection of the alleles of *P. falciparum*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Plasmodium* Species

Plasmodium falciparum is the major *Plasmodium* species infecting humans and is found worldwide in tropical and subtropical areas especially in Africa where this species predominates (CDC, 2016). *P. falciparum* can cause severe malaria because it multiplies rapidly in the blood, and can thus cause severe blood loss (anaemia). It is transmitted to humans by the female *Anopheles* species of mosquito. There are about 460 species of *Anopheles* mosquito, but only 68 transmit malaria. In fact, the infected parasites can clog small blood vessels. When this occurs in the brain, it could result to cerebral malaria, which can be lethal (CDC, 2016).

On the other hand, *P. vivax* is found predominantly in Asia, Latin America, and some parts of Africa. It is possibly the most prevalent human malaria parasite due

to population densities, particularly in Asia. As seen in table 2.1, *P. vivax* has a tertian two-day cycle and has a dormant liver stages ("hypnozoites") that can activate and invade the blood ("relapse") several months or years after the infecting mosquito bite (CDC, 2016).

P. ovale is found mostly in Africa, especially West Africa and the islands of the western Pacific. It is biologically and morphologically very similar to *P. vivax* (has a tertian, two-day cycle and also has a dormant liver stages) see table 2.1, but however, differently from *P. vivax*, it can infect individuals who are negative for the Duffy blood group, which is the case for many residents of sub-Saharan Africa.

This explains the greater prevalence of *P. ovale* (rather than *P. vivax*) in most of Africa (CDC, 2016).

P. malariae, found worldwide, is the only human malaria parasite species that has a quartan cycle (three-day cycle) as seen in table 2.1, however, the other three species have a tertian, two-day cycle. If *P. malariae* is left untreated, it triggers a long-lasting, chronic infection that may last a lifetime in some cases. *P. malariae* can cause serious complications in some chronically infected patients, such as the nephrotic syndrome (CDC, 2016).

P. knowlesi is found throughout Southeast Asia as a natural pathogen of long-tailed and pig-tailed macaques. In this region, particularly in Malaysia, it has recently been shown to be a significant cause of zoonotic malaria. *P. knowlesi* has a 24-hour replication cycle and so can rapidly progress from an uncomplicated to a severe form of infection (CDC, 2016).

In addition, malaria is classified as severe when there are any of the following criteria: reduced consciousness, fatigue, two or more seizures, low blood pressure (less than 70 mmHg in adults and 50 mmHg in children), circulatory shock, kidney failure or haemoglobin in the urine, bleeding problems, or hemoglobin less than 50 g/L (5 g/dL), pulmonary oedema, parasite level in the blood greater than 100,000 per microlitre (µL) in low-intensity transmission areas, or 250,000 per µL in highintensity transmission areas (WHO, 2010).

Furthermore, cerebral malaria is characterized as a severe *P. falciparum*-malaria with neurological symptoms, including coma, or with a coma that lasts longer than 30 minutes after a seizure (WHO, 2010). Table 2.1 below showed the various types of malaria, their pathogens and their symptoms.

Table 2.1: Types of malaria caused by Different *Plasmodium* Species

Name	Pathogen	Notes
Algid malaria	*Plasmodium falciparum*	severe malaria affecting the cardiovascular system and causing chills and circulatory shock
Bilious malaria	*Plasmodium falciparum*	severe malaria affecting the liver and causing vomiting and jaundice
Cerebral malaria	*Plasmodium falciparum*	severe malaria affecting the cerebrum
Congenital malaria	various plasmodia	plasmodium introduced from the mother via the fetal circulation
Falciparum malaria, *Plasmodium falciparum* malaria, pernicious malaria	*Plasmodium falciparum*	General symptoms associated with *P. falciparum* malaria
Ovale malaria, *Plasmodium*		

ovale malaria	*Plasmodium ovale*	General symptoms associated with *P. ovale* malaria
Quartan malaria, malariae malaria, *Plasmodium malariae* malaria	*Plasmodium malariae*	paroxysms every fourth day (quartan), counting the day of occurrence as the first day
Quotidian malaria	*Plasmodium falciparum*, *Plasmodium vivax*	paroxysms daily (quotidian)
Tertian malaria	*Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax*	paroxysms every third day (tertian), counting the day of occurrence as the first
Transfusion malaria	various plasmodia	Plasmodium introduced by blood transfusion, needle sharing, or needle stick injury
Vivax malaria, *Plasmodium vivax* malaria	*Plasmodium vivax*	General symptoms associated with *P. vivax* malaria

Source: Dorland's Illustrated Medical Dictionary, *Elsevier* (32nd edition)

2.2 Symptoms Associated with Malaria

Malaria is an acute febrile illness and symptoms appear 7 days or more in a nonimmune individual, after the infective mosquito bite. The first symptoms – fever, headache, chills and vomiting – may be mild and difficult to identify as malaria. *P. falciparum* malaria can progress to severe illness if not treated, which can lead to yellow skin, seizures, coma, or death (Caraballo, 2014; WHO, 2016). Children with severe malaria often experience one or more of the following symptoms: severe anaemia, respiratory distress, or cerebral malaria. In malaria endemic areas, people may develop partial immunity, allowing asymptomatic infections to occur (WHO, 2016). See Figure 2.1 below for the pictorial representation of malaria symptoms.

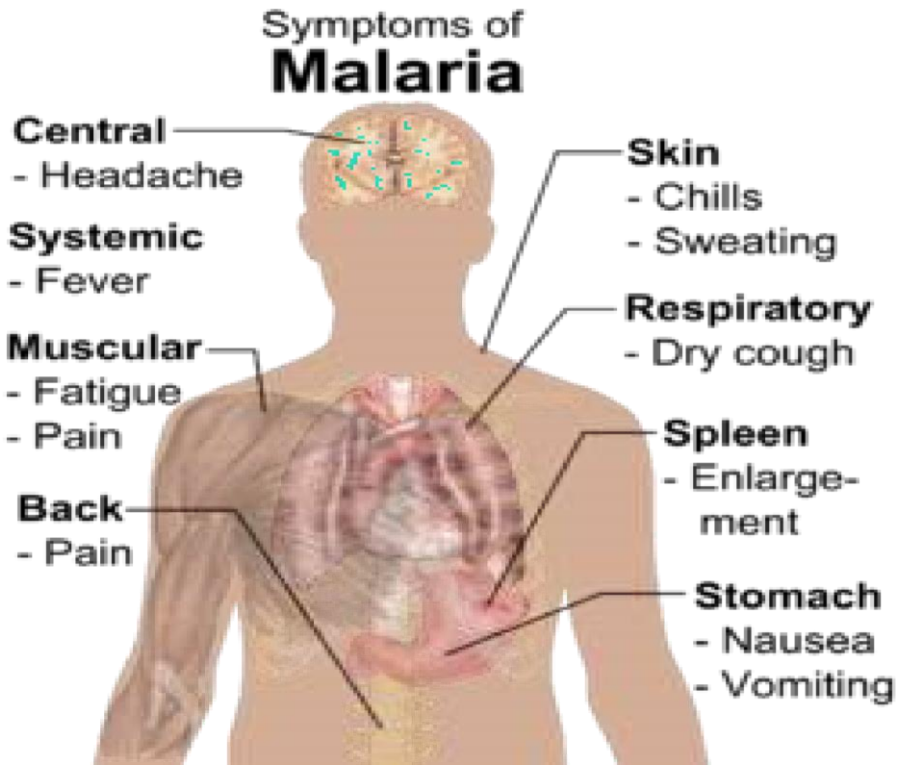


Fig 2.1: Main Symptoms of Malaria (Fairhurst and Wellens, 2010)

2.3 Disease Burden and Population at Risk of *P. falciparum*

Malaria is a significant public health problem, with a high global burden. SubSaharan Africa alone accounted for 90 % of the malaria cases and 92 % of malaria deaths worldwide in 2015 (WHO, 2015). Nigeria and Republic of Congo are two major African countries that contributes to the high malaria burden, with 36 % of the malaria cases worldwide occurring in these two countries (WHO, 2016).

Nigeria is reportedly a malaria endemic country with an entire population (186 million) at risk of contracting malaria, and a staggering 76 % of this population at high risk. In 2015, Nigeria contributed about 29 % of the malaria cases and 26% of malaria deaths worldwide (World Malaria Report, 2016). These large figures

imply that Nigeria's success in tackling malaria control will play a critical role in the actualization of the global goals.

Apart from infants and children under 5 years of age which have considerably higher risk of contracting malaria and developing severe disease, pregnant women and patients with HIV/AIDS, as well as non-immune migrants, mobile populations and travelers are also at higher risk (WHO, 2016).

Meanwhile, according to estimates published by the WHO in December 2016, there were 212 million cases of malaria in 2015 and 429 000 deaths, and between 2010 and 2015, malaria incidence among populations at risk fell by 21 % globally; during the same period, mortality rates among populations at risk fell by 29 %. An estimated 6.8 million malaria deaths have been averted globally since 2001 (WHO,

2016).

Sub-Saharan Africa continues to carry a disproportionately high proportion of the global burden of malaria. In 2015, the region was home to 90 % of malaria cases and 92 % of malaria deaths. About 13 countries, mostly in sub-Saharan Africa, account for 76 % of malaria cases and 75 % deaths globally (WHO, 2016). In this, more than two thirds (70 %) of all malaria deaths occur in children under 5 years of age but between 2010 and 2015, the under-5 malaria death rate fell by 29 % globally. Malaria, however, remains a major killer of children under the age of five, taking the life of a child every two minutes (WHO, 2016).

2.4 Selected Metabolic Pathways by *P. falciparum*

Some metabolic pathways of *Plasmodium falciparum* are explained below, its presence and components can be predicted through genomic analysis (Gardner *et al.,* 2002).

2.4.1 Haemoglobin metabolism

During the erythrocytic stage of the parasite's life cycle, it uses intracellular haemoglobin as a source of food. The protein is broken down into peptides, and the heme group is released and detoxified by biocrystallization in the form of hemozoin. Heme biosynthesis by the parasite has been documented (Bonday, 2002).

2.4.2 Carbohydrate metabolism

The parasite produces its energy during erythrocytic stages mainly through anaerobic glycolysis, with pyruvate being converted into lactate (Gardner *et al.,*

2002). Genes encoding for the TCA cycle enzymes are present in the genome, but it is unclear whether the TCA cycle is used for oxidation of glycolytic products to be used for energy production, or for metabolite intermediate biosynthesis (Gardner *et al.,* 2002). It has been hypothesized that the main function of the TCA cycle in

P. falciparum is for production of succinyl-CoA, to be used in heme biosynthesis (Gardner *et al.,* 2002).

2.4.3 Protein metabolism

It has been hypothesized that the parasite obtains all, or almost all of its amino acids by salvaging from the host or by haemoglobin degradation. This is supported

by the fact that no enzymes required for amino acid biosynthesis are found in genomic analysis except for interconversions between glycine-serine, cysteine-alanine, aspartate-asparagine, proline-ornithine and glutamine-glutamate (Gardner *et al.,* 2002).

2.4.4 Nucleotide metabolism

Biosynthesis of purines is not possible with *P. falciparum*, instead, the parasite is able to transport and interconvert host purines. Conversely, the parasite can produce pyrimidines *de novo* using glutamine, bicarbonate, and aspartate (Gardner *et al.,* 2002).

2.5 Anaemia in *P. falciparum* Infection

Parasite factors such as endemicity, drug resistance, *Plasmodium* species, parasite multiplication rates and antigenic polymorphism are of great relevance for anaemia development. Moreover, social-geographical factors such as access to treatment, cultural and economic factors, health policies and intensity of transmission contribute significantly to increased risk of anaemia (Quintero *et al.,* 2011). Malaria anaemia is usually normocytic and normochromic without spherocytes or

schistocytes (Phillips *et al*., 1986; Bashawri *et al.*, 2002). However, malaria-related anaemia may also be microcytic and hypochromic due to high haemoglobinopathy frequencies and iron deficiency in endemic countries (Bashawri *et al.*, 2002).

Another key feature of malaria anaemia is the presence of inadequate reticulocytosis, despite the degree of anaemia. In acute uncomplicated malaria due

to *P. falciparum,* haematocrit may be normal during the first 24 hours after the onset of fever, but afterwards there may be a steady drop in the haematocrit level (Wickramasinghe and Abdalla, 2000) despite the initiation of anti-malarial treatment (Phillips *et al.*, 1986) and even in the absence of parasites in the blood smear (English *et al.*, 2002) or the administration of blood transfusions.

During malaria infection, the parasite releases soluble derivatives that causes dysfunction of the bone marrow (BM). Therefore, these derivatives are therefore implicated in the pathogenesis of malarial anaemia (Silverman *et al.,* 1987; Miller *et al.*,1989; Jootar *et al.,* 1993). This is reinforced by the observation that, as a consequence of repeated infections or suboptimal treatment, children may be partially immunocompromised and so asymptomatic during chronic *P. falciparum* infections. Despite having very low Haemoglobin levels (Kurtzhals *et al*., 1999), these children show absolute reticulocyte counts lower than expected for the degree of the anaemia (Wickramasinghe and Abdalla 2000). These low counts might indicate some degree of bone marrow dysfunction (Kurtzhals *et al.*, 1997).

In addition, Anaemia due to acute malaria is usually normochromic normocytic in nature. The occurrence of microcytic hypochromic anaemia suggests either iron deficiency, nutritional anaemia or hemoglobinopathies; which are not uncommon in tropical countries. The pre-existing anaemia due to nutritional deficiency or haemogloboinopathy may be aggravated in malaria. Severe anaemia (Haemoglobin below 5 g/dl) may induce cerebral anoxia and cardiac failure. Patients with heavy parasitemia, pregnancy or delivery, and children are vulnerable to develop severe anemia (Mishra and Mohanty, 2002).

Anaemia was defined when Hb <12.0 g/dL and <13.0 g/dL in female and male, respectively. The degree of anaemia was further classified as described by Cheesbrough (2009), with little modification, as normal (12/13 g/dL -16/17 g/dL for female and male, respectively), high (>16 g/dL or >17 g/dL for female and male, respectively), severe (Hb < 7.0 g/dL), moderate (Hb 7.0 to 10.0 g/dL), and mild (Hb >10 g/dL <12 or 13 g/dL for females and males, respectively). Malarial anaemia in

children was defined as children with a malaria-positive smear for *P. falciparum* parasitaemia (of any density) and Hb < 12 g/dL and <13.0 g/dL in female and male, respectively.

The haematocrit measures the level of RBC compared to the total blood volume (RBC and plasma). The normal haematocrit volume for male is 40 to 54 % and 35 to 47 % for female. Normal RBC range for male and female are 4.0 to 7.0 and 3.6 to 6.0 cells/mcL. In addition, normal MCH is between 26 to 32 pg/cell. Normocytic anaemia was identified using the mean corpuscular volume (MCV) which is the average red blood cell size. The normal MCV on full blood count is between 7695fL (normocitic), with smaller cells (<76 fL) described as microcytic and larger cells (>95 fL) as macrocytic. Meanwhile, monochromic anaemia was identified using mean corpuscular haemoglobin concentration (MCHC). Erythrocytes containing the normal amount of haemoglobin (normal MCHC) are called normochromic and when the MCHC is abnormally low they are called hypochromic, and when the MCHC is abnormally high, hyperchromic. The normal MCHC is within the range of 31-33 g/dL.

2.5.1 Immunopathology of anaemia

Although several mechanisms tend to be involved in the generation of anaemia in individuals who are acutely or chronically infected with malaria, the mechanisms can nevertheless be grouped into two main categories: destruction of cells in the peripheral circulation; and a reduction in or alteration of the production of erythroid precursors (Quintero *et al.,* 2011). Although host genetics may exert some influence for these two mechanisms, its role in anaemia pathogenesis is still poorly understood. For instance, host genetic diversity may explain, to some extent, variations in the frequency of anaemia when distinct areas are compared worldwide (Quintero *et al.,* 2011). In addition, severe anaemia in malaria is due to Rupture of parasitized erythrocytes, Rupture of non-parasitized erythrocytes mediated though cytokines and reactive oxygen species (ROS), Bleeding from different sites, Bone marrow depression, disseminated intravascular coagulation (DIC) and Auto immune haemolytic anaemia (rarely) (Mishra and Mohanty, 2002).

2.5.2 Peripheral destruction of red blood cells (RBC) by *plasmodium* parasite

An important part of the *Plasmodium* life cycle occurs as an obligate intraerythrocytic parasite, where it differentiates and multiplies at the expense of the host cell's nutrients up to the induction of its burst. Each merozoite released either from the liver or from an erythrocyte invades a new erythrocyte; in the case of *P. vivax*, its merozoites have a preference for immature red cells (reticulocytes), whereas in the case *of P. falciparum,* its merozoites invade erythrocytes of any age (Simpson *et al.,* 1999; Rayner *et al.,* 2005). This parasite-specific invasion preference entails the expression of receptors on the erythrocyte surfaces that are

required for an invasion. An obvious consequence of the parasite multiplication and the periodic burst of schizonts is the rupture of infected erythrocytes. Although this clearly contributes to the development of anaemia, it does not appear to be sufficient to explain the levels of anaemia attained in individuals exposed to the infection. It has been established that levels of parasitaemia of $\geq$ 50,000 parasites/μL are indicative of severe *falciparum* malaria (WHO, 2000). An infection level that corresponds to the infection and destruction of approximately 1% of the total erythrocyte mass could be easily replaced by erythropoiesis under normal conditions. However, it seems that simultaneous to this mechanism, depuration of the parasitized erythrocytes also occurs as a consequence of the phenomenon of erythrocyte rigidity. This rigidity is induced by the transport of parasite antigens to the infected erythrocyte membrane and is followed by the deformation of the membrane, opsonisation by antibodies and complement and by macrophage activation (Wickramasinghe and Abdalla, 2000). However, in many instances, the severity of malaria anaemia does not correlate directly with the degree of circulating parasitaemia (e.g. $\leq$ 1%), though detected parasitaemia does not reflect the total parasite load, as it does not take into account parasites sequestered in the microvasculature (Nakazawa *et al.*, 1995). Additionally, erythrocytes of malaria patients have a decreased half-life compared to those of healthy individuals

(Looareesuwan *et al.*, 1991). Moreover, epidemiological and mathematical models have shown that between 8-12 non-parasitized erythrocytes may be destroyed for each *Pf*-RBC parasitized (Jakeman *et al.*, 1999; Price *et al.*, 2001).

Although the exact cause of the destruction of non-parasitized erythrocytes is unclear, several mechanisms have been postulated: (i) the formation of autoantibodies against the proteins that alter RBC membranes (Jakobsen *et al.*,

1995), (ii) antibody-independent phagocytosis of phosphatidyl-serine exposure, secondary to damage mediated by reactive oxygen species (Serghides *et al.*, 2003), (iii) recognition of malarial antigens on infected erythrocytes by immunoglobulins (Igs) and their further clearance by macrophages (Waitumbi *et al.*, 2000), (iv) complement-mediated phagocytosis and/or haemolysis (Ritter *et al*., 1993) and (v) loss of complement regulatory proteins (CD35, CD55 and CD59) (Waitumbi *et al.*, 2000; Stoute *et al.*, 2003). Furthermore, it has been documented that the activities and absolute numbers of macrophages are increased during infection; together with erythrocyte changes (decreased deformability and deposition of Igs), this could assist in the depuration of non-parasitized erythrocytes during infection (Mohan *et al.*, 1995).

2.6 Immunoglobulins

Immunoglobulins, often abbreviated as "Ig," antibodies are found in blood and other bodily fluids of humans and other vertebrate animals. They help identify and destroy foreign substances such as microbes (e.g., bacteria, protozoan parasites and viruses). Immunoglobulins are classified into five categories: IgA, IgD, IgE, IgG and IgM (Solomon and Weiss, 1995).

2.6.1 Immunoglobulin A (IgA)

IgA antibodies are found in areas of the body such the nose, breathing passages, digestive tract, ears, eyes, and vagina. It is secreted in milk and is also the most prevalent Ig in secretions. IgA antibodies protect body surfaces that are exposed to outside foreign substances. This type of antibody is also found in saliva, tears, and blood. About 10 % to 15 % of the antibodies present in the body are IgA antibodies. A small number of people do not make IgA antibodies. IgA is resistant

to digestion and can activate the complement pathway when aggregated. It should also be noted both subclasses of IgA (IgA1 and IgA2) bind fragment crystallization (Fc) receptors (Solomon and Weiss, 1995).

2.6.2 Immunoglobulin G (IgG)

IgG antibodies are the smallest but most common antibody (75 % to 80 %) of all the antibodies in the body and are found in all body fluids. They are very important in fighting bacterial and viral infections. Human IgG (immunoglobulin G) is expressed on the surface of mature B cells and is the most prevalent Ig in serum and the major Ig in extravascular spaces. IgG1, IgG2 and IgG3 are complement activators, with IgG3 being the strongest. Human immunoglobulin subclasses IgG1 and IgG3 tend to strongly bind fragment crystallization (Fc) receptors, whereas subclasses IgG2 and IgG4 bind weakly. IgG is also the only human

immunoglobulin to pass from mother to fetus to transfer immunity. IgG antibodies are the only type of antibody that can cross the placenta in a pregnant woman to help protect her baby (foetus) (Solomon and Weiss, 1995).

2.6.3 Immunoglobulin M (IgM)

IgM antibodies are the largest antibody. They are found in blood and lymph fluid and are the first type of antibody made in response to an infection. They also cause other immune system cells to destroy foreign substances. IgM antibodies are about 5 % to 10 % of all the antibodies in the body. IgM is expressed on the surface of immature and mature B cells as monomers. IgM is the third most abundant human immunoglobulin. IgM is also the first human immunoglobulin to be made by a fetus and virgin B cells which are challenged with antigen. IgM is a strong

complement activator and agglutinator due to its pentameric structure and binds fragment crystallization (Fc) receptors (Solomon and Weiss, 1995).

2.6.4 Immunoglobulin E (IgE)

IgE antibodies are found in the lungs, skin, and mucous membranes. They cause the body to react against foreign substances such as pollen, fungus spores, and animal dander. They are involved in allergic reactions to milk, some medicines, and some poisons. IgE antibody levels are often high in people with allergies. It is expressed on the surface of mature B cells. Human immunoglobulin IgE is the least abundant Ig in the serum and does not activate the complement pathway. Fragment crystallization (Fc) receptors for IgE are found on eosinophils and IgE binds Fc receptors on mast cells and basophils even before interacting with antigen. As a result of its binding to basophils and mast cells, IgE is involved in allergic reactions. This happens when allergen is bound to IgE on cells and releases various pharmacological mediators which cause allergies (Solomon and Weiss, 1995).

2.6.5 Immunoglobulin D (IgD)

IgD antibodies are found in small amounts in the tissues that line the belly or chest. Expressed on the surface of mature B cells, IgD works with IgM in B cell development. IgD is found in very low levels in serum and does not activate the complement pathway (Solomon and Weiss, 1995).

2.7 *Plasmodium falciparum* Antigens

A family of parasite proteins that are inserted into the infected erythrocyte surface is known as *Plasmodium Falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), (Bull *et al.,* 1999). These PfEMP1 are expressed on the surface of infected red blood cells (IRBCs) as antigens and has been shown to mediate adherence to a range of host receptors located on the endothelial lining of specific organs and on uninfected RBCs (Barragan *et al.,* 2000 and Robinson *et al.,* 2003). These antigens are expressed on the erythrocyte surface from about 18 hours into the asexual, erythrocytic phase of the parasite life cycle and undergo clonal antigenic variation. These antigens have been found to agglutinate infected erythrocytes in a variantspecific manner.

Agglutination has been similarly useful in describing antigenic variation in PfEMP1. Naturally induced antibodies agglutinate *P. falciparum*-infected erythrocytes in a predominantly variant-specific manner (Newbold *et al*., 1992), and switches in agglutinating phenotype can be correlated with switches in cytoadherence characteristics (Roberts *et al*., 1992).

Surveys of agglutination antibody responses to natural *P. falciparum* populations in Pakistan (Iqbal *et al*., 1993), Gambia (Marsh *et al*., 1986), and Papua New Guinea (Forsyth *et al*., 1989; Reeder *et al*., 1994) indicate that PfEMP1 antigens are very diverse since antibodies after an infection usually agglutinate only the homologous parasite isolate that caused that specifiic infection. This diversity together with their surface location and functional importance indicates that these molecules can be important targets for naturally acquired immunity, an idea supported by recent

epidemiological data that shows that anti-PfEMP1 antibodies provide variantspecific protection against malarial disease (Bull *et al*., 1998).

2.7.1 *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1)

Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) is a family of proteins present on the membrane surface of red blood cells (RBCs or erythrocytes) that are infected by the malaria parasite *Plasmodium falciparum* (Lalchhandama, 2017*)*. It is a parasite protein which is exported to the surface of the infected erythrocyte, where it is inserted into the red cell cytoskeleton in the second half of the parasite life cycle. It is however, encoded by a family of *var* genes, with each parasite genome containing approximately 60 different *var* genes (Gardner *et al.,* 2002; Su *et al.,* 1995). PfEMP1 undergoes antigenic variation (Smith *et al.,* 1995) caused by a switch in transcription between *var* genes. Each PfEMP1 molecule consists of a variable number of structurally unique domains. There are three types of domains: DBL, CIDR, and C2. Within the DBL category, there are six sequence classes (DBL-α, -β, -γ, -δ, -ε, and -X), while there are only two CIDR sequence classes (CIDR-α and CIDR-β), and the C2 domain is conserved (Gardner *et al.,* 2002). The different domains appear to have conserved but different functions:

CIDR-α binds CD36 (Baruch *et al.,* 1997; Smith *et al.,* 1995), DBL-γ binds to chondroitin sulfate A (Reeder *et al.,* 1999), and DBL-α is involved in rosetting (Chen *et al.,* 1998; Rowe *et al.,* 1997).

PfEMP1 is synthesized during the parasite's blood stage (erythrocytic schizogony) inside the RBC, during which the clinical symptoms of *P. falciparum* malaria are manifested. Acting as both an antigen and adhesion protein, it is believed to play a key role in the high level of virulence associated with *P. falciparum*. It was

discovered in 1984 when it was reported that infected RBCs had unusually largesized cell membrane proteins, and these proteins had antibody-binding (antigenic) properties. An elusive protein, its chemical structure and molecular properties were revealed only after a decade, in 1995. It is now established that there is not one but a large family of PfEMP1 proteins, genetically regulated (encoded) by a group of about 60 genes called *var*. Each *P. falciparum* is able to switch on and off specific *var* genes to produce a functionally different protein, rendering evasion from the host's immune system. RBCs carrying PfEMP1 on their surface stick to endothelial cells, which facilitates further binding with uninfected RBCs (through the processes of sequestration and rosetting), ultimately helping the parasite to both spread to other RBCs as well as bringing about the fatal symptoms of *P. falciparum* malaria (Lalchhandama, 2017).

The diversity of the PfEMP1 repertoire of parasites in a given geographic area is a key factor in the development of clinical immunity. Other factors that may also be important in determining the development and maintenance of clinical immunity are (i) the parasite density required to trigger an anti-PfEMP1 antibody response, (ii) the specificity and affinity of the anti-PfEMP1 immune responses, and (iii) the longevity of these antibodies (Krausel *et al.*, 2007).

Furthermore, PfEMP1 is a large family of proteins having high molecular weights ranging from 200 to 350 kDa (Pasternak *et al*., 2009). The wide range of molecular size reflects extreme variation in the amino acid composition of the proteins. But all the PfEMP1 proteins can be described as having three basic structural components, namely, an extracellular domain (ECD), a transmembrane domain (TMD) and an intracellular acidic terminal segment (ATS). The extracellular domain is fully exposed on the cell surface, and is the most variable region. It

consists of a number of sub-domains, including a short and conserved N terminal segment (NTS) at the outermost region, followed by a highly variable Duffybinding-like (DBL) domain, sometimes a Ca^{2+}-binding C2 domain, and then one or two cysteine-rich interdomain regions (CIDRs) (Bull and Abdi, 2016).

2.7.1.1 Function of *P. falciparum* erythrocyte membrane protein (PfEMP1)

The primary function of PfEMP1 is to bind and attach RBCs to the wall of the blood vessels. The most important binding properties of *P. falciparum* known to date are mediated by the head structure of PfEMP1, consisting of DBL domains and CIDRs (Crabb and Cowman, 2002). DBL domains can bind to a variety of cell receptors including thrombospondin (TSP), complement receptor 1 (CR1), chondroitin sulfate A (CSA) (Rowe *et al*., 2009), P-selectin (Senczuk *et al*., 2001), endothelial protein C receptor (EPCR) (Turner *et al.*, 2013), and heparan sulfate (Angeletti *et al*., 2015). The DBL domain adjacent to the head structure binds to ICAM1. CIDRs mainly bind to a large variety of cluster determinant 36 (CD36) (Pasternak *et al*., 2009; Kraemer *et al*., 2006). These bindings produce the pathogenic characteristics of the parasite, such as sequestration of infected cells in different tissues (Howell *et al*., 2007), invasion of RBCs (Cowman and Crab, 2006), and clustering of infected cells by a process called resetting (Miller *et al*., 1997).

CIDR1 protein in the semi-conserved head structure is the principal and best understood adhesion site of PfEMP1. It binds with CD36 on endothelial cells (Baruch *et al*., 1997; Hsieh *et al*., 2016). Only group B and C proteins are able to bind, and that too with only those having CIDRα2-6 sequence types. On the other hand, group A proteins have either CIDRα1 or CIDRβ/γ/δ, and they are

responsible for the most severe condition of malaria (Smith, 2014). Binding with ICAM1 is achieved through the DBLβ domain adjacent to the head structure. However, many PfEMP1s having DBLβ domain do not bind to ICAM1 (Howell *et al*., 2006), and it appears that only the DBLβ paired with C2 domain can to bind to ICAM1 (Howell *et al*., 2007). The DBLα-CIDRγ tandem pair is the main factor for resetting (ViganWomas *et al*., 2012), sticking together the infected RBC with the uninfected cells, and thereby clogging of the blood vessels. This activity is performed through binding with CR1 (Miller *et al*., 1997; Stoute, 2011).

The most dangerous malarial infection is in the brain and is called cerebral malaria. In cerebral malaria, the PfEMP1 proteins involved are DC8 and DC13. They are named after the number of domain cassettes they contain, and are capable of binding not only endothelial cells of the brain, but also in different organs including brain, lung, heart, and bone marrow (Avril *et al.*, 2013). Initially, it was assumed that PfEMP1 binds to ICAM1 in the brain, but DC8 and DC13 were found incompatible with ICAM1. Instead DC8 and DC13 specifically bind to EPCR using CIDRα subtypes such as CIDRα$_{1.1}$, CIDRα$_{1.4}$, CIDRα$_{1.5}$ and CIDRα$_{1.7}$ (Turner *et al.*, 2013). Nevertheless, it was later shown that DC13 can be bound to both ICAM1 and EPCR (Avril *et al.*, 2016). EPCR is therefore a potential vaccine and drug target in cerebral malaria (Lau *et al.*, 2015).

VAR2CSA is unique in that it is mostly produced by the placenta during pregnancy (the condition called pregnancy-associated malaria, PAM, or placental malaria).
The majority of PAM is therefore due to VAR2SCA (Hviid and Jansen, 2015). Unlike other PfEMP1, VAR2CSA binds to chondroitin sulphate-A present on the vascular endothelium of placenta. Although its individual domains can bind to

CSA, its entire structure is used for complete binding (Khunrae *et al*., 2010). The major complication in PAM is low-birth-weight babies. However, women who survived the first infection generally develop an effective immune response. In *P. falciparum*-prevalent regions in Africa, pregnant women were found to have high levels of antibody (IgG) against VAR2CSA, which protect them from placentaattacking malaria parasite (Salanyi *et al*., 2004).

2.7.1.2 Role of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1)

Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) plays an important role in the host–parasite interaction. During the asexual growth stage, members of this family of molecules are inserted into the surface of infected erythrocytes by parasites. PfEMP1 molecules are encoded by a family of about 60 highly diverse *var* genes (Gardner *et al.,* 2002) that undergo rapid switching in vitro and are thought to be largely responsible for the well characterized phenomenon of clonal antigenic variation (Roberts *et al*., 1992; Smith *et al*., 1995). In addition, they appear to be central to changes in cytoadherence properties that lead to the sequestration of infected erythrocytes in capillary beds, potentially a key step in the pathology of severe disease (MacPherson *et al*., 1995; Pongponratn *et al*., 2003).

The molecules are made up of combinations of different domains, each mediating a specific range of interactions on host endothelial cells (Baruch *et al*., 1996; Smith *et al*., 2000) platelets (Pain *et al*., 2001), uninfected erythrocytes (Rowe *et al*., 1997), and dendritic cells (Urban *et al*., 1999).

Therefore, in the life cycle of *Plasmodium* spp, proliferation and differentiation within erythrocytes are important steps. To accomplish this, the parasites transmit

polypeptides to the surface of infected erythrocytes, such as import of nutrients and to bind to other erythrocytes and the host microvasculature. Binding is mediated by the adhesive polypeptides *Plasmodium falciparum*-encoded repetitive interspersed families of polypeptides (RIFINs), subtelomeric variant open reading frame (STEVOR) and *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which are encoded by multigene families to ensure antigenic variation and evasion of host immunity. Such variant surface antigens are suggested to mediate the sequestration of infected erythrocytes in the microvasculature and block blood flow when binding is excessive (Mats *et al*., 2017).

2.8 Human Immune System Evasion

In a healthy human immune system, malaria parasite binding to RBCs stimulates the production of antibodies that target the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) molecules. Antibody binding with PfEMP1 disables the binding properties of DBL domains, causing loss of cell adhesion, and the infected RBC is destroyed, which in this scenario, malaria is avoided (Deitsch and Chitins, 2013). However, below are the gene families that help in immune system evasion by the parasite in humans

2.8.1 *var* gene family

The *var* genes encode the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins. The genes are located in the subtelomeric regions of the chromosomes. The genome includes an estimated 59 var genes (Gardner *et al.*, 2002). The proteins encoded by the *var* genes are transported to the erythrocyte membrane and cause the infected erythrocytes to adhere to host endothelial receptors. Antigenic variability occurs due to transcriptional switching between var

genes, which enables the parasite to escape the immune system. In other words, to evade the host's immune response, different *P. falciparum* switch on and off different *var* genes to produce functionally different (antigenically distinct) PfEMP1s. Each variant type of PfEMP1 has different binding property, and therefore antibodies are not always recognized (Deshmukh *et al*., 2013).

By default, all the *var* genes in the malaria parasite are inactivated and activation (gene expression) of *var* is initiated upon infection of the organs. In addition, only different var genes are triggered in each organ. The extent of the infection is determined by the type of organ in which infection occurs, thus activating the type of var gene. For example, only the var genes for the PfEMP1 proteins DC8 and DC13 are turned on in the most severe cases of malaria, such as cerebral malaria

(Avri *et al*., 2012; Claessens *et al*., 2012). Their CIDRα1 domains bind to EPCR after the synthesis of DC8 and DC13, which results in the onset of extreme malaria (Bernabeu and Smith, 2016). The abundance of the gene products (transcripts) of these PfEMP1 proteins (specifically the $CIDR_{\alpha 1}$ subtype transcripts) is directly related to the severity of the disease. This also means that preventing the interaction between $CIDR_{\alpha 1}$ and EPCR would be a good target for a potential vaccine (Jespersen *et al*., 2016; Mkumbaye *et al*., 2017). The gene for VAR2CSA (named var2csa) is triggered in the placenta in pregnancy-associated malaria, another extreme form of falciparum malaria. The primary cause of premature delivery, death of the foetus and severe anaemia in the mother is as a result of binding of

VAR2CSA to CSA (Khunrae *et al*., 2010). This means that drugs targeting

VAR2CSA will be able to prevent the effects of malaria, and for this reason VAR2CSA is the leading candidate for development of a PAM vaccine (Fried and Duffy, 2015).

2.8.2 r*if* gene family

The *rif* genes encode repetitive interspersed family (rifin) proteins. The genes are located in the subtelomeric regions of the chromosomes.

The genome includes an estimated 149 rif genes (Gardner *et al.,* 2002).

2.8.3 *stevor* gene family

The *stevor* genes encode the sub-telomeric variable open reading frame (stevor) proteins. The genes are also located in the subtelomeric regions of the chromosomes. There are an estimated 28 stevor genes in the genome and the function of the stevor proteins is currently unknown (Gardner *et al.,* 2002).

2.9 Merozoite Surface Proteins (MSP)

The genetic diversity of *P. falciparum* is usually achieved by genotyping the polymorphic region of antigenic markers such as block 2 of the merozoite surface protein 1 (MSP1), block 3 of the merozoite surface protein 2 (MSP2) and RII repeat region of the glutamate rich protein (GLURP) (Viriyakosol *et al.,* 1995). Another genotyping approach is the multilocus genotyping which targets non-antigenic markers such as asingle nucleotide polymorphisms (SNPs) (Nkhoma *et al.,* 2013) and simple sequence repeats of neutral microsatellites (Anderson *et al.,* 2000). Using these molecular genotyping approaches, the factors contributing to the *P. falciparum* population structure and diversity such as inbreeding, epidemic

population expansion, geographic isolation and gene flow or introgression of foreign parasites can be characterized (Abd Razak *et al.,* 2016). Many genetic measurements such as expected heterozygosity (*He*), the proportion of polymorphic loci, the number of alleles (*Na*), multiplicity of infection (MOI), linkage disequilibrium (LD) and F-statistics (F^{ST}) are used to estimate the factors affecting the malaria parasite population structure (Viriyakosol *et al.,* 1995; Anderson *et al.,* 2000 & Arnott *et al.,* 2012).

The malaria parasite, in its asexual, blood invading stage called the merozoite stage, infects red blood cells. The MSP1 complex of surface proteins likely mediates the first interactions of the parasite with red blood cells *(*Kadekoppala and Holder, 2010). Early electron microscope images of *Plasmodium* merozoites revealed that they were covered in a 'fuzzy' fibrillar coat of surface proteins; remarkably, this coat appeared to be shed during RBC invasion (Ladda *et al.,* 1969; Langreth *et al.,* 1978). Since these initial observations, the composition and function of merozoite surface proteins (MSPs) has been of great interest because of their role in RBC invasion and potential as vaccine candidates (Richards and Beeson,2009) and, more recently, as drug targets for inhibiting blood-stage replication (Boyle *et al.,* 2013;

Wilson *et al.,* 2015).
Merozoite Surface Protein 1 and 2 of *P. falciparum* are major blood-stage malaria vaccine targets and are also suitable markers for the identification of genetically distinct *P. falciparum* parasite sub-populations. MSP1 is major surface protein of approximately 190-kDa size. This plays a major role in the invasion of erythrocytes (Holder *et al.,* 1992) and is a major target of immune responses (Apio *et al.,* 2000).

MSP1 contains 17 blocks of sequence flanked by conserved regions (Takala *et al.*, 2002) Block 2, which is the most polymorphic part of MSP1, is grouped into three allelic families namely K1, MDA20, and RO33 type (Takala *et al.,* 2006). MSP2 is glycoprotein consisting five blocks where the central block is the most polymorphic

(Ferreira *et al.,* 2007). MSP2 alleles are grouped into two allelic families, FC27 and 3D7/IC1.

Proteins relevant to red blood cell (RBC) invasion are present on the merozoite surface, or contained within organelles known as rhoptries and micronemes at the apex of the merozoite. Merozoite surface proteins are tethered as either glycophosphatidylinositol (GPI)-anchored proteins, integral membrane proteins or as peripherally-associated proteins (held on the merozoite surface through interactions with membrane-bound proteins). Other merozoite proteins are maintained in the rhoptries and micronemes during schizont development, and then localize to the merozoite surface prior to, or soon after, merozoite egress from the schizont via a variety of mechanisms (Beeson *et al.*, 2016).

Once released from schizonts, merozoites may take several minutes before establishing contact with the surface of a RBC and commencing invasion. Following primary merozoite attachment to the RBC surface, *P. falciparum* invasion occurs within about 30 seconds (Gilson and Crabb 2009) and involves a sequence of extracelluar recognition events (Weiss *et al.,* 2015). The subsequent binding of merozoites is dependent on the erythrocyte binding antigen (EBA) and reticulocyte binding-like homologous (PfRH) protein families. Both EBA and PfRH ligands are integral-membrane proteins that allow a number of alternate

pathways for merozoite invasion (Tham *et al.*, 2012). The diversification of RBC binding pathways appears to have been driven by a combination of immune selection (Persson *et al.*, 2008) and human RBC polymorphisms (Maier *et al.*, 2003). Therefore, although their collective role in invasion is important, there is a level of redundancy among protein family members (Crosnier *et al*., 2011).

The kinetics of merozoite invasion is partly understood and largely limited to *in vitro* studies (Boyle *et al.*, 2013; Weiss *et al.*, 2015). From these studies, 80 % of merozoites invade within 10 minutes of co-incubation with RBCs, but the remainder can invade after prolonged periods post-egress (Boyle *et al.*, 2010). Likewise, the invasive capacity of merozoites appears to decline relatively quickly after schizont rupture with an estimated half-life of 8 mins at 37 °C and 15 mins at room temperature, however a subpopulation of merozoites can retain their invasive capacity for extended periods (Boyle *et al.*, 2013).

2.10 Natural Immunity against *P. falciparum*

Individuals living in malaria endemic areas develop naturally acquired immunity to *Plasmodium* spp. infections as they grow, but only slowly and only after repeated exposure (Marsh and Kinyanjui 2006; Doolan *et al*., 2009). The ability to control parasitemia in the blood is established at a later stage. The mechanisms underlying development of anti-disease immunity and the factors governing effective protection are still largely unknown (Reddy *et al.*, 2012). It is well known, however, that antibodies contribute to protection against clinical malaria due to *P. falciparum*. Passive immunoglobulin transfers from immune donors to *P. falciparum* infected individuals decreased parasitemia and clinical symptoms (Mayxay *et al*., 2001). Antibodies directed against cell surface proteins of either

the merozoite form of the parasite or of infected red blood cells have been shown to be important components of acquired protective immunity against malaria (Richards and Beeson, 2009).

Naturally acquired antibodies to sporozoites can protect against liver-stage infection and antibodies targeting blood-stage antigens can suppress high parasite densities and progression to symptomatic disease (Doolan *et al*., 2009). Breadth and magnitude of antibody responses are important, with responses to a repertoire of antigens associated with increased protection against disease (Osier *et al*., 2008; Richards *et al.,* 2010; Rono *et al.,* 2013), while also acting as biomarkers of past exposure (Elliott *et al.,* 2014).

2.11 Anti-malaria Vaccine

Over the past few years, there has been the rise of public-private collaborations and a significant increase in funding for malaria vaccine production (Guinovart and Alonso, 2007). Lessons have been learned on the various approaches to the clinical development plan for a vaccine candidate, including different clinical trial phases and endpoints and methods to conduct them. Improved understanding of mechanisms underlying naturally acquired immunity, definitions of surrogate markers of protection, including improved *in vitro* assays and animal models, and strengthened capacity in malaria-endemic countries to conduct clinical trials would accelerate the development of malaria vaccines (Guinovart and Alonso, 2007).

There are currently no licensed vaccines against malaria but the only approved vaccine as of 2015 is RTS,S. It requires four injections, and has a relatively low efficacy (about 26–50 %). Due to low efficacy, WHO does not recommend the use of RTS,S vaccine in babies between 6 and 12 weeks of age (WHO, 2016). The

vaccine was evaluated in a large clinical trial in 7 countries in Africa and received a positive opinion by the European Medicines Agency by 2015 (WHO, 2016). However, research still continues into recombinant protein and attenuated whole organism vaccines.

RTS,S is the most recently developed recombinant vaccine and it consists of the *P. falciparum* circumsporozoite protein (CSP) from the pre-erythrocytic stage. The CSP antigen induces the production of antibodies capable of preventing the invasion of hepatocytes and additionally elicits a cellular response enabling the destruction of infected hepatocytes. The CSP vaccine presented problems in trials due to its poor immunogenicity. RTS,S attempted to avoid these by fusing the protein with a surface antigen from hepatitis B, hence creating a more potent and immunogenic vaccine. When tested in trials an emulsion of oil in water and the added adjuvants of monophosphoryl A and QS21 (SBAS2), the vaccine gave protective immunity to 7 out of 8 volunteers when challenged with *P. falciparum* (Malaria Vaccine Initiative Path, 2013). RTS,S/AS01, was engineered using genes from the outer protein of *P. falciparum* malaria parasite and a portion of a hepatitis B virus plus a chemical adjuvant to boost the immune response. Infection is prevented by inducing high antibody titers that block the parasite from infecting the liver (Foquet *et al*., 2014).

As of October 2013 preliminary results of a phase III clinical trial indicated that RTS,S/AS01 reduced the number of cases among young children by almost 50 percent and among infants by around 25 percent. The study ended in 2014. The effects of a booster dose were positive, even though overall efficacy seem to wane with time. After four years reductions were 36 percent for children who received three shots and a booster dose. Missing the booster dose reduced the efficacy

against severe malaria to a negligible effect. The vaccine was shown to be less effective for infants. Three doses of vaccine plus a booster reduced the risk of clinical episodes by 26 percent over three years, but offered no significant protection against severe malaria (Borghino, 2015).

2.11.1 Potential targets of malaria vaccine

Parasite stage	Target
Sporozoite	Hepatocyte invasion; direct anti-sporozite
Hepatozoite	Direct anti-hepatozoite.
Asexual erythrocytic	Anti-host erythrocyte, antibodies blocking invasion; anti receptor ligand, anti-soluble toxin
Gametocytes	Anti-gametocyte. Anti-host erythrocyte, antibodies blocking fertilization, antibodies blocking egress from the mosquito mid-gut.

Plasmodium is a highly complex parasite with a complicated and multi-stage life cycle during which the parasite presents multiple antigens that show considerable variability (Guinovart and Alonso, 2007). This presents problems in vaccine development but also increases the number of potential targets for a vaccine. These have been outlined into the life cycle stage and the antibodies that could potentially elicit an immune response.

i. The initial stage in the life cycle, following inoculation, is a relatively short "pre-erythrocytic" or "hepatic" phase. Candidate vaccine antigens from the pre-erythrocytic stages may be the targets of antibodies that prevent sporozoite invasion of

hepatocytes or the targets of cellular immune responses that kill infected hepatocytes. A completely effective pre-erythrocytic vaccine would inactivate the parasite before it left the liver, leading to sterile immunity and prevention of disease (Schwartz *et al.*, 2012).

ii. The "erythrocytic" or blood phase is the second phase of the life cycle. Merozoite aggregation or invasion of red blood cells could be avoided by the vaccine. This approach is complicated by the absence of major histocompatibility complex molecule expression on the surface of erythrocytes. Rather, malarial antigens are expressed, and it is this towards which the antibodies could potentially be directed. Another approach would be to attempt to block the process of erythrocyte adherence to blood vessel walls. It is assumed that this process is accountable for much of the clinical syndrome associated with malarial infection; therefore, a vaccine administered during this stage would be therapeutic and hence administered during clinical episodes to prevent further deterioration.

iii. The last phase of the life cycle that has the potential to be targeted by a vaccine is the "sexual stage". This would not give any protective benefits to the individual inoculated but would prevent further parasite transmission by preventing the gametocytes from producing multiple sporozoites in the gut wall of the mosquito. This would therefore be used as part of a policy directed at eliminating the parasite from areas of low prevalence or to prevent

the development and spread of vaccine-resistant parasites. This type of transmissionblocking vaccine is potentially very important. The evolution of resistance in the malaria parasite occurs very quickly, potentially making any vaccine ineffective within a few generations. This approach to the prevention of spread is therefore essential.

iv. Another approach is to target the protein kinases that are present throughout the entire lifecycle of the malaria parasite. Research is underway on this, yet development of an effective vaccine targeting these protein kinases may still take a long time (Zhang *et al.*, 2012).

v. PfEMP1, one of the proteins known as variant surface antigens (VSAs) produced by *Plasmodium falciparum*, was observed to be a key target of the immune system's response against the parasite. Studies have showed that antibodies against PfEMP1 provide protective immunity, while antibodies developed against other surface antigens do not. Therefore, PfEMP1 could be a target to develop an effective vaccine which will reduce risk of developing malaria (Chan *et al.,* 2012; Parish, 2012).

2.12 Genetic diversity of *P. falciparum*

In a survey conducted in Lagos, Nigeria by Oyebola *et al*. (2014), on "Genetic diversity and complexity of *Plasmodium falciparum* infections in Lagos, Nigeria".

Results revealed that K1 (60/100) was the most predominant genotype of MSP1 allelic family followed by the genotypes of MAD20 (50/100) and R033 (45/100).

In the MSP2 locus, FC27 genotype (62/100) showed higher frequency than 3D7 genotype (55/100). The allelic families were detected either alone or in combination with other families. However, no R033/MAD20 combination was observed.

Multiplicity of infection (MOI) with MSP1 was higher in the locality of Ikorodu (1.50) as compared to Lekki (1.39). However, MOI with MSP2 was lower in the locality of Ikorodu (1.14) than in Lekki (1.76). There was no significant difference in the mean MOI between the two study areas (P=0.427). The observation of limited diversity of malaria parasites may imply that the use of antigenic markers as genotyping tools for distinguishing recrudescence and re-infections with *P. falciparum* during drug trials is subjective.

In a similar study conducted in South-west Nigeria by Amodu *et al.* (2005) which studied the association between diversity of the merozoite surface protein-1 (MSP1) locus and the severity of disease in childhood malaria in Ibadan. The distribution of MSP1 alleles was significantly different between asymptomatic malaria (ASM), acute uncomplicated malaria (UM) and severe malaria (SM). Asymptomatic malaria samples had a higher median number of alleles than the other two groups. The type of MSP1 allele detected was significantly associated with the clinical category of malaria. The absence of K1 alleles was associated with a three-fold increase risk of UM and a four-fold increased risk of SM when compared with asymptomatic malaria. The absence of MAD20 alleles was associated with a fivefold increase risk of UM and an eight-fold increase of SM. An association between the MSP1 locus of *P. falciparum* and clinical severity of malaria in a sample of

Nigerian children was observed from the study. The findings showed the presence of K1 and MAD20 alleles significantly associated with ASM and consequently a reduced risk of developing the symptomatic disease.

Olasehinde *et al.* (2000) in a study titled "Genetic diversity of *Plasmodium falciparum* field isolates from Ogun state, south western Nigeria". Majority of the patients showed monoclonal infections while multiplicity of the infection for MSP1 and MSP2 were 1.1 and 1.2 respectively. The estimated number of genotypes was 8 MSP1 (4 KI; 3 MAD; 1 RO33) and 6 MSP2 (3 FC27; 3 3D7) and about 80 % of the isolates coded for Glurp with allelic size ranges between 700 and 900 bp.

Similarly, in a survey conducted in India by Hussain *et al*. (2011) on "Genetic diversity in merozoite surface protein-1 and 2 among *Plasmodium falciparum* isolates from malarious districts of tribal dominant state of Jharkhand, India" the genetic diversity of *P. falciparum* population from low transmission area, Ranchi, Bokaro and Hazaribagh and highly malarious area, Latehar and Palamau districts of Jharkhand were evaluated by polymerase chain reaction-sequencing analyzing both MSP1 and MSP2 genes to explore the genetic structure of parasite from the region. Of the 134 *P. falciparum* isolates, the majority of patients from all the sites had mean monoclonal infections of 67.1 and 60.4 % of *P. falciparum* for MSP1 and MSP2, respectively, whereas, mean multiple genotypes of 32.8 % and 39.5 % for MSP1 and MSP2, respectively. Interestingly, it was observed that the higher multiclonal infection in low transmission area as compared to highly malarious area in the case of MSP1 genotypes, whereas in MSP2 higher multiclonal infection was observed in highly malarious area compared to low transmission area. The overall multiplicities of infection of MSP1 and MSP2 were 1.38 and 1.39, respectively.

The genetic diversity and allelic distribution found in this study is somewhat similar to other reports from India and Southeast Asian countries. However, *P. falciparum* infection can be highly complex and diverse in these disease-endemic regions of Jharkhand, suggesting continual genetic mixing that could have significant implications for the use of antimalarial drugs and vaccines.

In a survey conducted by Abdel Hamid *et al*. (2013) on "Genetic Diversity of *Plasmodium falciparum* field isolates in Central Sudan Inferred by PCR Genotyping of Merozoite Surface Protein 1 and 2" in which Characterization of *Plasmodium falciparum* diversity by amplification of the polymorphic regions of the Merozoite Surface Proteins 1 (MSP1) and 2 (MSP2) genes was done. From the results obtained, it was observed that the total number of alleles identified in MSP1 block 2 was 11, while 16 alleles were observed in MSP2 block 3. In MSP1, RO33 was found to be the predominant allelic type, carried alone or in combination with MAD20 and K1, whereas FC27 family was the most prevalent in MSP2. Sixty-two percent of isolates had multiple genotypes and the overall mean multiplicity of infection was 1.93. Age, however, was correlated with parasite density (P = 0.017), and a positive correlation was recorded between parasite densities and the number of alleles (P = 0.022). It was deduced that the genetic diversity in *P. falciparum* field isolates in central Sudan was high and consisted of multiple clones.

Likewise, in another study conducted in Ghana on "Genetic diversity of *Plasmodium falciparum* isolates from uncomplicated malaria cases in Ghana over a decade" by Duah *et al.* (2016), Archived filter paper blood blots were gathered from children aged nine years and below with uncomplicated malaria collected from different sites in Ghana were typed for the presence of the markers. From the total of 880 samples genotyped for MSP2 for the two major allelic families, both

of the major allelic families, 3D7 and FC27 were common in all population samples.

The highest multiplicity of infection (MOI) was observed in isolates from Begoro (forest zone, rural site): 3.31 for the time point 2007–2008. A significant variation was observed among the sites in the MOIs detected per infection (Fisher's exact test, $P < 0.001$) for the 2007 isolates and also at each of the 3 sites with data for three different years, Hohoe, $P = 0.03$; Navrongo, $P < 0.001$; Cape Coast, $P < 0.001$. Overall, there was no significant difference between the MOIs of the three ecological zones over the years ($P = 0.37$) and between the time points when data from all sites were pooled ($P = 0.40$). It was deduced from the study that the diversity and variation between isolates were observed to be profound; however, there was homogeneity throughout the three ecological zones studied. This is indicative of gene flow between the parasite populations across the country probably due to human population movements (HPM).

On the other hand, reviews of surveys on similar study to antibody responses were carried across different geographic locations. In a two cross-sectional survey carried out on three settings in Lake Victoria by Idris *et al.* (2017); antibody response to *P. falciparum* was determined in 4,112 individuals by ELISA using eluted dried blood from filter paper. The overall seroprevalence was 64.0 % for AMA-1, 39.5 % for MSP-119, and 12.9 % for CSP. Between settings, seroprevalences for merozoite antigens were similar between Ungoye and Mfangano, but higher when compared to the small islands. For AMA-1, the seroconversion rates (SCRs) ranged from 0.121 (Ngodhe) to 0.202 (Ungoye), and were strongly correlated to parasite prevalence. We observed heterogeneity in serological indices across study sites in

Lake Victoria. These data suggest that AMA-1 and MSP-119 sero-epidemiological analysis may provide further evidence in assessing variation in malaria exposure and evaluating malaria control efforts in high endemic area.

In another survey by Ismail *et al.* (2017), the researchers studied the quantity and quality of antibody responses against merozoite antigens, as well as multiplicity of infection (MOI), in children from Uganda. Higher levels of IgG antibodies was observed towards erythrocyte-binding antigen EBA181, MSP2 of *Plasmodium falciparum* 3D7 and FC27 (MSP2-3D7/FC27), and apical membrane antigen 1 (AMA1) in patients with uncomplicated malaria by enzyme-linked immunosorbent assay (ELISA) but no differences against EBA140, EBA175, MSP1, and reticulocyte-binding protein homologues Rh2 and Rh4 or for IgM against MSP23D7/FC27. Decreased invasion of two clinical *P. falciparum* isolates in the presence of patient plasma correlated with lower initial parasitemia in the patients, in contrast to comparisons of parasitemia to ELISA values or antibody affinities, which did not show any correlations. Analysis of the heterogeneity of the infections revealed a higher MOI in patients with uncomplicated disease, with the *P. falciparum* K1 MSP1 (MSP1-K1) and MSP2-3D7 being the most discriminative allelic markers. Higher MOIs also correlated positively with higher antibody levels in several of the ELISAs. It was observed that certain antibody responses and MOIs were associated with differences between uncomplicated and severe malaria. When different assays were combined, some antibodies, like those against AMA1, seemed particularly discriminative. However, only decreased invasion correlated with initial parasitemia in the patient, signaling the importance of functional assays in understanding development of immunity against malaria and in evaluating vaccine candidates.

Similarly, survey on evaluation of antibody response to *P. falciparum* in Senegalese children by Sarr *et al*. (2007) revealed specific IgG response increased

progressively from one-year to 5-year old children and then stayed high in children from five to nine years old. The children with *P. falciparum* infection had higher specific antibody responses compared to negative infection children, suggesting a strong relationship between production of specific antibodies and malaria transmission, rather than protective immunity. The study shows that anti-malaria response has been profoundly different in areas where malaria exposure depends on different Anopheles species. Such findings are discussed according to the use of immunological tool for the evaluation of malaria transmission; and the effect of Anopheles vectors on the regulation of antibody responses to *P. falciparum*.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the Study Area

The study was carried out in Minna, the capital city of Niger State, Nigeria, located at 9.62^0 latitude and 6.55^0 longitude and situated at elevation 243 meters above sea level, covering a land area of $88\,km^2$. Minna city comprises of two Local Government Areas: Bosso and Chanchaga LGA. The estimated population projection of Minna according to the National Population Commission of Nigeria and National Bureau of Statistics (2017) was 492,100 (Bosso- 208,100 and Chanchaga- 284,000) in 2016. However, by percentage, children 0-19 years has an estimated population of 57% (280,497) from the total population. The city has a tropical climate with two seasons, the rainy and the dry seasons, which start from May-October and December-March respectively. However, there is a transitional period of April and November. The rainy seasons here have a good deal of rainfall, while the dry seasons have very little or no rainfall. The average annual temperature is 27.5 °C in Minna, the rainfall averages 1229 mm and the relative humidity is

61.00%. The least amount of rainfall occurs in January with an average of 1 mm. Most of the precipitation falls in September, averaging 260 mm. The temperatures are highest on average in March, at around 30.5 °C. August is the coldest month, with temperatures averaging 25.3 °C.

3.2 Ethical Approval

Ethical approval for the study was obtained from the Niger State Ministry of Health after a research protocol and an application letter to that effect was tendered.

Following administrative clearance from the ministry, clearance was also obtained from the Director, Primary Healthcare, Chanchaga local government. Furthermore, informed consent was obtained from the parents or guardians of children captured in this study.

3.3 Sampling Sites

Two sets of study groups were adopted for this research, the first group were samples collected from children (with symptoms of fever) who attended selected healthcare facilities within the period of the study, otherwise known as the symptomatic cases. The selected healthcare facilities were: General Hospital,

Minna; Primary Healthcare, Old Airport Road, Limawa B; Primary Healthcare, Gwari Road, Nassarawa A; Family Support Programme, Limawa A; Primary Healthcare, Tunga and Primary Healthcare, Kpakungu. The other group were samples collected from children (with no signs of fever) within the residential communities in Minna. The communities were Kpakungu, Bosso, Fadikpe, Maitumbi, Shiroro (Saukekahuta) and Chanchaga Communities as indicated in Fig 3.1. However, there was an inclusion of the control group which were adults of 18 years and above.

3.4 Study Population and Sample Size

The sample size was estimated using the sample size formula $\frac{\frac{z^2 \times p(1-p)}{e^2}}{1+\frac{[z^2 \times p(1-p)]}{e^2 N}}$ where

z^2= z-score for 95% *i.e.* 1.96; p = standard of deviation 0.5 (50%); e = margin of error 95%; N = population size.

This was also reconfirmed by sample size calculator with 95 % precision, 95 % prevalence level and population of children in Minna which was 280,497 individuals and from all these the sample size for the study was calculated at approximately 300. A total of 316 venous blood samples were collected from children between 6 months and 17 years of age, and adult control group (>18 years), attending outpatients Department of Healthcare facilities and communities across Minna from February to July, 2018. Of the 316 blood samples collected, 181 (57 %) were males and 135 (43 %) were females. Meanwhile, 139 (44 %) of the children were less than 5 years of age; 112 (35 %) between 6 to 11 years; 50 (16 %) between 12 to 17 years; and 15 (5 %) of the population were adults above 18 years of age.

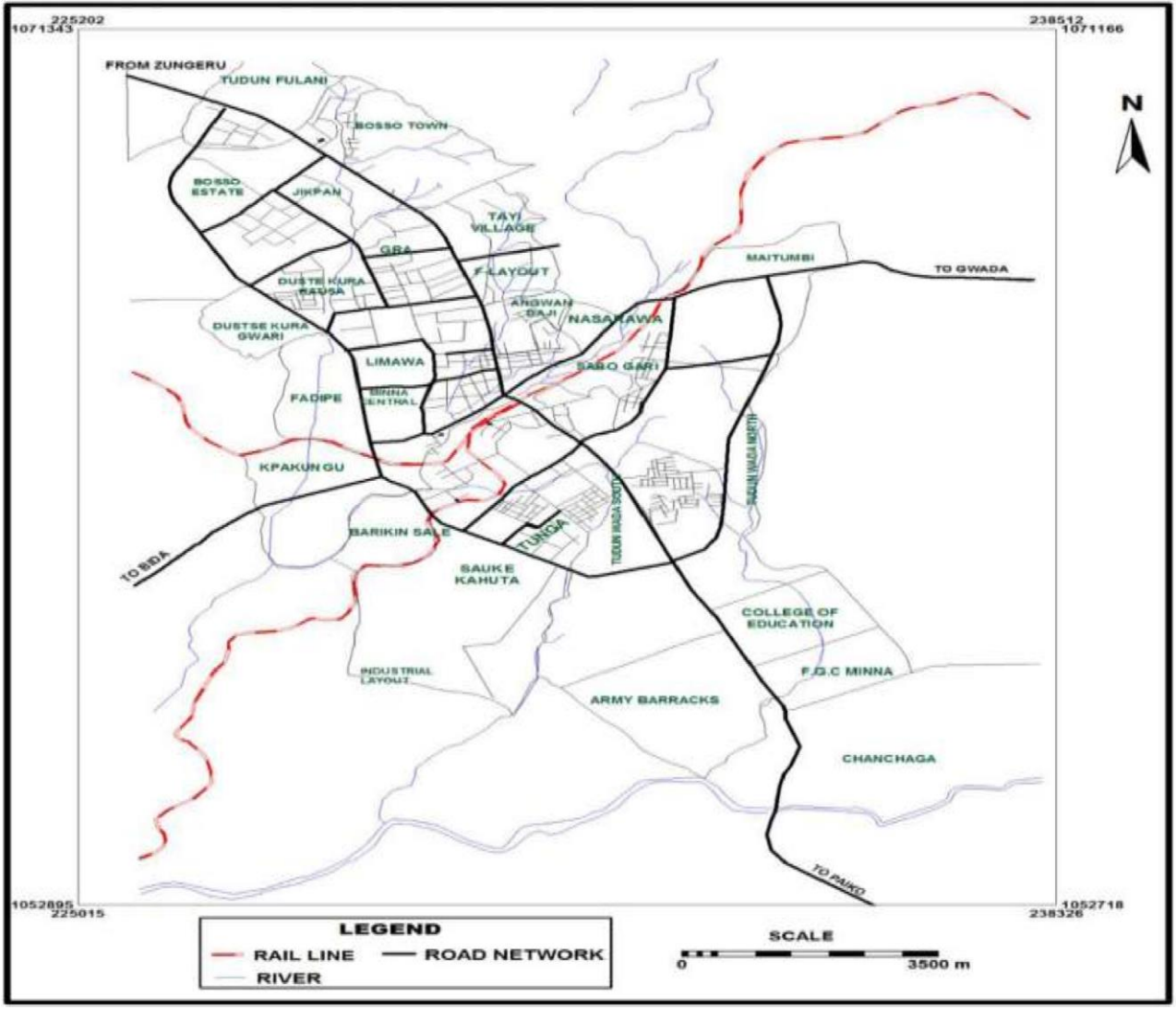


Fig 3.1: Locational Map of Minna showing the Sampling Sites (Source: Extract from Minna Metric Sheet (2015) as produced by Niger State Geographical Information System (NIGIS))

Healthcare facilities

Communities

3.5 Study Design

A cross sectional research (*i.e.* research that involves collecting data at one specific time) with observational/Quasi experimental design (which observes the study participants and measures variables without assigning any treatments or

design in which one or more key components of design is missing) was used for this research

(Levin, 2006).

3.6 Blood Sample Collection

Venipuncture technique as described by Epidi *et al.* (2008) was adopted-5ml of venous blood samples were taken using disposable syringes from children into a tube containing ethylene diamine tetra acetate (EDTA). Safety procedures was adopted by swabbing the area to be pricked with alcohol swab and then allowed to dry before collection. The tubes were properly labeled and transported to the laboratory in ice packs for further analysis.

3.7 Parasitological Examination

3.7.1 Thick and thin film

Thick and thin films of the blood samples were prepared as follows: for the thin film, a clean spreader slide was held at 45° angle, towards the drop of blood on the specimen slide until the blood spreads along the entire width of the spreader slide. The spreader slide was pushed forward rapidly and smoothly while it was held at the same angle. It was fixed with methanol and then stained with 10 % Giemsa stain pH 7.0 for 45 min. For the thick film, a drop of blood was spread in a circle like the size of a dime (diameter 1-2 cm) and was also stained with 10 % Giemsa stain pH

7.0 for 45 min after it was completely dry. The blood films were examined microscopically using X100 (oil immersion) objectives as described by Cheesbrough (2010).

3.7.2 Determination of parasite density

The parasite density was determined using the thick film while the parasite species and infective stage was identified using thin film. Parasite density per microliter of blood (parasitemia) was estimated from the thick film according to the method of counting parasites described by Trape (1985), based on the number of trophozoite parasites per 200 leukocytes on thick blood film with reference to participants white blood cell count. If gametocytes were seen, the count was extended to 500 leukocytes, this was then multiplied by an assumed WBC count of 8000.

Parasite Density/uL= (Number of parasite counted/ WBC counted) x 8000 /uL.

Parasitaemia was categorized as low (<1000 /μl), moderate (1001-10,000 /μl), high (>10,001 /μl) (Omalu, *et al.,* 2012), and hyper parasitemia (≥ 100,000) (Sumbele *et al.,* 2016).

3.8 Haematological Analysis

A complete blood count including values for white blood cell (WBC), red blood cell (RBC), platelet counts, haemoglobin concentration (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), were obtained using an autohaematology analyzer, the Beckman Coulter counter (URIT 3000), following the manufacturer's instructions.

3.9 Immunological Analysis

Measurement of Immunoglobulin (IgG) levels of antibodies against *P. falciparum* antigens was detected by performing a direct enzyme linked immunosorbent assay (ELISA) based on a protocol developed by Abcam. The Anti-Malaria ELISA kit ab178649 from abcam® was used following the manufacturer's instructions.

3.9.1 Reagent preparation for ELISA

All reagents, samples and controls were equilibrated to room temperature (18-25 ^{o}C) prior to use. 1X washing solution was prepared by diluting 20X washing solution with deionized water. In order to make 200 ml of the solution, 1X washing solution was combined with 10 ml of 20X washing solution with 190 ml deionized water and then mixed gently and thoroughly. In addition, all other solutions in the kit were supplied ready to use.

3.9.2 Sample preparation for ELISA

Before assaying, all samples were diluted 1:100 with IgG sample diluent. Sample (10 µL) was added to 990 µL IgG sample diluent to obtain a 1:100 dilution. It was mixed gently and thoroughly

3.9.3 Plate preparation for ELISA

The 96 well plate included with the kit were supplied ready to use, the plates were rinsed prior to adding reagents. A minimum of 1 well was left unused which served as a blank, here samples and conjugate were omitted from well addition

3.9.4 Principles of ELISA

The enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and color change to identify a substance. Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool for determining serum antibody concentrations. An ELISA assay is typically performed in a multi-well plate (96- or 384-wells).

Its basic principle is that antigens from the sample are attached to a surface. Then, a further specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate.

In addition, ELISA also called the double antibody sandwich technique, uses the following steps: Antibodies against the antigen to be measured are adsorbed to a solid support, in most cases a polystyrene microtiter plate. After coating the support with antibody and washing, the antigen is added and will bind to the adsorbed antibodies. Next, a conjugate that will also bind to the antigen is added. Conjugates are antibody molecules to which an enzyme is covalently bound (Clifton 1984).

3.9.5 Assay procedure for ELISA

i. Exactly 100 μL of sample was added into the wells. The plates were sealed with foil paper and incubated for 1 hour at room temperature.

ii. The seal was removed and the contents of the well were aspirated, each well was washed 3 times with 300 µL of 1X washing solution. Spill over into neighbouring well was avoided and the wash time between each wash cycle

was >5 sec. After the last wash, the remaining 1X washing solution was removed by decanting, the plate was inverted and then blotted against clean paper towels to remove excess liquid.

iii. A 100 µL of malaria anti-IgG conjugate was added into all wells except for the blank well. It was covered with foil and incubated for 30mins at room temperature.

iv. Step ii was repeated

v. A 100 µL of TMB (Tetramethyl benzidine) substrate solution was added into all wells and incubated at exactly 15 minutes at room temperature in the dark vi. Exactly 100 µL stop solution (0.16 M sulfuric acid) was added into all wells in the same order and at the same rate as for the TMB (Tetramethyl benzidine) substrate solution, until the blue colour developed during the incubation stage turned yellow vii. The absorbance of the specimen was measured at 450 nm within 30 minutes of the addition of stop solution

3.9.6 Calculation of IgG levels for ELISA

In order for an assay to be considered valid, the following criteria must be met:

i. Substrate blank: Absorbance value <0.100 ii.

Negative control: Absorbance value <0.200 and < cut-off

iii. Cut-off control: Absorbance value 0.150 - 1.300 iv.

Positive control: Absorbance value > cut-off

For the result calculation, the mean background subtracted absorbances for each sample was calculated and was compared to the mean cut-off control value. The cut-off control value was the mean absorbance value of the cut-off control wells.

$$\text{Results in Standard Units} = \frac{\text{Patients (mean) absorbance value} \times 10}{\text{Cut-off}} = \text{Standard Units}$$

3.9.7 Interpretation of results for ELISA

Samples were considered to give a positive signal, if the absorbance value was greater than 10 % over the cut-off value. Samples with an absorbance value of less than 10 % below the cut-off value were considered as negative.

3.10 Molecular Analysis for the Detection of *P. falciparum* MSP1 and MSP2 Genotypes

3.10.1 Principles of DNA extraction

DNA was extracted from clinical samples by using a lysis buffer (Buffer cartridge 2) to disrupt viral structure. The binding buffer contains guanidine thiocyanate, which act as a chaotropic agent. The binding buffer disassociated water molecules from nucleic acids and silica magnetic beads. This induced negative charge to nucleic acid and positive to silica magnetic beads. As a result, exposed DNA bind to the surface of beads. The washing buffer (Buffer catridge 1) rinses any impurities that may exist. The elution buffer dissolves pure DNA from the beads.

3.10.2 DNA extraction of *P. falciparum* antigens

ExiPrep^{TM}Dx Viral DNA/RNA kit version 3.0 (2017-02) by Bioneer Corporation was used for the extraction of *P. falciparum* DNA following the manufacturer's instructions. The ExiPrep^{TM}Dx Viral DNA/RNA kit are the total solution for the accurate and rapid extraction of DNA or RNA, delivering up to 16 extracted samples (including controls) within 1.5 hours from whole clinical samples.

i. Whole blood sample was transferred to a conical tube ii. The specimen was centrifuged at 3,000 rpm for 20 minutes and the supernatant were completely removed using a pipette iii. 1 ml of 1× PBS (Phosphate-buffered saline) buffer was added and vortexed for 30 seconds. The mixed solution was transferred into 1.5 ml microcentrifuge tube and centrifuged at 9,000 rpm for 5 minutes. The supernatant was completely removed by using a pipette and then mixed thoroughly with the addition of 400 µL of Re-suspension Buffer, 1× PBS or normal saline by using a pipette iv. The mixture was spinned down at 6,000 rpm for 5 seconds and the supernatant was used for ensuring nucleic acid extraction. For the nucleic acid extraction, 400 µL of the supernatant was needed on ExiPrep^{TM} 16 Dx v. The extracted DNA was loaded into the elution tubes and inserted into ExiPrep^{TM} 16 Dx following series of protocols as indicated by the manufacturer vi. After the nucleic acid extraction was completed the elution tube rack was removed from ExiPrep^{TM} 16 Dx

vii. The separation cover was removed according to protection cover separation tool utility method

3.10.3 Polymerase chain reaction (PCR) analysis

AccuPower®HotStart PCR PreMix by Bioneer was used according to the manufacturer's specifications. It was designed by Chemical-Mediated Hotstart method. The DNA polymerase was inhibited by pyrophosphate, but activated upon pyrophosphate hydrolysis by the thermostable pyrophosphatase. This prevents the formation of mis-primed products and primer-dimers during the reaction set up process resulting in improved PCR specificity.

3.10.3.1 Molecular identification using polymerase chain reaction (PCR)

Two polymorphic loci, MSP1 and MSP2, was used for the genotyping of the parasite population in this study. The regions of MSP1 and MSP2, which vary in repeat number and in adjacent sequence type, with three (MAD20, K1, RO33) and two (FC27, 3D7) allelic families, were analyzed by a nested PCR amplification. For the primary amplifications, outer primer pairs corresponding to the flanking sequence of the conserved regions of MSP1 and MSP2 was used. The second amplification reactions was based on the primary products using allelic-specific primers sets corresponding to K1, RO33 and MAD20 families of MSP1 and FC27, 3D7 families of MSP2. Primer solutions were thawed, the forward and reverse primer sequence used for this study are:

MSP1-A (AAG CTT TAG AAG ATG CAG TAT TGA C)

MSP1-B (ATT CAT TAA TTT CTT CAT ATC CAT C)

MSP2-A (ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA)

MSP2-B (ATA TGG CAA AAG ATA AAA CAA GTG TTG CTG)

FC27-A (GCA AAT GAA GGT TCT AAT ACT AAT AG)

FC27-B (GCT TTG GGT CCT TCT TCA GTT GAT TC)

3D7-A (GCA GAA AGT AAG CCT TCT ACT GGT GCT)

3D7-B (GAT TTG TTT CGG CAT TAT TAT GA)

K1-A (AAG AAA TTA CTA CAA AAG GTG CAA GTG)

K1-B (AGA TGA AGT ATT TGA ACG AGG TAA AGT G)

RO33-A (AGG ATT TGC AGC ACC TGG AGA TCT)

RO33-B (GAG CAA ATA CTC AAG TTG TTG CAA AGC)

MAD20-A (TGA ATT ATC TGA AGG ATT TGT ACG TCT TGA)

MAD 20-B (GAA CAA GTC GAA CAG CTG TTA)

3.10.3.2 Principles of PCR

PCR is a powerful amplification technique that can generate an ample supply of a specific segment of DNA (i.e., an amplicon) from only a small amount of starting material (i.e., DNA template or target sequence). It was first developed by Kary Mullis in the 1980s. It combines the principles of complementary nucleic acid hybridization with those of nucleic acid replication that are applied repeatedly through numerous cycles. It results in the exponential production of the specific

target DNA/RNA sequences by a factor of 10^7 within a relatively short period (Lorenz, 2012).

This in vitro amplification technique can amplify a single copy of nucleic acid target by using two synthetic oligonucleotide “primers” that bind to the target genomic sequence, which are extended by a Taq polymerase (a thermostable DNA polymerase). An automated process of repeated cycles (usually 25 to 40) of denaturation of the template DNA (at 94°C), annealing of primers to their complementary sequences (50°C), and primer extension (70°C) is employed for the amplification of target sequence (Lorenz, 2012).

3.10.3.3 Procedure for PCR

i. Exactly 10 µL of each primer was diluted with 90 µL of deionized water

ii. The diluted constituent explained above for both forward and reverse primers were added together totaling 200 µL of the mixture iii. This appropriate volume of diluted primer mix was distributed into each

PCR premix tubes as follows:

2 µL template DNA was added to the individual PCR tubes

2 µL primers constituent in ii above was then added to each tube 16 µL distilled water was added to the tubes until the total volume of the mixture becomes 20 µL. The lyophilized blue pellet was dissolved by vortexing and spin-down

NB: each pre-mix tube can only contain 20 µL PCR reactions with a final concentration of 1.5 mM $MgCl_2$, 250 µM of dNTP iv. Biorad thermal cycler was used and programmed according to the

manufacturer's instruction.

v. The PCR tubes were placed in the thermal cycler and the cycling program started.

vi. The first round of PCR reaction (primary PCR) was done with a mixture of forward and reverse reaction for both MSP1 and MSP2 with 2 positive (one for each) and negative controls with the following cycling conditions: initial denaturation for 5 minutes at 95 °C, then followed by 30 cycles of denaturing at 94 °C for 1 minute, annealing at 61 °C for 45 seconds, and extension cycle of 72 °C for 1.5 minute, and a final extension at 72 °C for 5 minutes (Ridzuan *et al*., 2016).

vii. The cycling conditions for nested PCR (second round) of MSP1 and MSP2 started with a single step of denaturation at 95 °C for 5 minutes followed by

30 cycles of denaturation at 94 °C for 1 minute, annealing temperature at 57 °C for 45 seconds for MSP2 allelic families (FC27 and 3D7); 62 °C and 58 °C annealing for 45 seconds for (K1, MAD20) and RO33 of MSP1 allelic families, respectively. This was then followed by an extension at 72 °C for 1.5 minute, and a final extension at 72 °C for 5 minutes (Ridzuan *et al*., 2016). Positive controls and negative controls were incorporated in each set of reaction. The parasite DNA from the *P. falciparum* isolates was analyzed for MSP1 and MSP2 genes.

3.10.3.4 Electrophoresis of the PCR product

A mixture of 2 % agarose powder (Nsobya *et al.*, 2004; Abdel Hamid *et al.*, 2013) in 100ml 1× TAE (Tris Acetate) buffer was made and then placed in a microwave

till it properly dissolved. It was left to cool and then stained with 10-12 µL ethidium bromide before pouring into a gel cast and to solidify, the combs were removed to form wells. Eight microliter of the ladder constituent and PCR products were loaded each, and the solidified agarose gel was gently removed and inserted in gel documentation system (E.C Apparatus Corporation) at 103 volts for 35 minutes and visualized by UV trans-illumination through a computer. The number and size of resulting DNA bands was analyzed using gene ruler (Abdel Hamid, *et al.*, 2013).

Alleles were considered the same if fragment sizes were within 10 bp (Gosi *et al.*, 2013) and 50 bp (Mwingira *et al.*, 2011) intervals for MSPs.

3.11 Estimation of Multiplicity of Infection (MOI) of *P. Falciparum* Antigens

The mean multiplicity of infection was estimated by dividing the total number of distinct MSP1 or MSP2 genotypes detected, by the number of positive samples detected by the marker. In other words, the number of genotypes per infection, or the multiplicity of the infection (MOI) was estimated by dividing the total number of fragments detected in the individual system by the number of samples positive in the particular system (either MSP1 or MSP2) and was also estimated by the average number of PCR fragments per infected individual, as described previously (Zwetyenga *et al*., 1998). Individual Isolates with more than one genotype were considered as polyclonal infection while the presence of a single allele was considered as monoclonal infection.

$$\text{MOI} = \frac{\text{No. of fragment}}{\text{Total no. of samples positive}}$$

3.12 Data Analysis

Data analysis was performed using SPSS version 23.0 statistical software programs. Descriptive statistics was used for calculation of mean and standard deviation values. Correlations were calculated by using Spearman correlation test for the relationships in age, sex, parasite density and haematological values. Differences in proportions of age, sex and parasite density were evaluated using Pearson chisquare. The attributable risk of anaemia due to malaria was determined using $[(n_1\ m_0 - n_0\ m_1) / n\ (n_0 + m_0)] \times 100$, where n_0 = anaemic children without malaria and n_1 = anaemic children with malaria, whereby $n_0 + n_1 = n$, m_0 = non-anaemic children without malaria, and m_1 = non-anaemic children with malaria, whereby $m_0 + m_1 = m$.

Non-parametrical test, Mann-Whitney *U* test statistic and Kruskall-Wallis *H* were used for analysis of level of antibody responses between two (sex) and multiple (age, parasite density) independent variables, respectively. Spearman's rank correlation coefficients was calculated to assess association between multiplicity of infection and parasite densities. The MSP1 and MSP2 allele frequencies were expressed as percentages. The proportions of alleles observed in each gene among the healthcare facilities and the studied communities was compared using the Chisquare test. The frequency of polyclonal infection was calculated using number of samples with more than one amplified fragment out of the total samples and a monoclonal infection produces a single amplified fragment per allele. Isolates with more than one allele of one or both genes were considered as a poly-allelic infection while the presence of a single allele was considered as single allelic infection. The correlation between multiplicity of infection (MOI) and percentages of *P. falciparum* positive cases were analyzed by using Spearman's correlation.

To determine allele frequencies, only the predominant allele was counted for population genetic analyses. The genetic diversity in each population was assessed by calculating both the mean number of alleles (A) and the mean expected heterozygosity (*He*) across loci in each population. Briefly, the allelic diversity (*He*) for each microsatellite loci or antigenic markers was calculated based on the allele frequencies, using the formula $He = n - \sum(1 - p)^2 \div (n - 1)$ where n is the number ofisolates analyzed and *p* represents the frequency of each different allele at a locus. *He* has a potential range from 0 (no allele diversity) to 1 (all sampled alleles are different) Abd Razak *et al.* (2016). The sample size was estimated using the sample size formula $\frac{\frac{z^2 \times p(1-p)}{e^2}}{1+\frac{\left[z^2 \times p(1-p)\right]}{e^2 N}}$ where z^2= *z*-score for 95 % *i.e.* 1.96; *p* = standard of

deviation 0.5 (50 %); *e* = margin of error 95 %; N = population size. The *P* value less than 0.05 was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Parasitological analysis of *P. falciparum* among children in Minna

4.1.1.1 Prevalence of *P. falciparum* among children from different healthcare facilities in Minna

A total of 316 (100 %) blood samples were screened for the presence of *P. falciparum*, comprising of 69 (21.83 %) from apparently healthy children from the community and 247 (78.16 %) from children attending the outpatients department of various healthcare facilities within the study areas as presented in Table 4.1.1. Of the 316 blood samples screened, 238 (75.37 %) were infected with *P. falciparum* which comprises of 39 (16.39 %) from apparently healthy children from the communities and 199 (83.61 %) from children with signs of fever attending the outpatient's departments of the six healthcare facilities. From this, General hospital, Minna had the highest infection rate of *P. falciparum* n=113 (87.57 %), followed by PHC, Kpakungu 8 (80.00 %) while Tunga had the least prevalence of n=21 (63.34 %). In general, a high prevalence rate of *P. falciparum* was observed in samples collected from all healthcare facilities as compared to samples collected from the community.

Analysis using the Mann-Whitney U test revealed a non-significant difference between *P. falciparum* infected and non-infected children from the study population (U=8125.50, *P*=0.084). However, Kruskal-Wallis H test was used for variation between the *P. falciparum* infections of the various sample collection sites and a significant difference was observed between *P. falciparum* infections and the various sample collection sites, χ^2 =27.385, *P*= 0.01 with a mean rank *P. falciparum* parasite of 128.80 for samples collected from the community, 177.90

for samples collected from General Hospital, Minna, 140.05 for Tunga PHC, 158.00 FSP,

165.90 for Kpakungu PHC, 141.07 for Gwari Road PHC, and 164.71 for Old airport PHC.

Table 4.1.1: Prevalence of *P. Falciparum* Infection among Children from Six Healthcare Facilities and Communities in Minna

	P. falciparum	
Study sites	**No. exam.**	**No. +ve (%)**
Res. Communities	69	39 (56.52)
General hospital	129	113 (87.59)
Tunga PHC	33	21 (63.63)
FSP Limawa	8	6 (75.00)
Kpakungu PHC	10	8 (80.00)
Gwari Road PHC	14	9 (64.29)
Old Airport PHC	53	42 (79.25)
Total	**316**	**238 (75.31)**

χ^2 = 27.385; *P*= 0.01 +ve

= positive

Res. = Residential

PHC = Primary Healthcare

FSP = Family Support Program

4.1.1.2: *Plasmodium falciparum* density of infected cases from the study sites

The *P. falciparum* density in children from different sampling collection sites is presented in Table 4.1.2. The result of the parasite density revealed an overall prevalence of 238 (75.32 %) from the total population sampled. *P. falciparum* parasite density was rated as low (<1000 /μl), moderate (1001-10,000 /μl) and high (>10,001 /μl). From the *P. falciparum* infected cases, it was deduced that samples collected from FSP Limawa and Tunga PHC observed a high prevalence of 3 (50.00 %) and 9 (42.86 %), respectively for cases with low parasite density. Meanwhile, cases with moderate parasite density were more prevalent for all the study sites.

However, only cases from general hospital recorded a prevalence rate of 11 (9.73 %) for high parasite density. The overall prevalence of parasite density revealed that outpatients with moderate parasite density had the highest parasitemia of 161 (67.65 %), followed by outpatients with low parasite density with 66 (27.73 %) while outpatients with high parasite density was observed with the least parasitaemia of 11 (4.62 %). However, high parasite density was not recorded from samples collected from apparently healthy children from the community. A significant correlation was observed (r =0.857, p =0.016) between the presence of *P. falciparum* and the intensity of the parasite. In addition, a significant correlation was also recorded between the parasite densities of all the healthcare facilities (r =0.025, p =0.055) at P<0.05.

Table 4.1.2: Parasite Density of Infected Cases among Children from Six Healthcare Facilities and Communities in Minna

Study sites	Sample size (n)	Parasite Density		
		Low PD (%) (<1000 /μl)	Mod PD (%) (1001-10,000 /μl)	High PD (%) (>10,001 /μl)
Res. Communities	39	10 (25.64)	29(74.36)	0 (0.00)
General Hospital	113	29 (25.66)	73 (64.60)	11 (9.73)
Tunga PHC	21	9 (42.86)	12 (57.14)	0 (0.00)
FSP Limawa	6	3 (50.00)	3 (50.00)	0 (0.00)
Kpakungu PHC	8	3 (37.50)	5 (62.50)	0 (0.00)
Gwari Road PHC	9	2 (22.22)	7 (77.78)	0 (0.00)
Old Airport PHC	42	10 (23.81)	32 (76.19)	0 (0.00)
Total	**238**	**66 (27.73)**	**161 (67.65)**	**11 (4.62)**

r =0.025, p =0.055 +ve

= positive

Res. = Residential

PHC = Primary Healthcare

FSP = Family Support Program

4.1.1.3 Prevalence of *Plasmodium falciparum* in relation to age and sex among children in Minna

The prevalence of *P. falciparum* parasite, with respect to sex and age group, among children in Minna is presented in table 4.1.3. Out of the 316 blood samples analyzed from the studied population, 232 (75.31 %) were infected with *P. falciparum* and the remaining 78 (24.68 %) were negative. Majority of the samples collected 139 (43.98 %) were from children age 6 months to 5 years with a *P. falciparum* prevalence of 102 (42.85 %). Between the ages 6 to 11 years, 82 (34.45 %) were infected with the *P. falciparum*. The lowest parasite prevalence was observed in children between ages 12-17 years with 42 (17.64 %). As well, the total number of males recorded was 181(57.27 %) with 126 (69.61 %) infected with *P. falciparum*. The remaining 135 (42.72 %) which were females recorded *P. falciparum* prevalence of 112 (82.96 %).

Malaria was more prevalent in children below the age of 5 with 102 (42.85 %) and observed most in the male category with 56 (44.44 %). The Male category had the highest prevalence rates of *P. falciparum* infection in all age groups except for the age group 12 to 17 years and the control group (>18 years), for which the females had a higher prevalence of 23 (20.53 %) and 6 (5.35 %) as against the 19 (15.07 %) and 6 (4.76 %) in the male category, respectively.

Data analysis shows that age was not a contributory factor to the presence of *P. falciparum* infection, in other words there was no significant difference (χ^2 =2.298, p=0.513) between the age of child and *P. falciparum* infection at (p >0.05). Pearson correlation was also used to determine the association between age and parasite prevalence (r =0.082, p = 0.148) and it revealed a non-significant weak

correlation between the age of child and positive *P. falciparum* cases ($p>0.05$). On the other hand, significant difference ($p<0.05$) was observed between sex and *P. falciparum* parasite ($\chi^2= 7.413$, $p = 0.006$) and a significant positive correlation ($r = 0.152$, $p =$

0.006) was also observed between the sex and *P. falciparum*.

Table 4.1.3: Prevalence of *P. falciparum* in Relation to Age and Sex among Children from Six Healthcare Facilities and Communities in Minna

	Overall		Male		Female	
Age	**Sample size (n)**	**No. +ve (%)**	**No. exam.**	**No. +ve (%)**	**No. exam.**	**No. +ve (%)**
6 mnths-5 yrs	139	102(42.85)	80	56(44.44)	59	46(41.07)
6 yrs-11 yrs	112	82(34.45)	70	45(35.71)	42	37(33.03)
12 yrs-17 yrs	50	42(17.64)	24	19(15.07)	26	23(20.53)
>18 yrs(control)	15	12(5.04)	7	6(4.76)	8	6(5.35)
Total	**316**	**238(75.31)**	**181**	**126(39.87)**	**135**	**112(35.44)**

Age χ^2 =2.298, P=0.513 Sex χ^2= 7.413, P = 0.006

4.1.1.4: Prevalence of *P. falciparum* density by age and sex among children in Minna

From the overall total of 238 positive *P. falciparum* cases recorded, 66 (27.73 %) had low parasite density, 161 (67.64 %) with moderate parasite density and 11 (4.62 %) recorded a high parasite density. *P. falciparum* cases with low, moderate and high parasite density was generally observed to decrease with age as presented in Table 4.1.4. *P. falciparum* was recorded at its peak in cases with moderate parasite density with 161 (67.64 %) and this was majorly found between the cohorts of 6 months to 5 years 66 (27.73 %). The lowest occurrence of parasite density was observed in cases with high parasite density with 11 (4.62 %). Males, especially those below the age of 5 recorded the highest parasite density of 56 (23.52 %). Meanwhile, hyper parasitemia was not recorded. Significant difference was not observed ($p > 0.05$) between parasite density and age of children, and also between parasite density and sex ($\chi^2=5.301$, $p = 0.807$ and $\chi^2=6.898$, $p = 0.075$, respectively).

Table 4.1.4: Prevalence of *P. Falciparum* Density in Relation to Age and Sex among Children from Six Healthcare Facilities and Communities in Minna

Sex	**Overall**			**Male**			**Female**		
Age/PD	**Low PD** (%)	**Mod PD** (%)	**High PD** (%)	**Low PD** (%)	**Mod PD** (%)	**High PD** (%)	**Low PD** (%)	**Mod PD** (%)	**High PD** (%)
6 mnths-5 yrs	31(13.02)	66(27.73)	5(2.10)	14(5.88)	39(16.38)	3(1.26)	17(1.26)	27(11.34)	2(0.84)
6 yrs-11 yrs	22(9.24)	57(23.94)	3(1.26)	14(5.88)	30(12.60)	2(0.84)	9(3.78)	27(11.34)	1(0.42)
12 yrs-17 yrs	9(3.78)	30(12.60)	3(1.26)	5(2.10)	13(5.46)	1(0.42)	4(1.68)	17(7.14)	2(0.84)
>18 yrs(control)	4(1.68)	8(3.36)	0(0.00)	3(1.26)	3(1.26)	0(0.00)	1(0.42)	5(2.10)	0(0.00)
Total	**66(27.73)**	**161(67.64)**	**11(4.62)**	**36(15.12)**	**82(34.45)**	**6(2.52)**	**31(13.02)**	**73(30.67)**	**5(2.10)**

Age χ^2=5.301, *P*= 0.807 Sex χ^2=6.898, *p*= 0.075

PD = Parasite density

Mod = Moderate

4.1.2 Haematological Analysis

4.1.2.1 Effect of *P. falciparum* on indices of anaemia among children in Minna

Indices of anaemia of haemoglobin, haematocrit, red blood cell count, mean cell volume, mean cell haemoglobin and mean corpuscular haemoglobin concentration was presented in Table 4.1.5. Cases with low levels of Hb, Hc, RBC and MCHC were observed to be higher than cases with normal levels with 205 (65.00 %), 194 (61.40 %), 216 (68.35 %) and 258 (81.64 %), respectively; however, cases with low levels of MCV and MCH recorded lower levels as compared to the normal range with 23 (9.30 %) and 94 (27.00 %), respectively. *P. falciparum* infected cases with low haemoglobin levels recorded 158 (77.00 %). Similarly, 150 (77.30 %), 18 (78.00 %), 74 (78.70%), 198 (76.74 %) and 163 (75.46 %) cases of low levels of Hc, MCV, MCH, MCHC and RBC were also observed in *P. falciparum* infected cases, respectively.

Furthermore, most *P. falciparum* positive cases recorded values lower and sometimes higher than the reference values as compared to negative *P. falciparum* cases. The mean and standard deviation (Mean ± SD) observed for each indices were 11.54±2.79, 35.13±8.34, 81.37±13.87, 22.95±3.70, 28.53±5.11 and 5.49±1.54 for Hb, Hc, MCV, MCH, MCHC and RBC, respectively.

The mean values for Hb, Hc, MCV, MCHC and RBC with low levels were significantly higher than samples with the normal levels (Hb χ^2=101.77, p =0.488; Hcχ^2= 55.65, p=0.093; MCV χ^2=64.329, p =0.361; MCHCχ^2=25.25, p =0.337 and RBC χ^2=49.70, p =0.773). However, the mean values of MCH in cases of low MCH, was significantly lower than samples with normal MCH (χ^2=34.388, p=0.045).

Table 4.1.5: Effect of *P. falciparum* on Indices of Anaemia among Children from Six Healthcare Facilities and Communities in Minna

		P. falciparum		
Anaemia indices	**Sample size (n)**	**No. -ve (%)**	**No. +ve (%)**	**Mean ±SD**
Normal Hb	111 (35.00)	31 (28.00)	80(72.00)	
Low Hb	205 (65.00)	47 (23.00)	158 (77.00)	
Total (%)	316	78 (24.68)	238(75.32)	11.54±2.79
Hb (g/dL) range	2.9-19.2	4.9-17.9	2.9-19.2	
Normal Hc	122 (38.60)	34 (28.00)	88 (72.00)	
Low Hc	194 (61.40)	44 (22.70)	150 (77.30)	
Total (%)	316	78 (24.68)	238 (75.32)	35.13±8.34
Hc (%) range	17-60	17-54	12-60	
Normal MCV	224 (90.70)	63 (28.00)	161 (72.00)	
Low MCV	23 (9.30)	5 (22.00)	18 (78.00)	
Total (%)	247	68 (27.54)	179 (72.45)	81.37±13.87
MCV (fL) range	0.9-18.0	3.3-9.1	0.9-18.0	
Normal MCH	180 (60.00)	53 (29.44)	127 (70.56)	
Low MCH	94 (27.00)	20 (21.30)	74 (78.70)	
High MCH	42 (13.00)	5 (12.00)	37 (88.00)	

Total (%)	316	78 (24.68)	238 (75.32)	22.95±3.70
	19-125	60-107	19-125	
MCH (pg/cell) range				
Normal MCHC	52 (16.45)	15 (29.00)	37 (71.00)	
Low MCHC	258 (81.64)	60 (23.26)	198 (76.74)	
High MCHC	6 (1.90)	3 (50.00)	3 (50.00)	
Total (%)	316	78 (24.68)	238 (75.32)	28.53±5.11
MCHC (g/dL) range				
	11-40	16-40	11-35	
Normal RBC	90 (28.48)	22 (24.44)	68 (75.56)	
Low RBC	216 (68.35)	53 (24.54)	163 (75.46)	
High RBC	10 (3.16)	3 (30.00)	7 (70.00)	
Total (%)	316	78 (24.68)	238 (75.32)	5.49±1.54
RBC (cells/mcL) range	10-81	20-48	10-81	

Normal haemoglobin Hb: 13-17 g/dL for male and 12-16 g/dL for female

Normal haematocrit Hc: 40-54 % for male and 35-47 % for female

Normal red blood cell RBC: 4.0-7.0 cells/mcL for male and 3.6-6.0 cells/mcL for female

4.1.2.2 Effect of *P. falciparum* on indices of anaemia among children from six healthcare facilities and communities across Minna

The effect of the presence or absence of *P. falciparum* infection on anaemia indices is presented on Table 4.1.6. It was generally observed that *P. falciparum* infected cases experienced more anaemia prevalence (low Hb, low Hc and Low RBC counts) in all the healthcare facilities and the communities. Only 24 cases (7.59 %) of low RBC count was recorded with 19 (79.16 %) infected with *P. falciparum.* Meanwhile, General hospital Minna recorded the highest prevalence of *P. falciparum* infected anaemia cases in low haemoglobin concentration, low haematocrit level and low RBC count with 88 (42.92 %), 87 (44.84 %) and 8 (33.33 %), respectively.

It was evident from this study that *P. falciparum* positive samples with low haematocrit level was significantly higher than *P. falciparum* positive cases with low Hb levels and low RBC count using Kruskal Wallis test (Hb χ^2=3.02, p=0.08; Hc χ^2= 3.71, p=0.05; RBC χ^2=1.419, p=0.234).

In addition, regression analysis was used to determine and quantify the association between anaemia and Parasite Density PD. The reference category was high parasite density of >10,000 g/uL. Odd Ratio OR (95 % CI) for anaemia among participants with low PD were 0.543 (0.14539.506) and 0.323 (0.250-67.438) for Hb and Hc anaemia, respectively, compared to high PD; while the OR (95 %) among the moderate PD were 0.646 (0.122 - 29.746) and 0.370 (0.226

- 54.468) for Hb and Hc anaemia, respectively, compared to high PD. The multinomial logistic regression model demonstrated that *P. falciparum* is not a significant predictor to anaemia.

The attributable risk of anaemia by malaria in this study was observed in low haemoglobin anaemia as 7.12 % which was lower than the low haematocrit anaemia with 8.11 %. In addition, the attributable risk of anaemia in relation to the moderate parasite density were 8.26 % and 8.33 % for both low hemoglobin and low haematocrit anaemia respectively.

Table 4.1.6: Effect of *P. falciparum* on Indices of Anaemia among Children from Six Healthcare Facilities and Communities across Minna

		Low haemoglobin concentration			Low haematocrit levels			Low red blood cell count		
Study sites	**No. exam.**	**-ve (%)**	**+ve (%)**	**Total (%)**	**-ve (%)**	**+ve (%)**	**Total (%)**	**-ve (%)**	**+ve (%)**	**Total (%)**
Res. Communities	69	20(9.75)	25(12.19)	45(21.95)	17(8.76)	18(9.27)	35(18.04)	2(8.33)	1(4.16)	3(12.50)
General Hospital	129	13(6.34)	88(42.92)	101(49.26)	13(6.70)	87(44.84)	100(51.54)	0(0.00)	8(33.33)	8(33.33)
Tunga PHC	33	8(3.90)	15(7.31)	23(11.21)	8(4.12)	15(7.73)	23(11.85)	1(4.16)	2(8.33)	3(12.50)
FSP Limawa	8	0(0.00)	3(1.46)	3(1.46)	0(0.00)	4(2.06)	4(2.06)	0(0.00)	1(4.16)	1(4.16)
Kpakungu PHC	10	2(0.97)	6(2.92)	8(3.90)	2(1.03)	6(3.09)	8(4.12)	0(0.00)	3(12.50)	3(12.50)
Gwari Rd PHC	14	1(0.48)	3(1.46)	4(1.95)	0(0.00)	2(1.03)	2(1.03)	0(0.00)	1(4.16)	1(4.16)
Old airport PHC	53	3(1.46)	18(8.78)	21(10.24)	4(2.06)	18(9.27)	22(11.34)	2(8.33)	3(12.50)	5(20.83)
Total	**316**	**47(22.92)**	**158(77.07)**	**205(64.87)**	**44(22.68)**	**150(77.31)**	**194(61.39)**	**5(20.83)**	**19(79.16**	**24(7.59)**

+ve = positive -ve
= negative
Res. = Residential
PHC = Primary Healthcare
FSP = Family Support Program

4.1.2.3 Degree of anaemia in *P. falciparum* infected children in Minna

The degree of anaemia in *P. falciparum* positive and negative cases from the study population has showed that only 2 cases (0.63 %) of severe anaemia was observed in *P. falciparum* negative blood samples, as presented in Table 4.1.7. Moderate and mild anaemia were recorded with 10 cases (3.16 %) and 34 cases (10.75 %), respectively in *P. falciparum* negative blood samples. On the contrary, *P. falciparum* positive samples showed the highest prevalence in all the degree of infection with the highest prevalence observed in mild anaemia 103 cases (32.59 %), followed by moderate anaemia with 43 cases (13.60 %) and the least was observed in severe anaemia with 11 cases (3.48 %). Meanwhile, more than 111 cases (35.00 %) of the entire study population had normal Hb levels with 80 cases (25.31 %) of *P. falciparum* infected samples and 31 cases (9.81 %) of negative samples. Interestingly, one case each 0.32 % for both *P. falciparum* infected, and negative blood sample recorded greater than the normal Hb levels. There was however, no significant correlation (r= 0.026, p= 0.644) between the different intensities/degrees of anaemia and *P. falciparum* infection.

4.1.2.4 Prevalence of anaemia subtypes in *P. falciparum* infected children in Minna

P. falciparum positive normocytic hypochromic anaemia recorded the highest of 86 (27.21 %) followed by macrocytic hypochromic, microcytic hypochromic and microcytic normochromic anaemia type with 36 (11.39 %), 31 (9.81%) and 30 (9.49 %). The least prevalence rates of anaemia infected with *P. falciparum* were found in macrocytic hyperchromic, macrocytic normochromic, microcytic hyperchromic and normocytic hyperchromic with 1 (0.31 %), 6 (1.89 %), 7 (2.21 %) and 10 (3.16 %), respectively. It was observed that the normochromic anaemia were the most prevalent types while the hyperchromic types showed low prevalent rates.

In general, normocytic anaemia was observed as 180/316 (56.96 %) from the total population, out of which, 59/180 (32.77 %) were not infected with *P. falciparum* while the remaining 121/180

(67.22 %) were infected with *P. falciparum*. Likewise, in macrocytic anaemia, a total of 51/316 (16.13 %) from the total population was observed with anaemia and from which, only 8/51 (15.68 %) were negative to *P. falciparum* while the remaining 43/51 (84.31 %) were infected with *P. falciparum*. In addition, microcytic anaemia showed a well-defined picture of 84/316 (26.58 %) from the total population.

From this, 16/84 (19.04 %) were negative to *P. falciparum* parasite while the remaining 68/84 (80.95 %) were infected with *P. falciparum*. Furthermore, *P. falciparum* positive normocytic normochromic anaemia had a prevalence of 25 (7.91 %) and this is the anaemia that occurs due to acute malaria as presented on Table 4.1.8.

Significant difference $p<0.05$ was observed between *P. falciparum* positive cases and the anaemia subtypes ($\chi^2 = 17.411$, $p = 0.043$) and a significant, weak correlation exists between them ($r = 0.151$, $p = 0.007$). In addition, significant differences was not observed between parasite density of *P. falciparum* parasite and the different anaemia subtypes ($\chi^2 = 35.833$, $p = 0.119$) while a significant, weak correlation was observed between them ($r= 0.171$, $P= 0.002$).

Table 4.1.7: Degree of Anaemia in *P. falciparum* Infected Children from Six Healthcare Facilities and Communities in Minna

		***P. falciparum* parasite infection**	
Anaemia severity	**No. exam.**	**No. -ve (%)**	**No. +ve (%)**
Normal (12/13-16/17 g/dL)	111	31(9.81)	80(25.31)
Mild anaemia (10-12 g/dL	137	34(10.75)	103 (32.59)
Moderate anaemia (7.0-10.0 g/dL)	53	10(3.16)	43 (13.60)
Severe anaemia (< 7.0 g/dL)	13	2(0.63)	11 (3.48)
High anaemia (>16/17 g/dL	2	1(0.32)	1 (0.32)
Total	**316**	**78(24.68)**	**238 (75.31)**

r= 0.026, p= 0.644

Table 4.1.8: Prevalence of Anaemia Subtypes in *P. falciparum* Infected Children from Six Healthcare Facilities and Communities in Minna

		P. falciparum	
Anaemia subtypes	**No. exam.**	**No. -ve (%)**	**No. +ve (%)**
Normocytic hypochromic	133	47 (14.87)	86 (27.21)
Normocytic hyperchromic	12	2 (0.63)	10 (3.16)
Normocytic normochromic	35	10 (3.16)	25 (7.91)
Macrocytic hypochromic	44	8 (2.53)	36 (11.39)
Macrocytic hyperchromic	1	0 (0.00)	1 (0.31)
Macrocytic normochromic	6	0 (0.00)	6 (1.89)
Microcytic hypochromic	37	6 (1.89)	31 (9.81)
Microcytic hyperchromic	7	0 (0.00)	7 (2.21)
Microcytic normochromic	40	10 (3.16)	30 (9.49)
Total	**316**	**84 (26.58)**	**232 (73.41)**

$\chi^2 = 35.833$, $p = 0.119$

+ve = positive

-ve = negative

4.1.3 Immunological analysis of *P. falciparum* antigen using ELISA

4.1.3.1 Seroprevalence of antibody IgG response to *P. falciparum* antigen

From the ninety-three 93 samples screened by enzyme linked immuno sorbent assay (ELISA) for the measurement of Immunoglobulin IgG levels, there was a high seroprevalence of antibodies in general against *P. falciparum* antigens tested with 69 (74.20 %). The frequency of a non-reactive response to an antigen was 17(18.27 %) *i.e.* there was no production of antibody, which was an indication of the absence of *P. falciparum.* Furthermore, an indeterminate group- the grey zone, recorded 7 (7.52 %) as presented on Table 4.1.9. This effect could be as a result that either the patients were recently infected with the *P. falciparum* pathogen or they possess the low strength of the pathogen (Singh *et al.,* 2015). The mean levels of IgG antibodies against *P. falciparum* was

35.32±23.01.

Table 4.1.9: Seroprevalence of IgG Antibody Response to *P. falciparum* Antigen

Antibody IgG response	**Seroprevalence (%)**
Non-reactive <9 %	17 (18.27)
Grey zone 9-11 %	7 (7.52)
Positive >11 %	69 (74.19)
Total	**93 (100.00)**

4.1.3.2 Seroprevalence of IgG antibody response to *P. falciparum* antigen in relation to *P. falciparum* microscopy detection

From the total of 93 samples exposed to ELISA, 84 (90.32 %) of the samples were infected with *P. falciparum* and about 9 (9.67 %) of the samples were tested negative with microscopy. Interestingly, of the 9 cases (9.67 %) tested negative with microscopy, more than half of the samples (5) produced IgG antibodies against the antigens. In contrast, as seen in Table 4.1.10, cases with *P. falciparum* infection recorded higher specific antibody IgG responses 64 (68.81 %) as compared to negative *P. falciparum* cases, suggesting a strong relationship between production of specific antibodies and *P. falciparum* transmission, rather than protective immunity. There was no statistical significant difference and a non-significant negative correlation between the negative and positive *P. falciparum* parasite and the production of antibody IgG as measured by ELISA (χ^2=0.896, p =0.979; r = -0.028, p = 0.793), respectively. However, analysis using the MannWhitney U test revealed a non-significant difference of IgG antibody response to *P. falciparum* infection between negative *P. falciparum* cases and *P. falciparum* infected cases (U=304.00, p=0.816).

Table 4.1.10: Seroprevalence of IgG Antibody Response to *P. falciparum* Antigen in Relation to *P. falciparum* Microscopy Detection

Antibody IgG response	**No. exam.**	***P. falciparum* negative (%)**	***P. falciparum* positive (%)**
Non-reactive (<9 %)	17	3(3.22)	14(15.05)
Grey zone (9-11 %)	7	1(1.07)	6(6.45)
Positive (>11 %)	69	5(5.37)	64(68.81)
Total	**93**	**9(9.67)**	**84(90.32)**

χ^2=0.896, p =0.979; r = -0.028, p = 0.793

4.1.3.3 Seroprevalence of antibody IgG response to *P. falciparum* antigen according to sex and age

It was recorded from this study for both male and female category, prevalence of reactive samples *i.e.* samples that produced IgG antibody against *P. falciparum* antigen, were quite similar with 35 (37.63 %) and 34 (36.55 %), respectively. The highest seroprevalence was observed in children below the age of five, 24 (25.80 %), most especially the male 14 (15.05 %) as against the female category with 10 (10.75 %). The frequency of a non-reactive response to an antigen was 17 (18.27 %) with 8 (8.60 %) from male and 9 (9.67 %) from the female categories. Furthermore, an indeterminate group- the grey zone also recorded a total 7 cases (7.53 %) with male category having 2 (2.15 %) and female category with 5 (5.37 %). This effect could be as a result that either the patients were recently infected with the *P. falciparum* pathogen or they possess the low strength of the pathogen (Singh *et al.,* 2015). In general, 69 (74.19 %) produced IgG antibody against *P. falciparum* antigen and about 17 (18.27 %) non-reactive *i.e.* no production of antibody against *P. falciparum* as presented on Table 4.1.11.

Differences in antibody response were not significant for both male and female (p >0.05) and there was no significant correlation between the sexes and the presence of IgG as measured by ELISA (χ^2= 1.264, p = 0.532; r = 0.050, p = 0.632). Analysis using the Mann-Whitney U test, however, revealed IgG antibody response to *P. falciparum* infection in the male was non-significant to the female category (U=741.50, p =0.500). In addition, the differences in the seroprevalence of *P. falciparum* antibodies in response to the antigen was not significant (p >0.05) with age and no significant association exists between them (χ^2= 6.740, p = 0.346; r = 0.034, p = 0.750). Furthermore, Kruskal-Wallis H test was used for variation between the age groups and it showed a non-significant difference in IgG antibody response between the age

groups, χ^2 =3.370, p= 0.338, with mean rank parasite densities of 37.53 for age group6 months to 5 years, 44.36 for age group 6 to 11 years, and 47.20 for age group 12 to 17 years.

Fig 4.1.1a shows a boxplot of the distribution of IgG absorbance values at 450 nm in *P. falciparum* for male and female. The mean absorbance recorded were 45 SU and 25 SU; the lower quartiles of 5 SU and 5 SU; and an upper quartiles of 65 SU and 63 SU for both male and female groups, respectively. Meanwhile, Fig 4.1.1b also showed a box plot of the distribution of IgG absorbance values at 450 nm in *P. falciparum* for different cohorts. The horizontal bars for mean absorbance were recorded as 25 SU, 32 SU, 50 SU and 48 SU; the lower quartiles of 2 SU, 4 SU, 5 SU and 24

SU; and an upper quartiles of 70 SU, 67 SU, 69 SU and 68 SU for age groups 6 months to 5 years,

6 to 11 years, 12 to 17 years, and >18 years, respectively.

Table 4.1.11: Seroprevalence of Antibody IgG Response to *P. falciparum* Antigen According to Sex and Age

Sex		**Overall**			**Male**			**Female**	
Age/ IgG response	**Non-reactive <9 (%)**	**Grey zone 9-11(%)**	**Positive >11(%)**	**Non-reactive <9 (%)**	**Grey zone 9-11(%)**	**Positive >11(%)**	**Non-reactive <9 (%)**	**Grey zone 9-11 (%)**	**Positive >11(%)**
6mnths-5yrs	9 (9.67)	3 (3.22)	24 (25.80)	5 (5.37)	2 (2.15)	14 (15.05)	4 (4.30)	1 (1.07)	10 (10.75)
6yrs-11yrs	4 (4.30)	4 (4.30)	21 (22.58)	1 (1.07)	0 (0.00)	10 (10.75)	3 (3.22)	4 (4.30)	11 (11.82)
12yrs-17yrs	4 (4.30)	0 (0.00)	19 (20.43)	2 (2.15)	0 (0.00)	8 (8.60)	2 (2.15)	0 (0.00)	11 (11.82)
>18yrs(control)	0 (0.00)	0 (0.00)	5 (5.37)	0 (0.00)	0 (0.00)	3 (3.22)	0 (0.00)	0 (0.00)	2 (2.15)
Total	**17 (18.27)**	**7 (7.52)**	**69 (74.19)**	**8 (8.60)**	**2 (2.15)**	**35 (37.63)**	**9 (9.67)**	**5 (5.37)**	**34 (36.55)**

Sex χ^2= 1.264, P = 0.532; r = 0.050, p = 0.632; U=741.50, p =0.05 and Age χ^2= 6.740, p = 0.346; r = 0.034, p = 0.750

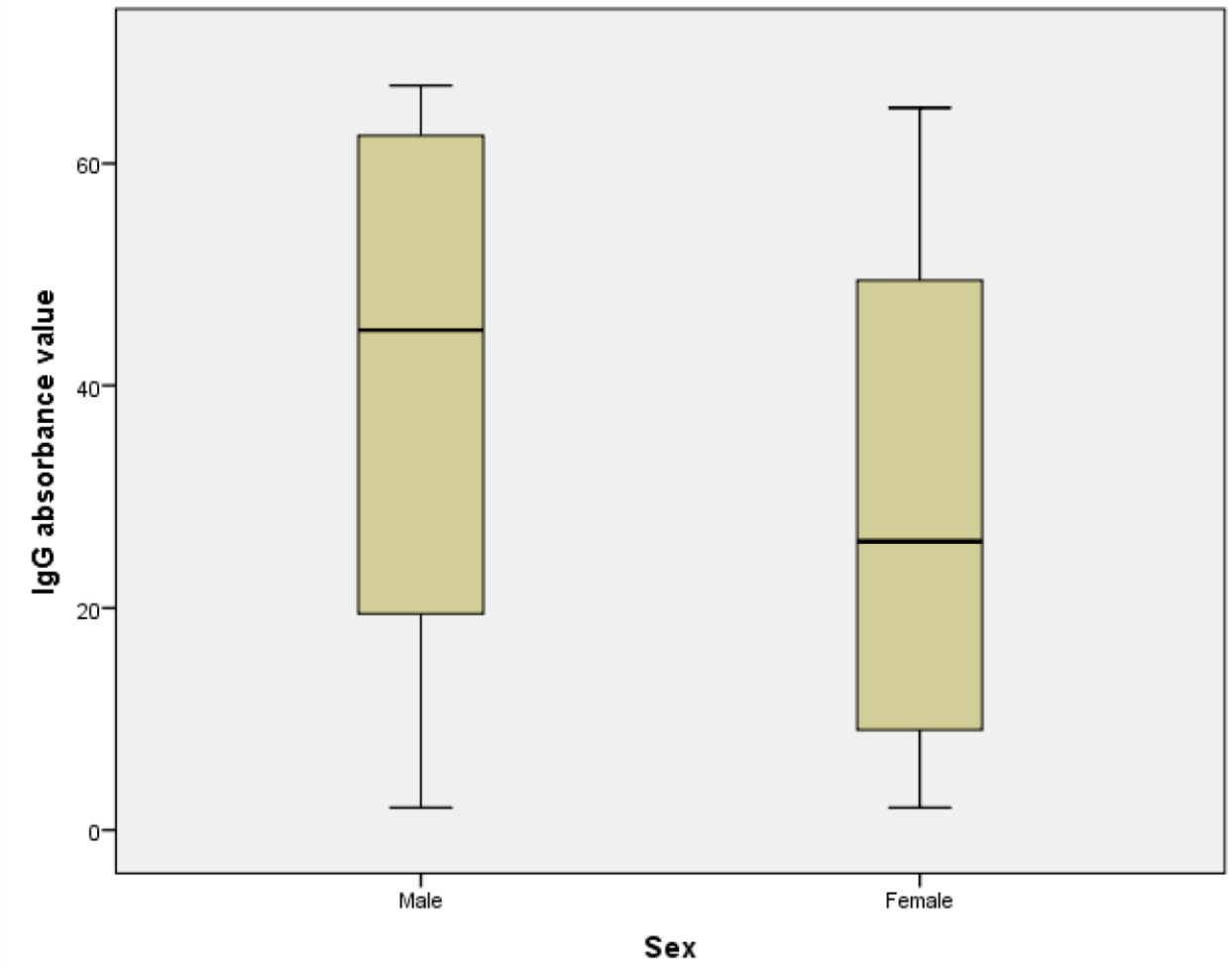


Fig 4.1.1a: Boxplot of the Distribution of IgG Absorbance Values at 450 nm in *P. falciparum* Infection for Male and Female

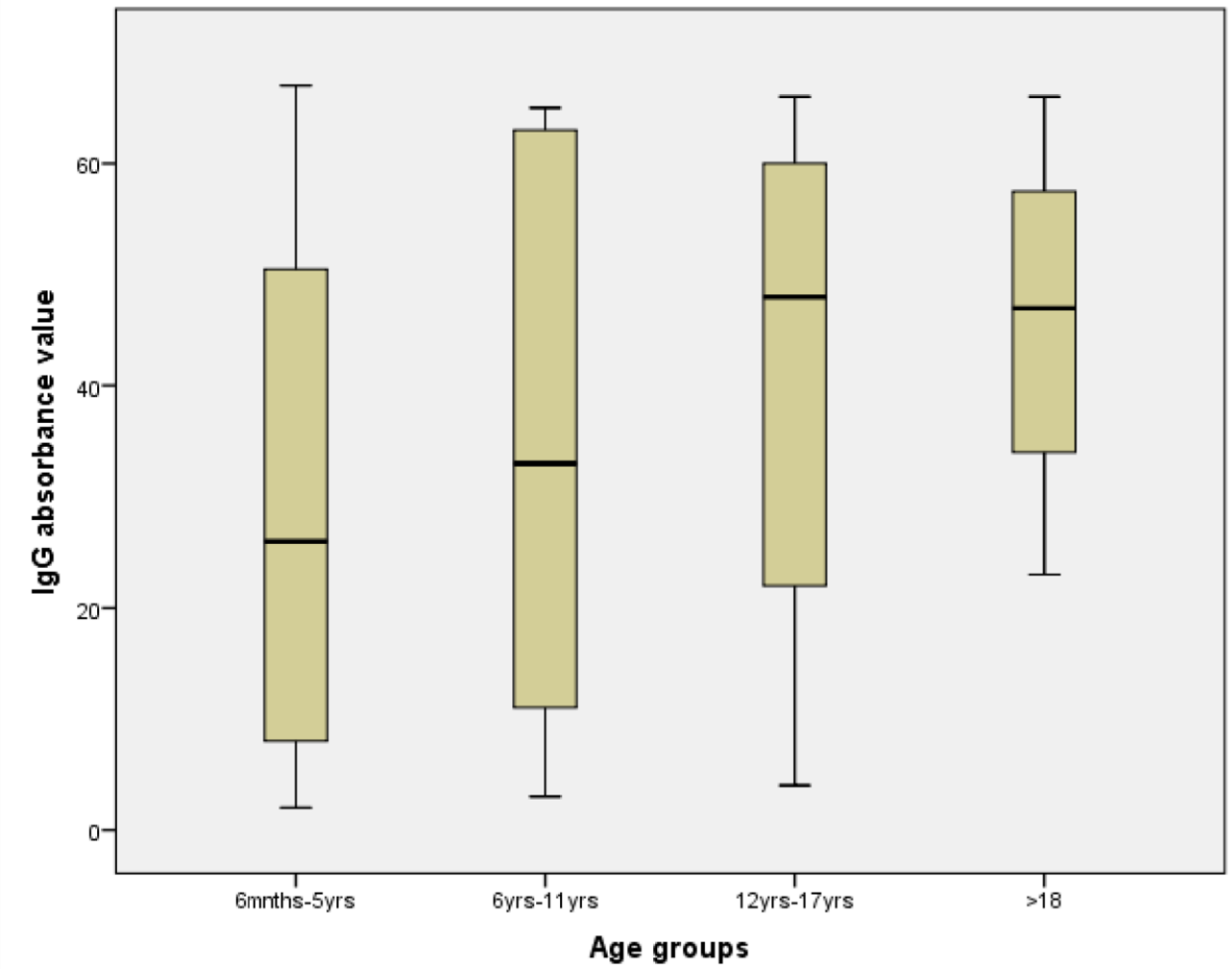


Fig 4.1.1b: Boxplot of the Distribution of IgG Absorbance Values at 450 nm in *P. falciparum* Infection for Different Age Groups

4.1.3.4 Seroprevalence of IgG antibody response to *P. falciparum* mild to severe infection

The prevalence of *P. falciparum* antibody responses to the antigen was compared to seek for the association or correlation with the parasite densities. The highest seroprevalence of *P. falciparum* IgG antibodies response to antigen 9 (90.00 %) was detected in plasma samples with high parasite density (PD) (severe malaria) followed by those with low PD 13 (81.25 %) as presented on Table 4.1.12. The least observed Seroprevalence of *P. falciparum* IgG antibody was observed in samples with moderate PD 42 (72.41 %).

The differences in the seroprevalence of *P. falciparum* IgG antibody was not significant (p> 0.05) between all the parasite densities (low, moderate and high parasitemia) and non-significant association exists between them (χ^2= 3.721, p = 0.714; r = 0.040, p = 0.706) as measured by ELISA. For variation between the IgG antibody and the PD, significant difference (p >0.05) was not observed between *P. falciparum* infected erythrocyte and parasite density using Analysis of Variance (ANOVA) (F=0.408, p =0.516). Furthermore, Kruskal-Wallis H test was used for variation between the parasite densities in the IgG antibody response. It showed a statistically nonsignificant difference in IgG antibody response between the parasite densities, χ^2 =0.473, p= 0.789, with mean rank parasite densities of 43.73 for low PD, 39.34 for moderate PD and 42.05 for high PD.

Fig 4.1.2 shows a boxplot of the distribution of IgG absorbance values at 450 nm in *P. falciparum* for parasite density. The mean absorbance represented in the horizontal bars were recorded as 45 SU, 46 SU, 25 SU and 30 SU; the lower quartiles of 5 SU, 4 SU, 3 SU and 3 SU; and an upper quartiles of 65 SU, 67 SU, 70 SU and 0 SU for negative, Low PD, moderate PD and high PD groups, respectively.

Table 4.1.12: Seroprevalence of IgG Antibody Response to *P. falciparum* Antigen in Mild to Severe Infection

		Antibody IgG response		
Parasite density	**No. exam.**	**Non-reactive <9 (%)**	**Grey zone 9-11 (%)**	**Positive >11 (%)**
Negative	9	3(33.33)	1(11.11)	5(55.55)
Low PD (<1000 /μl)	16	2 (12.50)	1(6.25)	13(81.25)
Moderate PD (1001-10,000 /μl)	58	11(18.96)	5(8.62)	42(72.41)
High PD (>10,001 /μl)	10	1(10.00)	0(0.00)	9(90.00)
Total	**93**	**17(18.27)**	**7(7.52)**	**69(74.19)**

PD- Parasite density

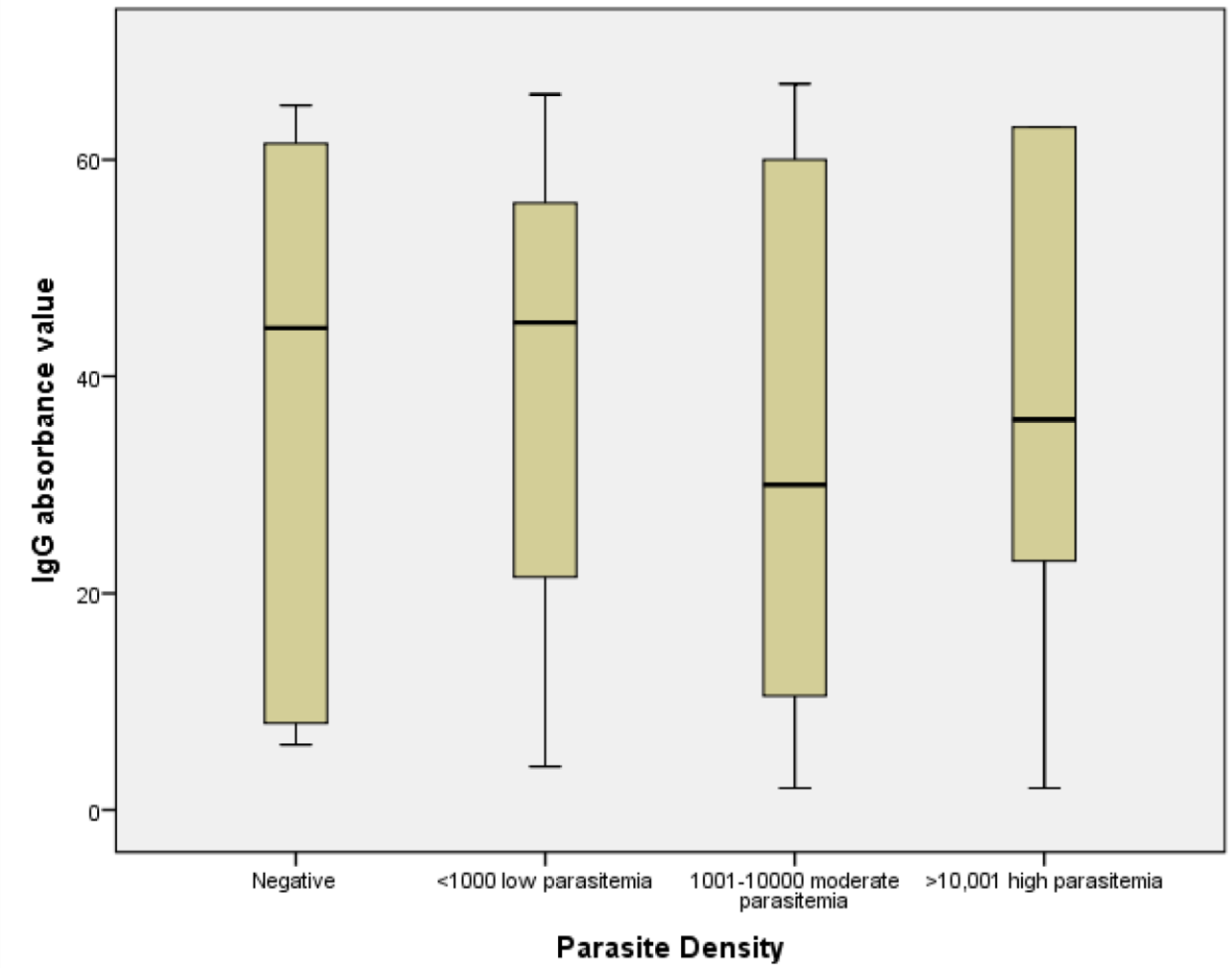


Fig 4.1.2: Boxplot of the Distribution of IgG Absorbance Values at 450 nm in *P. falciparum* for Parasite Density

4.1.3.5: Antibody IgG responses to *P. falciparum* antigen in plasma samples from infected children with anaemia

It was recorded that samples with low haemoglobin and haematocrit anaemia both had a very similar antibody IgG response rate of 59 (63.44 %) and 58 (62.36 %), respectively. All Plasma samples with normal haemoglobin and haematocrit produced antibody IgG response rate of 10 (10.75 %) and 11 (11.82 %), respectively. Interestingly, all plasma samples (also microscopically positive for *P. falciparum*) with normal haemoglobin and haematocrit level produced antibodies IgG as measured by ELISA against *P. falciparum* antigens. In the other way round, not all plasma samples with low haemoglobin and haematocrit anaemia produced antibodies IgG as measured by ELISA against *P. falciparum* antigens. However, the differences in the seroprevalence of *P. falciparum* antibodies was not significant and non-significant negative correlations exists (P> 0.05) between both haemoglobin anaemia (χ^2= 3.897, P = 0.142; r = -0.075, P = 0.474) and low haematocrit anaemia (χ^2= 4.339, P = 0.114; r = -0.079, P = 0.450) as measured by ELISA. However, analysis using the Mann-Whitney U test revealed IgG antibody response to *P. falciparum* infection in children with low haemoglobin was non-significant to children with normal Hb (U=308.00, p =0.513); and this was also observed in cases with low haematocrit anaemia where children with low Hc anaemia were non-significant to children with normal Hc (U=329.50, p =0.426). On the other hand, Kruskal-Wallis H test was conducted to determine the relationship between IgG antibody responses to *P. falciparum* and the severity or degree of anaemia. It showed a non-significant difference in IgG antibody response between the degree of anaemia, χ^2 =1.729, p = 0.421, with a mean rank of 44.92 for severe anaemia, 36.06 for moderate anaemia and 42.51 for mild anaemia.

Table 4.1.13: Antibody IgG Responses to *P. falciparum* Antigen in Plasma Samples from Infected Children with Anaemia

		Antibody IgG response		
Anaemia type	**No. exam.**	**Nonreactive <9 (%)**	**Grey zone 9-11 (%)**	**Positive >11 (%)**
Normal Hb	10	0(0.00)	0.00(0)	10(10.75)
Low Hb	83	17(18.27)	7(7.52)	59(63.44)
Total	**93**	**17(18.27)**	**7(7.52)**	**69(74.19)**
Normal Hc	11	0(0.00)	0(0.00)	11(11.82)
Low Hc	82	17(18.27)	7(7.52)	58(62.36)
Total	**93**	**17(18.27)**	**7(7.52)**	**69(74.19)**

Haemoglobin Hb anaemia (χ^2= 3.897, p = 0.142; r = -0.075, p = 0.474) Haematocrit Hc anaemia (χ^2= 4.339, p = 0.114; r = -0.079, p = 0.450)

4.1.3.6: Antibody IgG responses to *P. falciparum* antigen in plasma samples from infected children with anaemia subtypes

The seroprevalence of IgG as measured by ELISA for different anaemia subtypes was presented on Table 4.1.14. The most frequent seroprevalence was observed in plasma samples with microcytic normochromic anaemia 15 (16.12 %) followed by normocytic normochromic, normocytic hypochromic, and microcytic hypochromic anaemia with 13 (13.97 %), 11 (11.82 %) and 10 (10.75 %), respectively. The least seroprevalence after exposure to ELISA were observed in plasma samples with macrocytic hyperchromic, macrocytic normochromic, microcytic hyperchromic, macrocytic hypochromic and normocytic hypochromic with 1 (1.07 %), 3 (3.22 %), 3 (3.22 %), 6 (6.45 %) and 7 (7.52 %), respectively. When considering the seroprevalence of antibodies IgG as measured by ELISA separately, it was observed for all the samples of macrocytic hypochromic, macrocytic hyperchromic, and macrocytic normochromic to have 100 %

seroprevalence *i.e.* all the plasma samples that belonged to this category produced antibodies IgG

against *P. falciparum* antigen. In addition, normocytic hyperchromic, normocytic normochromic, normocytic hypochromic, microcytic hypochromic, microcytic normochromic and microcytic hyperchromic produced the seroprevalence of 7/8 (87.50 %), 13/16 (81.25 %), 11/14 (78.57 %), 10/14 (71.42 %), 15/25 (60.00 %) and 3/6 (50.00 %), respectively.

However, the differences in the seroprevalence of *P. falciparum* antibodies IgG was not significant in all the anaemia subtypes and non-significant negative correlations exists ($p > 0.05$) between them ($\chi^2 = 12.863$, $p = 0.683$; $r = -0.090$, $p = 0.392$). Furthermore, Kruskal-Wallis H test was used for variation between anaemia subtypes in the IgG antibody response. It showed a non-

significant difference in IgG antibody response between the anaemia subtypes, χ^2 =7.987, *P*=0.435, with a mean rank of 46.15 for normocytic hypochromic, 51.00 for normocytic hyperchromic, 51.34 for normocytic normochromic, 45.58 for macrocytic hypochromic, 62.00 for macrocytic hyperchromic, 50.83 for macrocytic normochromic, 47.36 for microcytic hypochromic, 22.83 for microcytic hyperchromic and 38.61 for microcytic normochromic.

Fig 4.1.3 shows a boxplot of the distribution of IgG absorbance values at 450 nm in *P. falciparum* for anaemia subtypes. The horizontal bars for mean absorbance were observed as 45 SU, 55 SU, 50 SU, 28 SU, 0, 43 SU, 30 SU, 10 SU and 20 SU; the lower quartiles of 2 SU, 5 SU, 8 SU, 10

SU, 0, 40 SU, 2 SU, 0 and 5 SU; and an upper quartiles of 65 SU, 65 SU, 70 SU, 63 SU, 0, 45 SU, 63 SU, 55 SU and 64 SU for normocytic hypochromic, normocytic hyperchromic, normocytic normochromic, macrocytic hypochromic, macrocytic hyperchromic, macrocytic normochromic, microcytic hypochromic, microcytic hyperchromic and for microcytic normochromic,

respectively.

Table 4.1.14: Antibody IgG Responses to *P. falciparum* Antigen in Plasma Samples from Infected Children with the Anaemia Subtypes

		Antibody IgG response		
Anaemia subtypes	**No. exam.**	**Non-reactive <9 (%)**	**Grey zone 9-11 (%)**	**Positive >11 (%)**
Nmt hypo	14	2(2.15)	1(1.07)	11(11.82)
Nmt hyper	8	1(1.07)	0(0.00)	7(7.52)
Nmt nmc	16	2(2.15)	1(1.07)	13(13.97)
Macro hypo	6	0(0.00)	0(0.00)	6(6.45)
Macr hyper	1	0(0.00)	0(0.00)	1(1.07)
Macro nmc	3	0(0.00)	0(0.00)	3(3.22)
Micro hypo	14	2(2.15)	2(2.15)	10(10.75)
Micro hyper	6	3(3.22)	0(0.00)	3(3.22)
Micro nmc	25	7(7.52)	3(3.22)	15(16.12)
Total	**93**	**17(18.27)**	**7(7.52)**	**69(74.19)**

χ^2= 12.863, p = 0.683; r^2 = -0.090, p = 0.392

Keys

Nmt hypo = Normocytic hypochromic Macro hypo = Macrocytic hypochromic Micro hypo = Microcytic hypochromic

Nmt hyper = Normocytic hyperchromicMacro hyper = Macrocytic hyperchromic Micro hyper = Microcytic hyperchromic

Nmt nmc = Normocytic normochromic Macro nmc = Macrocytic normochromic Micro nmc = Microcytic normochromic

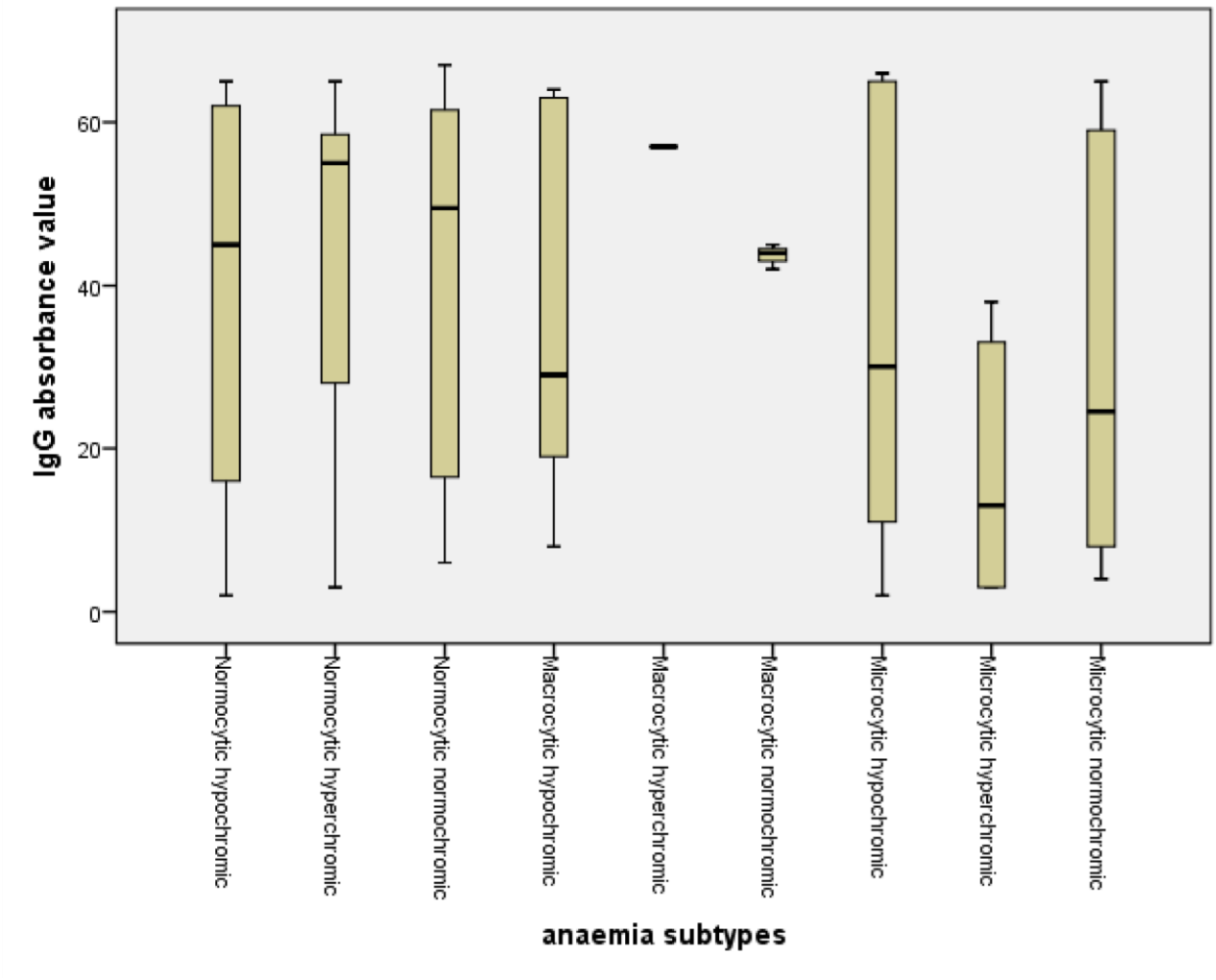


Fig 4.1.3: Boxplot of the Distribution of IgG Absorbance Values at 450 nm in *P. falciparum* for Anaemia Subtypes

4.1.4 Genetic Diversity of *P. falciparum* Isolated from Children in Minna

4.1.4.1 Allele diversity of *P. falciparum* MSP1 and MSP2 among children in Minna

From the total of 50 samples presented for PCR, 49 (98.00 %) were microscopically positive with only 1 (2.00 %) microscopically negative sample as presented on Table 4.1.15a. A total of 46 (92.00 %) were confirmed *P. falciparum* positive by nested-PCR *i.e.* samples were positive for at least one allele which were typed for MSP1 or MSP2 genes. A total of 111 band sizes was recorded for all the alleles of MSP 1 and 2, of which 57/111 (51.35%) and 54/111 (48.64%) of them were successfully examined for the presence of MSP1 (MAD20, K1, RO33) and MSP2 (3D7, FC27), respectively. In addition, Table 4.1.15b shows number of *P. falciparum* alleles and base pair ranges observed per allelic family in MSP1 and MSP2. MAD20 was the predominant allele observed in

MSP1 with 31/50 isolates (62.00%) and yielded four (4) fragments (within a range of 100-500bp).

Amplification of the allelic family K1 was positive for 21/50 isolates (42.00%) and yielded three (3) fragments (within a range of 250-1000bp). The lowest allele frequency observed for MSP1 gene was RO33 with only 5/50 isolates (10.00%) and yielded two (2) fragments (within a range of 200-500bp). MSP2 gene recorded FC27 as the predominant allele with 43/50 isolates (86.00%) and produced up to eight (8) fragments (within a range of 100-900bp) and 3D7 produced three (3) fragments (within a range of 400-800bp) from 11/50 isolates (22.00%).

Table 4.1.**15a: Allele Diversity of *P. falciparum* MSP1 and MSP2 among Children in Minna**

	MSP1	MSP2			
PCR status	**K1 (%)**	**MAD 20 (%)**	**RO33 (%)**	**3D7 (%)**	**FC27 (%)**
Negative	29(58.00)	19(38.00)	45(90.00)	39(78.00)	7(14.00)
Positive	21(42.00)	31(62.00)	5(10.00)	11(22.00)	43(86.00)
Total	**50(100)**	**50(100)**	**50(100)**	**50(100)**	**50(100)**

Table 4.1.15b: Number of *P. falciparum* Alleles and Base Pair Ranges Observed per Allelic Family in MSP1 and MSP2

Gene/Allele	PCR Positive samples (%)	PCR band size (bp)	Number of observed bands
MSP1			
KI	21(18.91)	250-1000	3
MAD20	31 (27.92)	100-500	4
RO33	5(4.50)	400-500	2
MSP2			
3D7	11(9.90)	100-800	4
FC27	43(38.73)	100-900	8
TOTAL	**111 (100)**		

4.1.4.2 Monoclonal and polyclonal infections of the different allelic families of MSP1 and MSP2 among children in Minna

In MSP1 gene, monoclonal infections (only one band size or fragment produced per allele) of 47 (82.46 %); and a polyclonal infection (more than one band size produced per allele) of 10 (17.54 %) was recorded. On the other hand, MSP2 recorded 83 (74.77 %) and 28 (25.23 %) for monoclonal and polyclonal infections, respectively. The overall monoclonal infections recorded during the study was 83 (74.77 %) and a polyclonal infection of 28 (25.23 %). Cases with monoclonal infections from K1 recorded a fragment of either 300 or 1000 bp while the polyclonal infections observed more than one fragments between 250 to 1000 bp. MAD20 on the other hand, recorded fragments of 100, 300 or 400 bp for monoclonal infections and a combination of more than one fragments of between 100 to 500 bp for polyclonal infections. RO33 did not record a monoclonal infection, while 400 to 500 bp was observed for a polyclonal infection. For MSP2, all the alleles of 3D7 had monoclonal infections of either 100, 400, 450 or 800 bp. FC27 observed for monoclonal infection 100, 600 or 900 bp while a combination of different fragments between 100900 bp were observed for polyclonal infections as presented on Plates I-V.

Furthermore, of the 46 PCR positive samples, 41 (89.13%) of the samples had poly-allelic infection *i.e.* they harbored more than one parasite genotype identified by the presence of two or more alleles of one or both genes, and 5 (10.87 %) had single allele (mono-allelic) of MSP2 genes only. However, the combination of the poly-allelic features differ accordingly with a total of 20

(18.00%) with a combination of two (2) allele types in the following order: RO33/FC27 1 (1.00%);

MAD20/FC27 15 (13.00%); MAD20/3D7 2 (2.00%) and KI/FC27 2 (2.00%). Whereas 17

(15.00%) contained three (3) allele combination types with K1/RO33/FC27 2 (2.00%);

K1/MAD20/FC27 10 (9.00%); MAD20/3D7/FC27 2 (2.00%) and K1/3D7/FC27 3 (3.00%). In addition, a combination of four (4) allele types was observed: K1/RO33/3D7/FC27 2 (2.00%) and

K1/MAD20/3D7/FC27 2 (2.00%)

Table 4.1.16: Monoclonal and Polyclonal Infections of the Different *P. falciparum* Allelic Families of MSP1 and MSP2 among Children in Minna

	MSP1							MSP2				
Infections	**KI (%)**	**Size (bp)**	**MAD20 (%)**	**Size (bp)**	**RO33 (%)**	**Size (bp)**	**Total MSP1 (%)**	**3D7 (%)**	**Size (bp)**	**FC27 (%)**	**Size (bp)**	**Total *MSP2* (%)**	**TOTAL (%)**
Monoclonal infections	17 (80.95)	1000/300 /400	28 (90.32)	100/300 /400	0 (0.00)		47 (82.46)	11 (100.00)	100/400/450 /800	25 (58.14)	100/600/ 900	36 (66.67)	83 (74.77)
Polyclonal infections	4 (19.04)	250-1000	3 (9.67)	100-500	5 (100.00)	400-500	10 (17.54)	0(0.00)		18 (41.86)	100-900	18 (33.33)	28 (25.23)
Total	**21 (36.84)**	**31 (54.38)**	**5 (8.77)**	**57 (100)**	**11 (20.37)**	**43 (79.62)**	**54 (100)**	**111 (100)**					

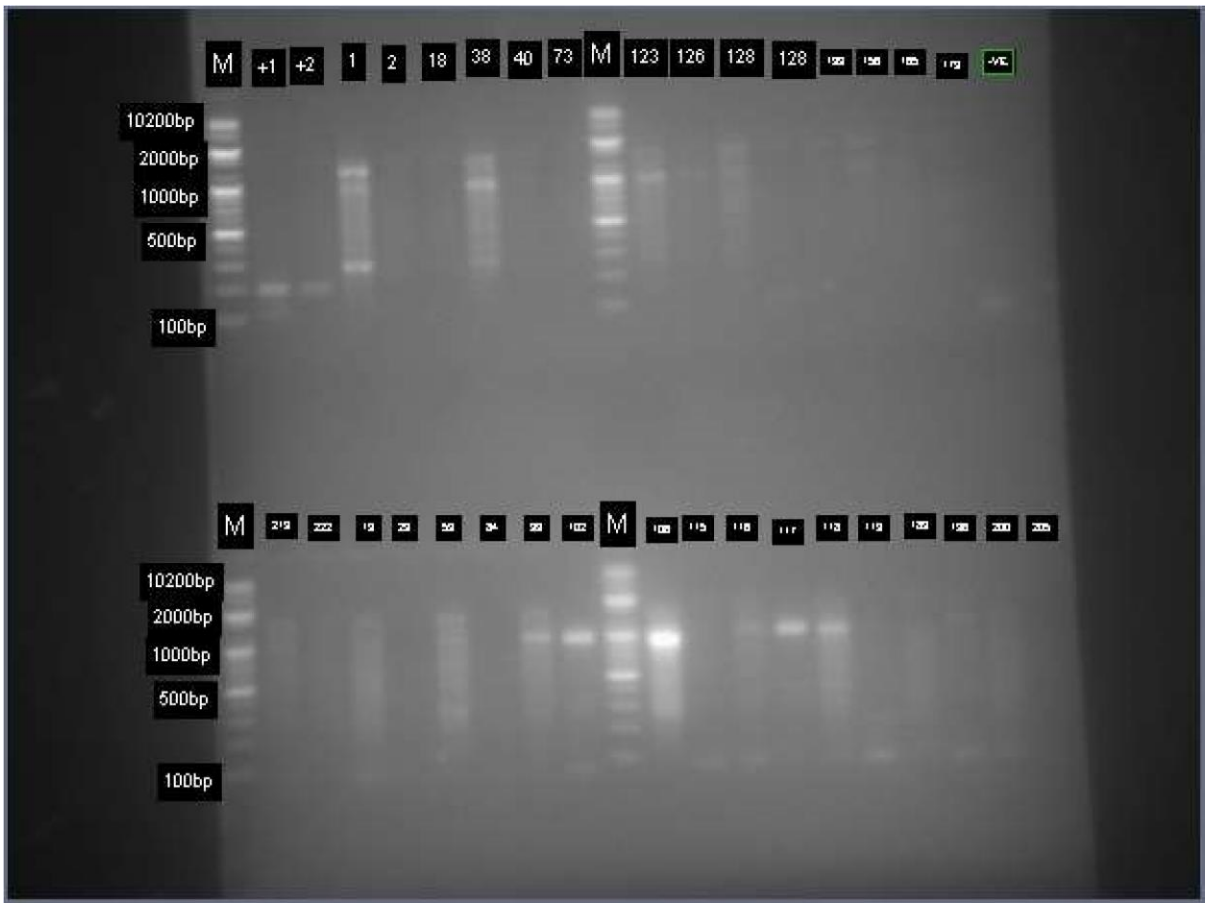


Plate I: PCR Typing of K1 (MSP1) with Band Size (250-1000 bp)

M- Marker

+1- Positive control 1

+2- Positive control 2

-ve- Negative

Bp- Base pair

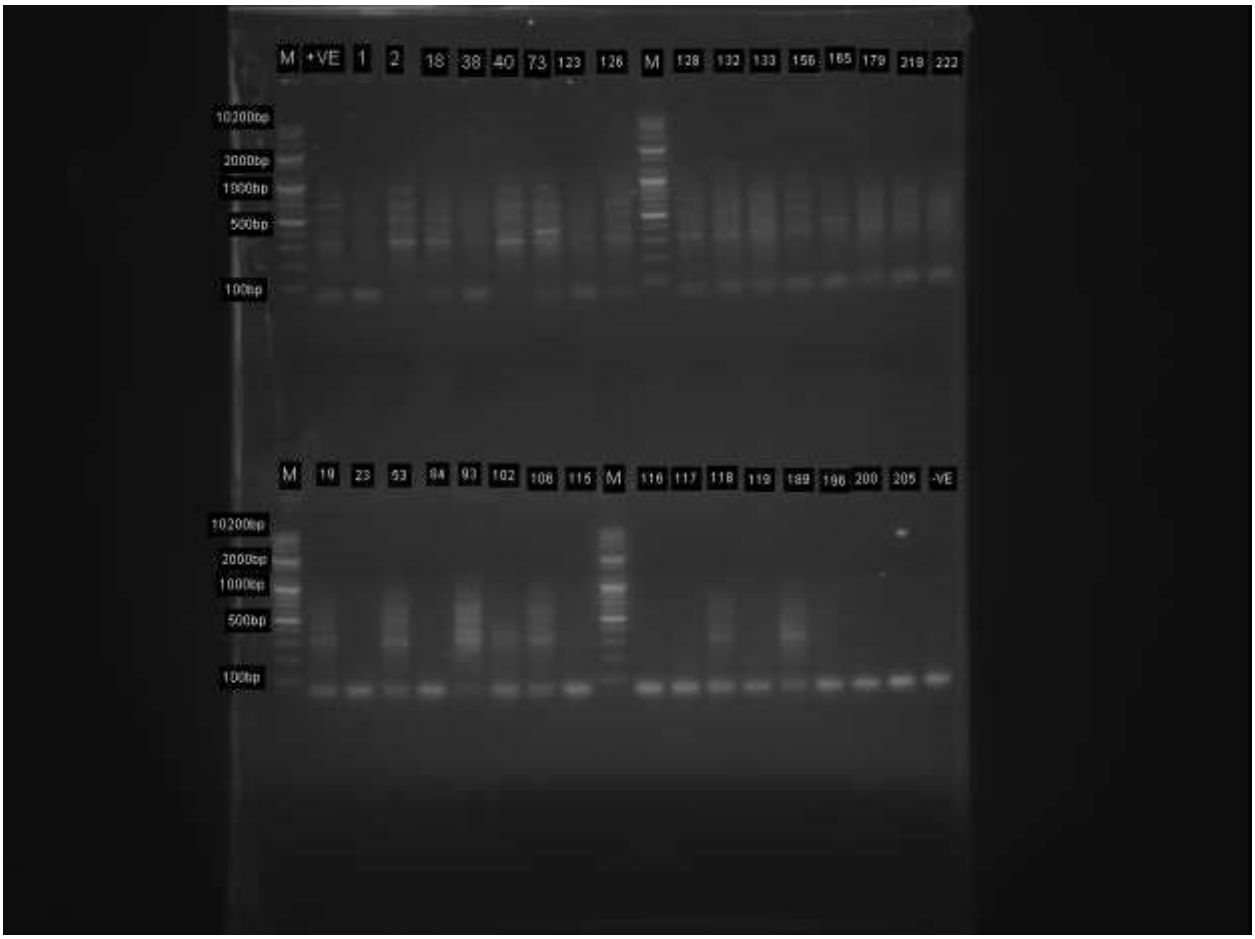


Plate II: PCR Typing of MAD20 (MSP1*)* with Band Size (100-500 bp)

M- Marker

+1- Positive control 1

+2- Positive control 2

-ve- Negative

Bp- Base pair

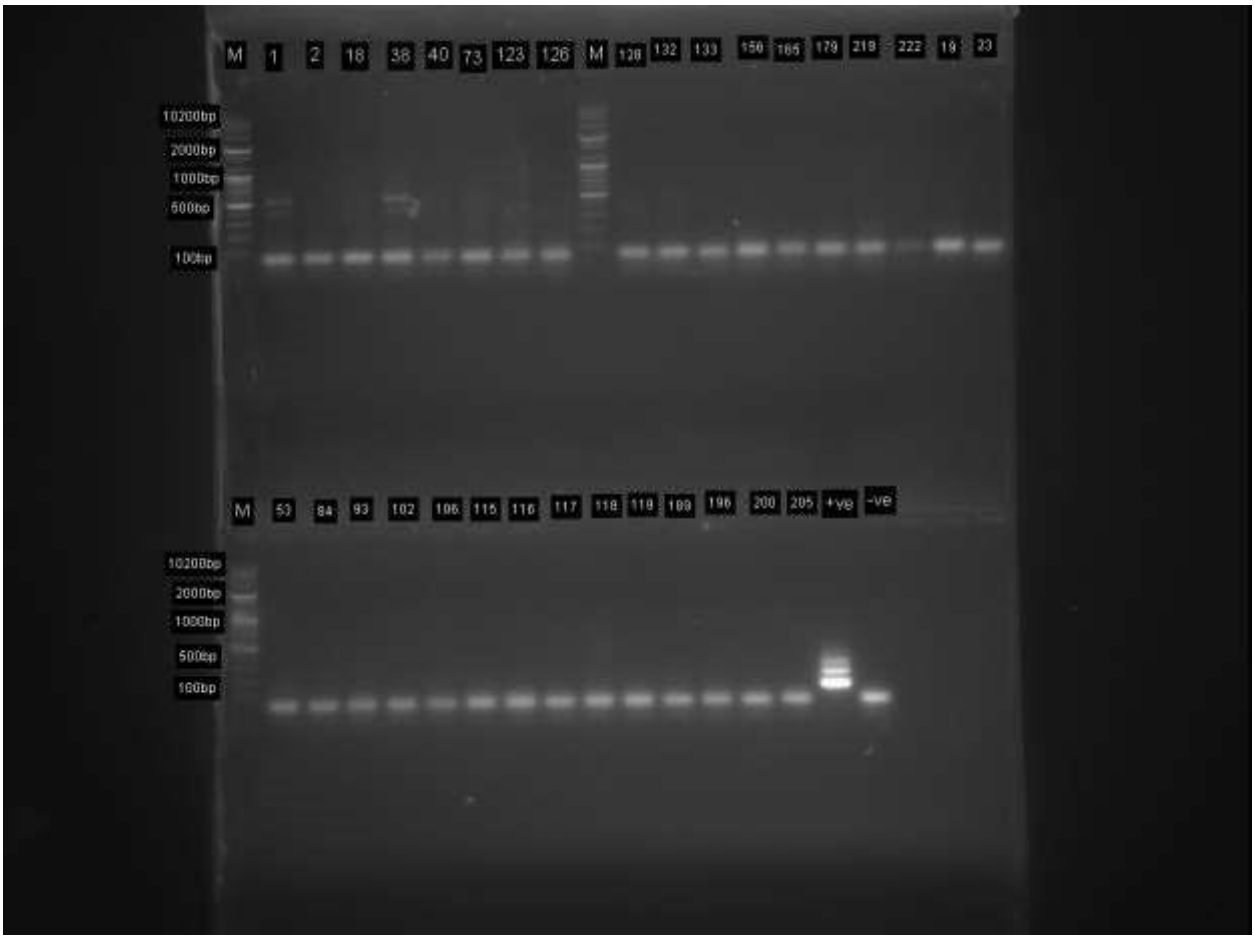


Plate III: PCR Typing of RO33 (MSP1*)* with Band Size (400-500 bp)

M- Marker

+1- Positive control 1

+2- Positive control 2

-ve- Negative

Bp- Base pair

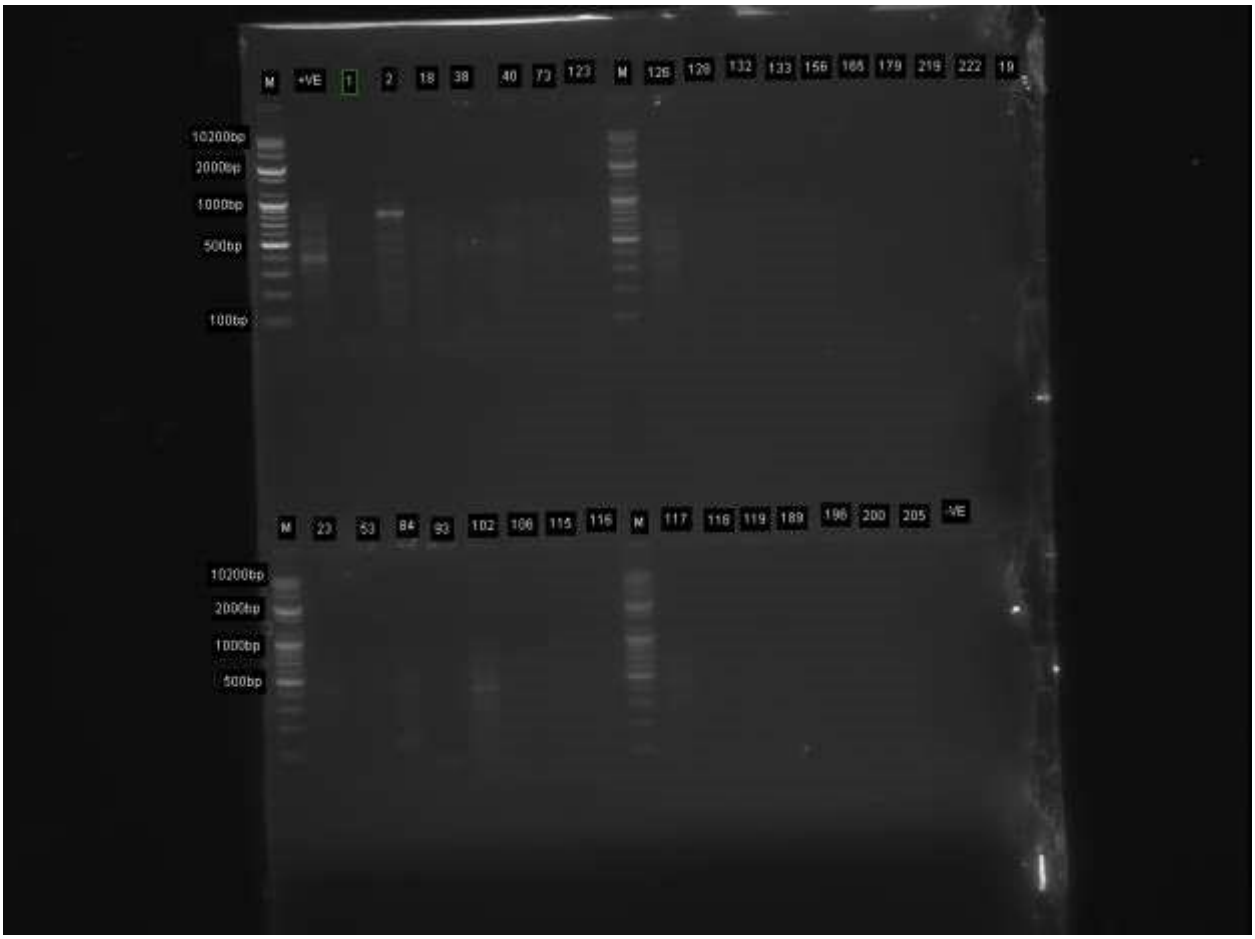


Plate IV: PCR Typing of 3D7 (MSP2*)* with Band Size (100-800 bp)

M- Marker

+1- Positive control 1

+2- Positive control 2

-ve- Negative

Bp- Base pair

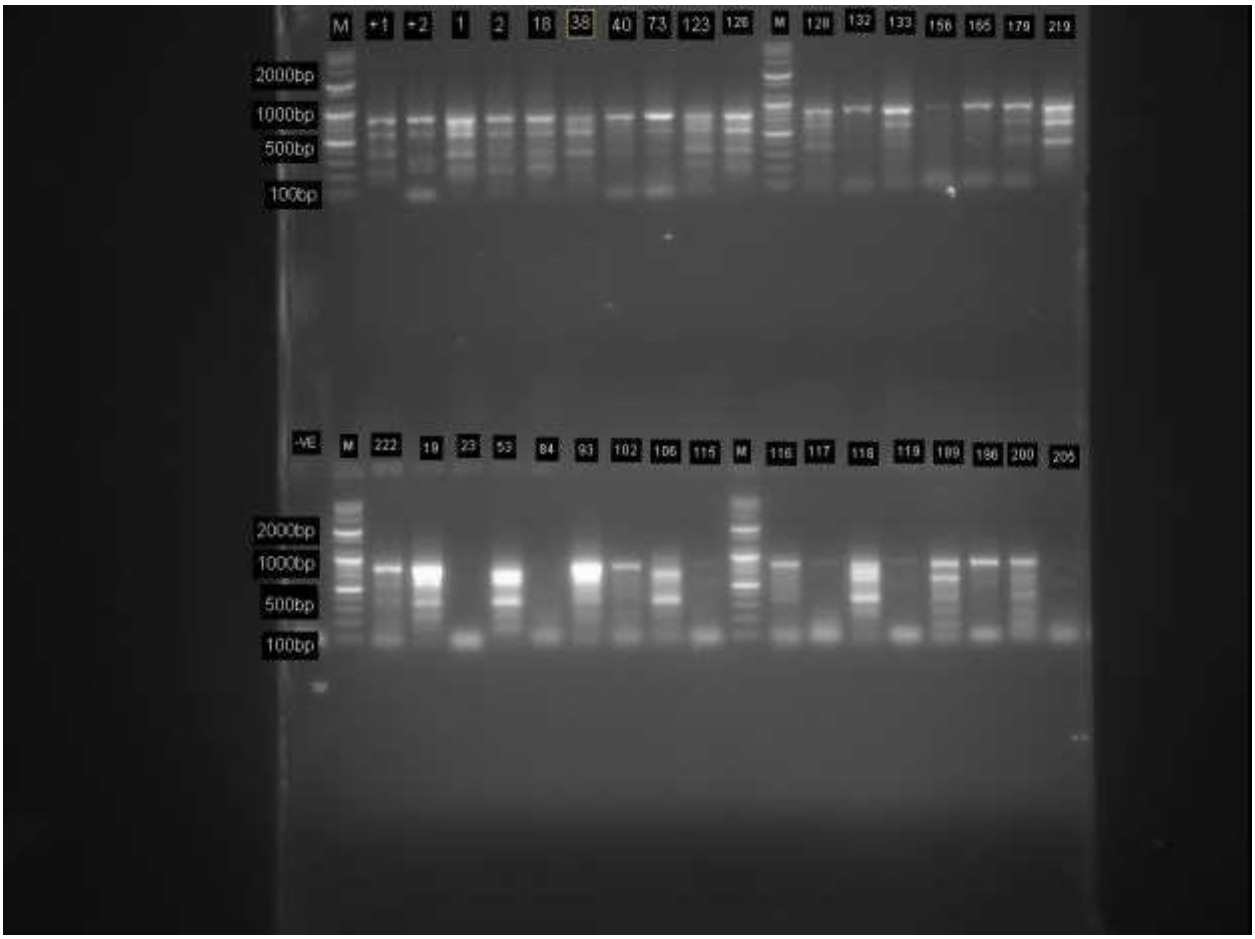


Plate V: PCR Typing of FC27 (MSP2*)* with Band Size (100-900 bp)

M- Marker

+1- Positive control 1

+2- Positive control 2

-ve- Negative

Bp- Base pair

4.1.4.3: Age and sex distribution of the different *P. falciparum* allelic types of MSP1 and MSP2 among children in Minna

The male category from this study recorded the highest distribution of all the allelic families of MSP1 and MSP2 with 20 (40.00 %) for K1, 18 (36.00 %) for MAD20, 5 (10.00 %) for RO33, 7(14.00 %) for 3D7 and 31 (62.00 %) for FC27, as against what was recorded in the female category with 1 (2.00 %), 13 (26.00 %), 0 (0.00 %), 4 (8.00 %) and 12 (24.00 %) for K1, MAD20, RO33, 3D7 and FC27, respectively. However, no single allele distribution was observed in RO33 in the female category as presented on Table 4.1.17. Furthermore, children between 6 months to 5 years recorded more allele distribution than other cohorts with 7 (33.33 %) for K1, 13 (41.93 %) for MAD20, 5(100 %) for RO33, 6 (54.55 %) for 3D7 and 17 (39.53 %) foe FC27; and in the case of RO33 allele, the distribution was only seen in this category. In the adult control group (>18 years), allele was only observed in MAD20 with 2 (6.45 %) and FC27 with 2 (4.65 %).

The dominant allele observed for MSP1 and MSP2 was MAD20 with 31 (62.00 %) and FC27 with 43 (86.00 %). Majority of the fragments observed for FC27 were 900 bp followed by 600 bp while 42.00 % were polyclonal with more than one fragments. Subsequently, MAD20 had the most dominant allele in the female category with all having band size of 300 bp and interestingly, all are monoclonal. RO33 and K1 alleles were virtually not detected in the female category.

Significant difference was not observed between age and all the allelic families of both MSP1 and MSP2 using Kruskal-Wallis H test to test for variation between the age groups for the alleles (K1: χ^2= 4.996, p = 0.172) with a mean rank age groups of 22.95 for age group 6 months to 5 years, 26.84 for age group 6 to 11 years, 33.86 for age group 12 to 17 years, and 15.00 for the

control group >18 years; MAD20: χ^2= 1.671, p = 0.643 with a mean rank age groups of 24.77 for age group 6 months to 5 years, 24.47 for age group 6 to 11 years, 27.86 for age group 12 to 17 years,

Table 4.1.17: Age and Sex Distribution of the Different *P. falciparum* Allelic Types of MSP1 and MSP2 among Children in

Minna

Sex	**Overall**	**Male**				**Female**											
Age	K1 (%)	MAD20	RO33	3D7	FC27 (%)		K1 (%)	MAD20	RO33	3D7	FC27 (%)		K1 (%)	MAD20	R033	3D7 (%)		FC27 (%)	
grps/alleles		(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)										
6 mnts-5 yrs		7(33.33)		13(41.93)		5(100)	6(54.55)		17(39.53)		7(35.00)		7(38.89)		5(100)	2(28.57)		13(41.94)	0(0.00)
6(46.15)		0 (0.00)	4 (100)	4 (33.33)															
6 yrs-11 yrs		13(61.90)		11(35.48)		0(0.00)	5(45.45)		17(39.53)		13(65.00)		9(50.00)		0(0.00)	5(71.43)		14(45.16)	0(0.00)
2(15.38)		0 (0.00)	0 (0.00)	3 (25.00)															
12 yrs-17 yrs		1 (4.76)	5(16.13)		0(0.00)	0(0.00)	7(16.28)		0(0.00)	2(11.11)		0(0.00)	0(0.00)	4(12.90)		1(100)	3(23.07)		0 (0.00)	0 (0.00)	3
(25.00)																			
>18 yrs	0 (0.00)	2(6.45)	0(0.00)	0(0.00)	2(4.65)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	0(0.00)	2(15.38)		0 (0.00)	0 (0.00)	2 (16.67)			
Total	**21(42.00)**	**31(62.00)**	**5(10.0)**	**11(22.0)**	**43(86.00)**	**20(40.00)**	**18(36.0)**	**5(10.0)**	**7(14.00)**	**31(62.00)**	**1(2.00)**	**13(26.0)**	**0 (0.00)**	**4 (8.00)**	**12 (24.00)**				

and 35.00 for the control group >18 years; RO33: χ^2= 6.929, p = 0.074with a mean rank age groups of 28.68 for age group 6 months to 5 years, 23.00 for age group 6 to 11 years, 23.00 for age group 12 to 17 years, and 23.00 for the control group >18 years; 3D7: χ^2= 3.039, p = 0.386with a mean rank age groups of 26.82 for age group 6 months to 5 years, 26.58 for age group 6 to 11 years, 20.00 for age group 12 to 17 years, and 20.00 for the control group >18 years; and FC27: χ^2= 2.986, p = 0.394)with a mean rank age groups of 23.32 for age group 6 months to 5 years, 26.37 for age group 6 to 11 years, 29.00 for age group 12 to 17 years, and 29.00 for the control group >18 years. It was also observed that no significant correlation at p >0.05 between age and the allelic families (K1: r = 0.119, p = 0.410; MAD20: r = 0.146, p = 0.311; 3D7: r = -0.214, p = 0.136; FC27: r = 0.237, p = 0.098) except for RO33 (r = -0.300, p = 0.035) which showed significant negative weak correlations with age.

In addition, analysis using the Mann-Whitney U test revealed for both K1 and MAD20 alleles, the male category was significantly higher than the female group with U=129.00, p =0.001 and U=195.00, p =0.057, respectively. However, RO33, 3D7 and FC27 alleles showed the male category was not significantly higher than the female group with U=232.00, p=0.109, U=260.00, p=0.728 and U=228.00, p =0.128, respectively. Furthermore, there was a significant negative weak correlation and on the other hand a significant positive weak correlation between the males and females that are positive to K1 and MAD20 isolates (KI: r = -0.497, p = 0.000; MAD20: r = 0.272, p = 0.056), respectively. The remaining isolates- RO33, 3D7 and FC27 had no significant associations- RO33: r =-0.229, p =0.110; 3D7: r = 0.050, p = 0.723; and FC27: r = -0.217, p = 0.129, respectively.

4.1.4.4: Distribution of *P. falciparum* alleles of MSP1 and MSP2 among children from the communities and healthcare facilities

The spatial distribution of the different allelic families of MSP1 and MSP2 genes from symptomatic children in different healthcare facilities and apparently healthy children

(asymptomatic) from the community was presented on Table 4.1.18. Old airport Primary Healthcare (PHC) had the highest distribution of 43 alleles (38.73 %) with MAD20 as the dominant allele in that group 15 (34.88 %). MAD20 frequently observed a 300bp band size of up to 25.00 % and FC27 observed about 62.00 % of 600 and 900 bp (polyclonal). Interestingly, a very good number of positive isolates 29 (26.13 %) were observed from the blood samples collected from apparently healthy children from the studied communities, and though RO33 was not observed from this category, allele FC27 was the dominant allele 12 (41.37 %) and about 75.00 % observed band size 900bp: followed by K1 with 9 (31.03 %) and 33.00 % with 1000 bp fragments. Meanwhile, Kpakungu PHC, FSP, and Gwari Road PHC had lower allele distribution of 2 (1.80

%), 5(4.50 %) and 7 (6.31 %), respectively. For the Kpakungu PHC, no allele was observed for K1, RO33 and 3D7; likewise, in FSP, no allele was observed in RO33 and 3D7, and only a single allele of MAD20 was not observed in Gwari Road PHC. No significant (p>0.05) difference was observed between the sites of sample collection and all the allelic families of both MSP1 and MSP2.

Table 4.1.18: Distribution of *P. falciparum* Alleles of MSP1 and MSP2 among Children from Six Healthcare Facilities and Communities in Minna

Sample site/ allelic dist.	Res. Comm. (%)	Gen hosp. (%)	Tunga PHC (%)	FSP Limawa (%)	Kpakungu PHC (%)	Gwari Rd PHC (%)	Old airport PHC (%)
K1	9(31.03)	2(14.28)	0(0.00)	1(20.00)	0(0.00)	2(28.57)	7(16.28)
MAD20	6(20.68)	4(28.57)	3(27.27)	2(40.00)	1(50.00)	0(0.00)	15(34.88)
RO33	0(0.00)	0(0.00)	1(9.09)	0(0.00)	0(0.00)	2(28.57)	2(4.65)
3D7	2(6.89)	2(14.28)	2(18.18)	0(0.00)	0(0.00)	1(14.28)	4(9.30)
FC27	12(41.37)	6(42.85)	5(45.45)	2(20.00)	1(50.00)	2(28.57)	15(34.88)
Total allelic distribution (n=111)	**29(26.13)**	**14(12.61)**	**11(9.90)**	**5(4.50)**	**2(1.80)**	**7(6.31)**	**43(38.74)**

4.1.4.5: Distribution of alleles of MSP1 and MSP2 genes in relation to *P. falciparum* parasite density among children in Minna

The level of parasitemia in relation to MSP1 and MSP2 alleles among children in Minna was presented on Table 4.1.19. The distribution of alleles was higher in cases with moderate parasite density (1001-10000 µL) and with a total of 99/111 (89.19 %), meanwhile, FC27 (MSP2) and

MAD20 (MSP1) observed the highest burden of 38/99 isolates (38.38 %) and 29/99 isolates (29.29 %) from this category, respectively. FC27 was the only isolate detected in the *P. falciparum* negative sample with a band size of 900 bp. For cases with low parasite density, FC27 and K1 were the dominant alleles with 4 (36.36 %) and 3 (27.27 %), respectively.

Analysis using the Mann-Whitney U test revealed a non-significant difference between moderate parasite density and cases with low parasite density for all the alleles of MSP1 and MSP2, with K1: U=58.50, p=0.180; MAD20: U=77.00, p=0.570; RO33: U=80.00, p=0.486; 3D7: U=63.00, p=0.173 and FC27: U=76.00, p=0.399. There prove to be no significant correlation at P>0.05 between parasite densities and the alleles of MSP1 and MSP2 (K1: r = -0.051, p = 0.725),

(MAD20: r = 0.186, p = 0.197), (RO33: r = 0.105, p = 0.469), (3D7: r = -0.086, p = 0.552) and (FC27: r = -0.127, p = 0.380).

Table 4.1.19: Distribution of Alleles of MSP1 and MSP2 Genes in Relation to *P. falciparum* Parasite Density among Children in Minna

		Parasite Density PD	
Allele dist.	**Neg. sample (%)**	**Low PD (%) (<1000 µL)**	**Mod. PD (%) (1001-10000 µL)**
K1	0(0.00)	3(27.27)	18(18.18)
MAD20	0(0.00)	2(18.18)	29(29.29)
RO33	0(0.00)	0(0.00)	5(5.05)
3D7	0(0.00)	2(18.18)	9(9.09)
FC27	1(100.00)	4(36.36)	38(38.38)
Total allelic distribution (n=111)	**1(0.90)**	**11(9.90)**	**99(89.18)**

4.1.4.6: Distribution of MSP1 and MSP2 alleles in relation to anaemia among children in Minna

For both alleles of MSP1 and MSP2, more than half of the population recorded a normal haemoglobin level, with K1, MAD20, RO33, 3D7 and FC27 recorded 7 (13.46 %), 18(34.61 %),

1 (1.92 %), 5 (9.61 %) and 21 (40.38 %), respectively. The allele distribution was higher, 59 alleles

(53.15 %) in cases of low haemoglobin than in cases with normal haemoglobin, 52 alleles (46.84 %). For samples with low haemoglobin levels, FC27 had the highest allele frequency distribution of 22 (40.38 %), followed by K1, MAD20, 3D7, and RO33 with 14 (23.72 %), 13 (22.03 %), 6 (10.16 %) and 4(6.77 %), respectively. For cases with low haematocrit anaemia, a little more than half of the population 26 (52.00 %) for alleles of MSP1 and MSP2 had a normal haematocrit level, with only 8 (14.54 %), 18 (32.72 %), 1 (1.81 %), 7 (12.72 %) and 21 (38.18 %) positive for K1, MAD20, RO33, 3D7 and FC27, respectively. The allele distribution was higher in haematocrit anaemia category 56 (50.45 %) with FC27 having the highest allele frequency distribution of 22

(39.28 %), K1 and MAD20 equally recorded 13 (23.21 %), while 3D7 and RO33 equally had 4 (7.14 %).

Allele distributions were quite similar for cases with both haemoglobin and haematocrit anaemia. Half of the studied population for MSP1 and MSP2 had normal Hb and Hc levels but the predominant allelic type observed was the FC27, while RO33 observed the least allelic distribution. For MAD20, RO33, 3D7 and FC27, significant differences and correlations were not observed at $p > 0.05$ between these alleles and haemoglobin levels.

Table 4.1.20: Distribution of MSP1 and MSP2 Alleles in Relation to Anaemia among Children in Minna

		Haemoglobin		Haematocrit	
Anemia/ allelic dist.	**No. of alleles observed**	**Normal Hb (%)**	**Low Hb (%)**	**Normal Hc (%)**	**Low Hc (%)**
K1	21	7(13.46)	14(23.72)	8(14.54)	13(23.21)
MAD20	31	18(34.61)	13(22.03)	18(32.72)	13(23.21)
RO33	5	1(1.92)	4(6.77)	1(1.81)	4(7.14)
3D7	11	5(9.61)	6(10.16)	7(12.72)	4(7.14)
FC27	43	21(40.38)	22(37.28)	21(38.18)	22(39.28)
Total allelic distribution (n=111)	**111**	**52(46.84)**	**59(53.15)**	**55(49.54)**	**56(50.45)**

Hb- Haemoglobin

Hc- Haematocrit

4.1.4.7 Genetic diversity analysis of *P. falciparum* populations in Minna

A comparison between the expected heterozygosity (*He*) index for different sample collection sites showed that a very low level genetic diversity was detected in samples collected from the apparently healthy children from the communities, and the healthcare facilities with expected heterozygosity (*He*) value of 0.071 and 0.025, respectively. The *He* level was also determined for antigenic markers, MSP1 and MSP2 and an intermediate genetic diversity was observed for both MSP1 and MSP2 with expected heterozygosity (*He*) value of 0.714 and 0.830, respectively which signifies a wide range of allele diversity. Low levels of allele diversity *He* levels of 0.085, 0.219 and 0.063 were detected from children in different age groups of 6 months to 5 years, 6 to 11 years and 12 to 17 years, respectively. Meanwhile, the *He* values for both male and female were similar and was quite low, with 0.200 and 0.206, respectively. In the overall, a very low level of genetic diversity was detected in Minna with expected heterozygosity (*He*) value of 0.0636.

4.1.5 Estimation of multiplicity of infections (MOI) of alleles of MSP1 and MSP2 genes

4.1.5.1: Multiplicity of infection (mean MOI) for spatial distribution of alleles of MSP1 and MSP2 among children in Minna

The contribution from each site to the total number of samples and all the three reported allelic families of MSP1 (K1, MAD20 and RO33) and MSP2 (FC27 and 3D7) were observed among the isolates from the six healthcare facilities and samples collected from the communities, as shown in Table 4.1.21. Gwari Road PHC had the highest MOI of 3.50, and then FSP Limawa, and Old airport PHC had 2.55 and 2.53, respectively. The lowest was observed in Kpakungu with 1.00.

Apparently healthy children from the community recorded 2.07 MOI. In addition, the MOIs for MSP1 and MSP2 were 1.23 and 1.17, respectively, and the overall MOIs was 2.22.

There was however, no statistical significance (P>0.05) between MOI and the different study sites (χ^2= 27.442, p = 0.284) and no significant correlations exists between the two (r = 0.169, p =

0.241).

4.1.5.2 Multiplicity of infection (mean MOI) of age and sex distribution in relation to MSP1 and MSP2 genes among Children in Minna

The mean multiplicity of infection for both male and female were 2.61 and 2.14, respectively. The males had the highest MOIs of 2.61 as compared with the female category with 2.14.

Meanwhile, the MOIs of age groups within alleles of MSP1 and MSP2 was also shown in Table 4.1.22. The highest MOI was observed in children age groups of 6 months to 5 years with 2.52, followed by age groups 6 to 11 years and 12 to 17 years with 2.47 and 2.42, respectively. It was observed that MOI decreases with age. There was however, no statistical significance difference between MOI and sex of the child (χ^2= 5.613) and no significance difference between MOI and age of the child (p>0.05).

Table 4.1.21: Multiplicity of Infection (mean MOI) for Spatial Distribution of Alleles of MSP1 and MSP2 among Children in Minna

	Sample collection sites							
Allele dist.	**Res. Comm.**	**Gen. hospital**	**Tunga PHC**	**FSP Limawa**	**Kpakungu PHC**	**Gwari Rd PHC**	**O/Aairport PHC**	**Total**
K1	9	2	0	1	0	2	7	21
MAD20	6	4	3	2	1	0	15	31
RO33	0	0	1	0	0	2	2	5
3D7	2	2	2	0	0	1	4	11
FC27	12	6	5	2	1	2	15	43
Total	**29**	**14**	**11**	**5**	**2**	**7**	**43**	**111**
MOI	**2.41**	**2.33**	**2.20**	**2.55**	**2.00**	**3.50**	**2.53**	**2.22**

Table 4.1.22: Multiplicity of Infection (mean MOI) of Age and Sex Distribution in Relation to MSP1 and MSP2 among Children in Minna

	Sex		Age groups				
Allele dist.	**Male**	**Female**	**6 mnths-5 yrs**	**6 yrs-11 yrs**	**12 yrs-17 yrs**	**>18yrs (control)**	**Total**
K1	20	1	7	9	5	0	21
MAD20	18	13	13	11	5	2	31
RO33	5	0	5	0	0	0	5
3D7	7	4	6	5	0	0	11
FC27	31	12	17	17	7	2	43
Total	**81**	**30**	**48**	**42**	**17**	**4**	**111**
MOI	**2.61**	**2.14**	**2.52**	**2.47**	**2.42**	**2.00**	

4.1.5.3 Multiplicity of infection (mean MOI) of parasite density with alleles of MSP1 and MSP2 among children in Minna

The mean MOIs for low and moderate parasite densities were presented in Table 4.1.23. The negative sample by microscopy was used as a negative control, and interestingly FC27 allele was observed after exposure to PCR with a MOI of 1.00. Samples with low PD had the highest MOIs of 2.75 compared with those of moderate PD with 2.47. There was a significant difference between MOI and the parasite densities (χ^2= 15.076, p = 0.058), but no significant correlation exist between them (r = 0.004, p = 0.977).

4.1.5.4: Multiplicity of infection (mean MOI) of Anaemia with Allelic Families of MSP1 and MSP2

The mean MOIs between low haemoglobin and haematocrit anaemia for both MSP1 and MSP2 was presented in Table 4.1.24. Cases of low haemoglobin anaemia recorded the highest MOI of 2.56 as compared with the normal haemoglobin. Meanwhile, the MOI was higher in cases with low haematocrit anaemia (2.43) as compared to those with normal haematocrit level with MOI 2.39.

There was however, no statistical significance and no significant correlation between MOI and low haemoglobin anaemia (χ^2= 4.337, p = 0.362; r = 0.099, p = 0.496); and there was also no statistical significance and significant correlation between MOI and low haematocrit anaemia (χ^2=

2.707, p = 0.608; r = 0.066, p = 0.647) at p>0.05.

Table 4.1.23: Multiplicity of Infection (mean MOI) of Parasite Density with Alleles of MSP1 and MSP2 among Children in Minna

	Parasite Density			
Allelic dist.	**Neg.**	**Low PD**	**Mod. PD**	**Total**
K1	0	3	18	21
MAD20	0	2	29	31
RO33	0	0	5	5
3D7	0	2	9	11
FC27	1	4	38	43
Total	**1**	**11**	**99**	**111**
MOI	**1.00 (n=1)**	**2.75 (n=4)**	**2.47 (n=40)**	

Table 4.1.24: Multiplicity of Infection (mean MOI) of Anaemia with Allelic Families of MSP1 and MSP2

	Anaemia			
Allelic dist.	**Normal Hb**	**Low Hb**	**Normal Hc**	**Low Hc**
K1	7	14	8	13
MAD20	18	13	18	13
RO33	1	4	1	4
3D7	5	6	7	4
FC27	21	22	21	22
Total	**52**	**59**	**55**	**56**
MOI	**2.26**	**2.56**	**2.39**	**2.43**

Keys

Hb= Haemoglobin Hc=Haematocrit

4.2 Discussion

Malaria is a major global public health concern especially in countries where transmission occur regularly. Infection with *Plasmodium falciparum* is the major cause of morbidity and mortality especially among the vulnerable groups to which children, especially those whose age are less than 5 years.

This study reported a very high prevalence of *P. falciparum* in Minna with 75.00 % from which children below the age of 5 recorded a prevalence of 42.00 %. Recent malaria risk maps estimated that malaria prevalence in Nigeria varied from less than 20 % to over 70 % depending on the location (Onyiri, 2015). The high prevalence observed from this report was consistent with previous work in Minna, Niger state and other Nigerian states where 65.50 % of samples collected from two locations (Mekunkele and Gidan kwano) in Minna were infected with *P. falciparum* (Omalu *et al.,* 2014); meanwhile, about 60.60 % of the participants were found positive for *P. falciparum* in Kano state (Dawaki *et al.,* 2016). Previous studies from Minna showed that, of the 152 pregnant women screened for malaria infection, 21 (13.82 %) of them were infected with malaria parasites (Omalu *et al.,* 2012). In another study conducted by Olasehinde *et al.* (2015), in Ogun state, Southwestern Nigeria, it also observed a high *P. falciparum* prevalence of 61.10 %. However, the prevalence of *Plasmodium species* recorded by Umaru and Gabriel (2015), in Makarfi, Kaduna state was much lower 35.70 % and inconsistent with the observations from this study. The similarities and variations in the prevalence rates could be attributed to spatial distribution and temporal variations of the parasite over space and time, and moreover, different climatic conditions, more or less rainfall, and surface water that served as mosquito breeding sites could also contribute to its prevalence.

In addition, prevalence of *P. falciparum* from the different sample collection sites was observed for symptomatic children from the healthcare facilities as 62.97 % with General hospital, Minna; Tunga primary healthcare; Family support programme (FSP), Limawa; Kpakungu PHC; Gwari road PHC; Old airport PHC recording 35.75 %, 6.64 %, 1.89 %, 2.53 %, 2.84 % and 13.29 %, respectively. Only 12.32 % of blood samples collected from asymptomatic children from the community were infected with *P. falciparum*. Nevertheless, the confirmed malaria case in symptomatic children (62.97 %) was similar but higher to the observation of Sumbele *et al*. (2016) with 38.20 %. But however, of the 69 asymptomatic cases recorded from the communities, more than half 39 (56.52 %) were infected with *P. falciparum,* which indicates a large proportion of asymptomatic infections which may likely act as a reservoir for transmission of the parasite. The high prevalence of asymptomatic plasmodial infection recorded in this study was quite similar to the observation of Starzengruber *et al.* (2014), which stated that 53.60 % of at least one member tested from 416 households tested positive for malaria. Furthermore, significant difference was observed between *P. falciparum* infections and the various sample collection sites of this study and this was inconsistent with the observation of Dawaki *et al.* (2016), that there were no significant differences in the prevalence of malaria among the districts studied.

Malariometric parameters such as transmission based on age and gender, parasite density and anaemia were also determined. It was observed that the prevalence of *P. falciparum* decreases with age in the study population and so, the highest prevalence was observed in children below the age of 5 with 32.27 % which was in consonance with a previous report by Olasehinde *et al.* (2015), which recorded the highest prevalence in children 1-5 years with 70.80 %. Umaru and Gabriel (2015), however, reported a different results with the highest prevalence observed in

children between 5-15 years age group 19.30 %, followed by the under 5 group with 11.70 %. There was a non-significant difference between age and the presence of *P. falciparum* infection (P> 0.05) but this was not in accordance with previous work from Umaru and Gabriel (2015), which observed the difference in proportion of positive cases in each age group were statistically significant (Chisquare *P*<0.0001). The variations obtained between the results could be due to a presumed shift in prevalence among <5 age group in Makarfi Community and due to the fact that more attention and care were given to children < 5 years of age than the older children. However, the observation from this study was similar to the previous study by Sumbele *et al.* (2016), which recorded no significant differences between age and positive *P. falciparum* cases.

Findings from this study revealed a slightly higher overall prevalence of *P. falciparum* in male 39.87 % (126/316) than in female 35.44 % (112/316) participants which was consistent with previous study in Kaduna and Kano States by Umaru and Gabriel (2015), and Dawaki *et al.* (2016), in which they also observed a higher prevalence rate in the male than the female participants. The high prevalence in the male participants could be due to exposure, and inherent and cultural determinants (Bates *et al.,* 2004; Al-Mekhlafi *et al.,* 2011; Winskill *et al.,* 2011 and Loha and Lindtjørn, 2012). Coinciding with this, greater and repeated exposure to malaria among males may result in development of partial immunity that renders them at lower risk of clinical malaria compared to females (Smith *et al.,* 1999). However, from this study, Significant difference (P <0.05) was observed between sex and *P. falciparum* parasite. This was not in line with the observation of Sumbele *et al.* (2016), which observed the absence of significant difference between sex and positive *P. falciparum* samples.

P. falciparum positive asymptomatic cases recorded more of moderate parasite density (100110,000 /µl) of 29/69 (42.02 %). This is evident from this study that apparently healthy children from the studied communities have a high level of immunity against *P. falciparum* parasite, because immunity is the factor that most strongly determines whether a malaria infection produces symptoms (Lindblade *et al.*, 2014). An individual's level of immunity to infection is determined by past exposure history and age. Increased immunity leads to improved control over parasite multiplication and decreased parasite density, which in turn lessens the severity of symptoms (Lindblade *et al.*, 2014). However, some asymptomatic infections may be residual or recrudescent parasitemia remaining after treatment for a clinical episode of malaria. Symptomatic cases on the other hand observed a moderate parasite density of 53.44 % (132/247), however, only 4.45 % (11/247) recorded a high parasite density.

As observed from this study, *P. falciparum* parasite was observed at its peak in cases with moderate parasite density of 49.05 % (155) and this was majorly observed in cohorts of 6 months to 5 years. The lowest frequency was observed in cases with high parasite density. This was not in consonance with the previous study conducted by Sumbele *et al.* (2016), in Mount Cameroon, which stated that, a greater proportion of the children had high parasite densities 45.20% (33) while low, moderate, and hyperparasitemia occurred in 28.80 % (21), 17.80 % (13), and 8.20 % (6), respectively. This discrepancy could be ascribed to the peculiar geographic and weather features, time and the fact that their study was conducted during the peak of malaria transmission season (between May and August, 2014). A non-significant correlation was observed between the presence of febrile illness and the intensity of the parasite $P>0.05$ from this study.

This study has confirmed that anaemia is a severe public health problem in children from this study with 50.00 % and 47.46 % of the population being anaemic for low haemoglobin and haematocrit, respectively. This is based on the World Health Organization (WHO), criteria which states that anaemia prevalence of over 40 % is considered as a major public health problem, between 20 and

40 % as a medium-level public health problem, and between 5 % and 20 % as a mild public health problem. However, the overall prevalence observed in this study was much higher than the 33.80 % obtained from Mount Cameroun by Sumbele *et al.* (2016). For both low levels of haemoglobin and haematocrit anaemia, there prove to be no statistical significant correlation $p>0.05$ between anaemia and *P. falciparum* negative and positive samples. This was, however, in discordance with a study conducted in Ibadan, Southwest Nigeria by Mockenhaupt, *et al*. (1999), which observed the influence of *P. falciparum* infection on haemoglobin levels differed significantly between *P. falciparum* positive and negative samples. Also, in another study conducted on pregnant women from Niger Delta, south-south Nigeria, a positive and significant correlation between the level of parasitaemia and anaemia among *P. falciparum* parasitized pregnant women (Erhabor *et al.*, 2010). These discrepancies may be due to the seasons of conducting the research and a different subjects used for the study.

This study shows that anaemia is more pronounced in children particularly those infected with *P. falciparum* with an overall malarial anaemia burden from the total study population as 48.73 % (154/316) and 46.51 % (147/316), while the burden for population with anaemia was 75.49 % (154/204) and 63.36 % (147/232) for both low haemoglobin and haematocrit anaemia, respectively. Umaru and Gabriel (2015), observed a quite similar prevalence of 54.30 % of malaria positive patients with low haematocrit levels.

For both low levels of haemoglobin and haematocrit anaemia, there prove to be statistical nonsignificant differences between anaemia and *P. falciparum* positive and negative cases and no significant correlation exists between them $p>0.05$. This was consistent with the previous work of Umaru and Gabriel (2015), in Makarfi, Kaduna, North-western Nigeria, which observed anaemia to be associated with malaria infection and was inconsistent with previous studies of

Sumbele *et al.* (2010), and Sumbele *et al.* (2016), in Mount Cameron, Gabonese Children, western Kenya and also Mount Cameron, respectively. This similarity and discrepancies could be as a result of the diverse weather conditions.

For both low Hb and Hc levels, males had the highest prevalence rate of 44.11 % (90/204) and 38.36 % (89/232) as compared to the females 31.37 % (64) and 25.00 % (58/232), respectively. The prevalence rates was not quite similar to what was observed in a study conducted by Jamal *et al.* (2015), which recorded 120 cases (41.50 %) in males and 516 cases (94.64 %) in females, show low level of haemoglobin which implies females had the highest prevalence rate. However, statistical significant difference was observed between sex and parasitized Hb anaemia and a significant positive correlation was also observed. For low Hc anaemia, significant difference was observed between sex and parasitized Hc anaemia and a significant negative correlation was observed.

The highest anaemia prevalence of 42.82 % was observed in children less than 5 years. This was in accordance with the study of Sumbele *et al.* (2016) and Kawo *et al.* (2018), who recorded more anaemia among children less than 5 years in Mount Cameroon and Ethiopia, respectively. In another study conducted in 2 different locations in Mali by Koita *et al.* (2012), who observed children less than 9 years of age were frequently exposed than older subjects during the dry season. Moreover, significant difference was not observed between age and parasitized Hb anaemia, but a significant positive association was observed. In addition, significant difference was not observed between age and parasitized Hc anaemia, but a significant positive association was observed. This was in discordance with the work of Koita *et al.* (2012), which observed no difference among agegroups after the rainy season. The difference in significance could be as a

result of varying seasons in the conduct of the study and a sustained malaria control measures in Northern Mali.

However, from this study, it was observed that apparently healthy children (asymptomatic) from the communities constitute just 14.24 % and 11.07 % for both haemoglobin and haematocrit anaemia from the entire study population, respectively. Meanwhile, more than 50.00 % (160 and

159 of both haemoglobin and haematocrit) of the samples collected from outpatients (symptomatic) were anaemic. The higher prevalence of anaemia in symptomatic children compared to asymptomatic ones is in line with previous studies of Sumbele *et al.* (2016), who recorded a significantly higher prevalence of anaemia in feverish children compared to nonfeverish ones.

The highest prevalence observed was in mild anaemia with 32.59 %, followed by moderate anaemia with 13.60 % and the least was observed in severe anaemia with 3.48 %. Meanwhile, 35.00 % of the entire study population had normal Hb levels with 25.31 % of *P. falciparum* infected cases and the remaining 9.81 % of negative samples which was lower to the observations of Sumbele *et al.* (2016), which revealed a 38.00 % prevalence of moderate to severe anaemia not associated with malaria. This was not unusual as fever is not specific to infection with malaria parasite only but could be indicative of other anaemia-causing infections (Ngnie-Teta *et al.*, 2007). There was however, no significant correlation between the different intensities/degrees of anaemia and *P. falciparum* infection $P>0.05$, this was in accordance with previous work of Sumbele *et al.* (2016), which observed from their study, no significant differences in the categorization of anaemia (severe, mild, and moderate).

In addition, microcytic anaemia showed a well-defined picture of 26.58 % (84/316) from the total population. From this, 19.04 % (16/84) were negative to *P. falciparum* parasite while the remaining 80.95 % (68/84) were infected with *P. falciparum*. This was also observed in a study conducted by Mockenhaupt, *et al.* (1999), that states that 18 % of children had microcytosis and 72 % of the children were infected with *P. falciparum*, compared with 79 % of children with normal MCV. The most frequent causes of microcytosis are iron deficiency anaemia and haemoglobinopathies (Oski, 2003). Similarly, from this study, *P. falciparum* positive normocytic normochromic anaemia had a prevalence of 7.91 % (25) and this is the anaemia that occurs due to acute malaria while the presence of microcytic hypochromic anaemia indicates either iron deficiency, nutritional anaemia or hemoglobinopathies; which are not uncommon in tropical countries (Mishra, *et al.*, 2002).

In comparison, Normocytic hypochromic anaemia had the highest prevalence rates and macrocytic hyperchromic anaemia had the lowest in both males and females, though the males were found to have greater prevalence as compared to the females. This findings were not in consonance with the study of Jamal *et al.* (2015), which states that of the 120 cases (41.50 %) in males that show low level of haemoglobin, 40.00 % among them show the well-defined picture of hypochromic microcytic anaemia, while 72 cases (60.00 %) give the picture of normochromic normocytic and in females, 516 cases (94.64 %) show the low level of hemoglobin (9.00 %) and 396 cases show Hypochromic microcytic anaemia, while 120 cases (23.21 %) shows the normocytic normochromic blood picture.

The multinomial logistic regression model demonstrated that anaemia is not a significant predictor to *P. falciparum* parasite density. The multinomial logistic regression model also

demonstrated that no single anaemia subtype showed any significant predictor to parasite density *i.e.* anaemia is not a predictor of parasite density.

The attributable risk (AR) of anaemia by malaria in this study was observed in low haemoglobin anaemia with 7.12 % which was lower than the low haematocrit anaemia with 8.11 %. Why low haematocrit had a higher attributable risk of anaemia is unclear as no significant differences were observed in their prevalence. Though, Sumbele *et al.* (2016), observed a similar AR of 7.6 % in anaemia caused by malaria in the study population. However, the AR of anaemia in relation to the moderate parasite density were 8.26 % and 8.33 % for both low haemoglobin and low haematocrit anaemia, respectively. The low estimate of the risk of haemoglobin and haematocrit anaemia that may be attributed to malaria in the children clearly indicate the important contribution of other inflammatory infections or diseases (Sumbele *et al.*, 2016). Though, studies regarding AR of anaemia due to malaria have seldom been reported out in Nigeria.

In this study, the antibody responses to *P. falciparum* antigen and the polymorphic markers in MSP1 and MSP2 isolates were used to examine genetic diversity and complexity of parasite populations in children with malaria infections in Minna, Niger State, Nigeria. There was a high seroprevalence of antibodies in general against *P. falciparum* antigens tested with about 74 %. The findings from this study was supported by previous work which reported a high seroprevalence and protective role of antibodies in the endemic Dielmo area (Diop *et al.*, 2014), and also in another study by Nazareth *et al.* (2017), where a synthetic malaria antigen was used, with about threequarters (78.80 %) of the participants positive for total IgG antibodies to the antigen (AS202.11 peptide).

Samples detected by microscopy with *P. falciparum* had higher specific antibody IgG responses compared to negative *P. falciparum* samples, suggesting a strong relationship between production of specific antibodies and *P. falciparum* transmission, rather than protective immunity, in other words, the studied specific antibody IgG responses appeared thus to be associated with malaria infection rather than anti-malarial protective immunity and this could also be a proof that the antibody served as a potential markers of both exposure to *P. falciparum* and protection against disease. This was discussed by Sarr *et al.* (2007), in their study which states that children with *P. falciparum* infection had higher specific antibody responses compared to negative infection children. Meanwhile, of the 9 % of the samples tested *P. falciparum* negative by microscopy, more than half 5 % produced antibodies to *P. falciparum* antigens. This could also be a proof of a submicroscopic parasite and is evident for a boosting immune responses by these sub-microscopic parasites. This observation was in line with other studies from low transmission settings (Shekalaghe *et al*., 2009 and Giha *et al.,* 2010), but was not in agreement with a previous study in an intense malaria transmission setting in Uganda (Proietti *et al.,* 2011), and also in Kenya, Lake Victoria (Idris *et al.,* 2017).

However, associations between antibody responses to *P. falciparum* antigen and the risk of *P. falciparum* infection have shown to be statistically non-significant. This observation was inconsistent in multiple studies (Fowkes *et al.,* 2010). Potential reasons for these inconsistencies may include differences in the intensity and stability of transmission, allelic variation of specific antigens and IgG subclass switching (Marsh *et al.,* 2006).

Seroprevalence to *P. falciparum* antibodies was, however, fairly similar for all the cohorts of children (between 20 % and 25 %), with no statistical significance between them. There were

conflicting results as to whether the production of antibodies as a result of *P. falciparum* infection increases or decreases with age. This was observed in a previous study by Nazareth *et al.* (2017), which observed a statistical difference in the seropositivity between the youngest (≤12 years) and the other age categories; but did not differ significantly between the middle age (13–40 years) and the older (>40 years) categories. In another previous studies conducted by Niang *et al.* (2017), they observed a low (47.19 %) and high (89.45 %) seroprevalences to Pfsch07/03 (*P. falciparum* schizonts) antibodies respectively, in children and older adults. This was further elucidated from this study where all control samples (which were adults) produced antibodies against *P. falciparum* antigens and thus could also be as a result of more previous exposures to malaria infection in the adults. In contrast, it was also reported from a previous study that specific IgG responses increased progressively in children aged from one- to five years and then stay high until eight. In another study by Idris *et al.* (2017), it states that *P. falciparum* antibody prevalence increased with age. In the present study, the fact that seroprevalence to *P. falciparum* antibodies was however fairly similar for all the cohorts of children, *P. falciparum* did not appear to be a factor of variation of the age-dependent development of anti-malarial IgG responses.

Seroprevalence to *P. falciparum* antibodies observed between male and females were similar (36 % and 37 %, respectively) with no statistical significance between them, indicating similar exposure between the two groups.

The influence of microscopically detectable Parasite Densities PD to prevalence of antibody responses in this study shows that samples with high PD showed the highest seroprevalence, with 90% that produced antibodies against the *P. falciparum* antigens. This was then followed by samples with low PD with 81 % and the least seroprevalence in this category was found in

samples with moderate PD 72 %. Contrary to previous findings in Lake Victoria, Kenya by Idris *et al.* (2017), an increase IgG antibody levels was observed with parasite densities. Also, in line with previous studies by Idris *et al.* (2017), the differences in the seroprevalence of *P. falciparum* antibodies from this study was not significant (P>0.05) between parasite densities (low, moderate and high parasitemia) and no significant association exists between them as measured by ELISA; and this was also as observed in a similar work conducted by Ismail *et al.* (2017), which states that there was no correlation between initial parasitemia and the presence of either IgG or IgM with any of the tested antigens, as measured by ELISA. The result obtained from this study was quite similar with a previous work conducted between two villages by Sarr *et al.* (2007), that states that a correlation was observed between the intensity of malaria infection (number of *P. falciparum* parasite/µl of blood) and Antibody responses in Mboula (r = 0.332, P = 0.02), whereas in Gankette Balla the correlation (r = 0.047) was not significant.

Significant difference was not observed between IgG antibody response and anaemia with both low haemoglobin and haematocrit anaemia having a very close antibody response rate to *P. falciparum*. This was not in line with a previous study of Idris *et al.* (2017), which observed antibody responses, negatively associated with anaemia in different locations in their study (Mfangano, Takawiri and Ngodhe). Plasma samples with normal haemoglobin and haematocrit had antibody IgG response rate of 10.75 % and 11.82 %, respectively. Interestingly, all plasma samples (also microscopically positive for *P. falciparum*) with normal haemoglobin and haematocrit level produced antibodies IgG as measured by ELISA against *P. falciparum* antigens, the reason for this remain obscured. In the other way round, not all plasma samples with low haemoglobin and haematocrit anaemia produced antibodies IgG as measured by ELISA against *P. falciparum* antigens.

Furthermore, for MSP1 allelic family, MAD20 which had the highest allele frequency in this gene recorded 31 isolates (62.00 %) and yielded four fragments within the range of 100-500 bp. Amplification of the allelic family K1 recorded 21 isolates (42.00 %) and yielded three bands within the range of 250-1000 bp. Allelic family RO33 which was the lowest in MSP1 gene was recorded only 5 isolates (10.00 %) and yielded two fragments 400 and 500 bp. This was not quite similar with the observations of Olasehinde *et al.* (2012) which detected proportion of isolates with K1 family was 68.00 % with 4 alleles in the range of 100 to 300 bp. Proportion of isolates with

MAD20 family was 40.00 % and a total of 3 alleles were observed within 100 to 300 bp. RO33 proportion was 20.00 % and the family was observed to be monomorphic with an allele size of 200 bp.

The predominant allele types for this study was MAD20 (MSP1) and FC27 (MSP2) with more than half of the study population infected. This observation was not in conformity with previous works conducted in South-west Ethiopia, Sudan and Malaysia, which detected the K1, RO33 and

RO33 allelic family as predominant (Abdel Hamid *et al.,* 2013; Atroosh *et al.,* 2011 and Mohammed *et al*, 2015). However, this was in conformity with a previous work of Soe *et al.* (2017) which observed MAD20 type as the most prevalent for MSP1. Meanwhile, previous works from Ehiopia, Kenya, Congo Brazzaville Myanmar and other sub-Saharan-African countries detected 3D7 as the predominant allele in the MSP2 gene (Mayengue *et al.,* 2011; Mwingira *et al.,* 2011 and Soe *et al.* 2017), which was contrary to what was observed from this study with FC27 as the predominant allele.

Indeed, each of the sample collection sites of *P. falciparum* population were carrying different predominant allele. FC27 and K1 were the predominant allele for samples collected from

apparently healthy children from the community and RO33 was not observed in this group. On the other hand, FC27 and MAD20 were predominant for samples collected from the six healthcare facilities. These findings was in line with a previous study conducted by Abd Razak *et al*. (2016), which highlighted the occurrence of predominant family genotype of MSP1 and MSP2 (K1 and FC27 for Kalabakan population and MAD20 and 3D7 for Kota Marudu population). However, significant difference was not observed between the sites of sample collection and all the allelic families for both MSP1 and MSP2 except for RO33 which observed a significant difference. The observation of lack of differences in gene variants across almost all the sites may be due to the reported case of population migration into Niger state, and Minna in particular (Daniyan and Muhammed, 2018). Migrants from the rural areas move to Minna especially during the dry season to become migrant workers for economic reasons before the rain begins, to go back to their farm; or for visiting extended families which is an important cultural practice. As such parasites genes are easily carried from one place to the other which results in variants with non-significant differences in alleles investigated across the Minna.

Only one-third of the population (35.00 %) harboured multiple genotypes of 2, 3 or 4 from a single isolate of either or both MSP1 and MSP2 alleles; and about one-fourth (25.00 %) polyclonal. The high level of mixed infections indicates a high transmission intensity in Minna. The frequency was quite lower than what was obtained from a previous study by Abdel Hamid *et al.* (2013), and

Mohammed *et al.* (2015), who reported that almost two-third of the samples (62.00 %) and (59.00 %) harboured multiple genotypes, respectively.

Generally, the determined multiplicity of infections (MOIs) between the different sites was not significant among all the study sites. This was also obtained in the previous study of Oyebola *et al.* (2014), in two different locations (Ikorodu and Lekki) in south-west Nigeria where no significant difference in the MOI between those two study areas were observed. Also, in another similar study conducted in Ghana by Duah *et al.* (2016), using only the polymorphic antigenic marker, MSP2, they observed generally no significant difference in the MOIs between the different ecological zones of Ghana.

The overall mean multiplicity of infection observed in this study were 1.23 and 1.17 for the genetic markers MSP1 and MSP2, respectively which was lower than that observed by Mohammed *et al.* (2015) with 1.37 and 1.20 for MSP1 and MSP2, respectively, and also from results in Côte d'Ivoire, where their MOI was found to be 2.88 MSP2 (Silue *et al.*, 2006). This discrepancy may be due to differences in geographical areas and their transmission patterns and may also be due to differences in sample population. Generally, however, the higher the malaria transmission level, the greater becomes the tendency to get a higher MOI and mean number of alleles per locus (Mohammed *et al.*, 2015). Epidemiologic data from some study sites in Africa suggest that the multiplicity of *P. falciparum* infection may be directly related to the intensity of transmission.

The overall MOI for samples collected from the hospital (symptomatic) was lower 2.21 than what was observed from apparently healthy children from the communities (asymptomatic) 2.41. This observation was in conformity with the observation of Farnert *et al*. (1999), which indicate that symptomatic infection generally appears to have a lower MOI as compared to asymptomatic patients living in high transmission areas.

Meanwhile, samples with low parasite density had the highest MOIs of MSP1 and MSP2 compared with those of moderate parasite density; and a significant difference between MOI and the parasite densities was observed but no significant correlation exist between them. This finding was in line with a previous study conducted by Mohammed *et al.* (2015), in a study, which states there was no any significant correlation between multiplicity of infection and parasite density of patients

(Spearman rank coefficient = 0.03; $P = 0.8$), and was not in line with the study by Ghanchi *et al.* (2010), which also observed no significant differences between parasite density and mean multiplicity of infection in MSP1 and MSP2. Likewise, when considering the MOIs of samples with malarial anaemia, cases of low haemoglobin and haematocrit anaemia had the highest MOI as compared with samples with normal haemoglobin and haematocrit. Statistical significant difference was not observed and no significant correlation ($P>0.05$) between MOI and low haemoglobin anaemia haematocrit anaemia. On the other hand, the present study found a nonsignificant association between MOI and age. In a similar study conducted by Abdel Hamid *et al.* (2013), they unfolded that the association between age and multiplicity of infection was not well understood.

From this study, it was observed generally that *P. falciparum* populations were not genetically diverse (0.0636) but findings from this study also revealed a high level of genetic diversity within the antigenic markers- MSP1 and MSP2 (0.714 and 0.830, respectively) indicating a larger genotype diversity within the MSP1 and MSP2 loci. This was common to the genotyping of antigenic marker in African regions such as Malaysia, Burkina Faso, Sao Tome, Malawi, Uganda and Tanzania which have identified *P. falciparum* populations with alleles occurring at a

very high *He* level (0.78 to 0.99) (Mwingira *et al.,* 2011 and Abd Razak *et al.,* 2016). Mohammed *et al.*

(2015), also observed a high *He* level of 0.79 for MSP1 and was observed higher than the moderate *He* levels observed in MSP2 (0.54).

Meanwhile, low level genetic diversity of less than 0.2 was observed in all the different age groups and between male and female groups. However, the lack of major differences in parasite variants across Minna may be as a result of a high level of gene flow due to human population movements. Moreover, malaria control program across Niger state by the distribution of long lasting insecticide treated nets (LLINs) across the 555 health facilities and improved service delivery to control and treat malaria may cause the genetic drift and decrease the level of *He* and MOI.

This study shows a close link between the presence of *P. falciparum* infection and the level of antibody IgG response and genetic diversity. Moreover, as the genetic diversity is expected to decline in the face of malaria elimination, the information provided herein may serve as the baseline data for ongoing epidemiological monitoring in this endemic area. The limitation of this study was the small sample size obtained.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

A high prevalence of *P. falciparum* of more than 70 % has been reported in Minna from this study. Children below the age of five especially in the male category recorded the highest prevalence rates of *P. falciparum.* Meanwhile, a half of the blood samples collected from the asymptomatic children were infected with *P. falciparum.* Though, symptomatic cases observed the highest prevalence as compared to the asymptomatic cases, the high prevalence of asymptomatic cases was quite disturbing due to the large proportion of asymptomatic plasmodial infections that are likely to act as a transmission reservoir. This has significant implications for ongoing malaria control programs based on treatment of symptomatic cases. The relationship between age and parasite prevalence was similar in both male and female category, decreasing with age and the peak observed in children below the age of five.

Findings from this study has confirmed that anaemia is a severe public health problem in children. Moreover, the indices of anaemia revealed from this study an appreciable prevalence levels of malarial anaemia. Interestingly, asymptomatic children from the community also experienced an appreciable number of low haemoglobin and haematocrit anaemia with positive *P. falciparum* infection, and children below the age of five observed the highest prevalence. Subsequently, *P. falciparum* positive cases with mild anaemia observed the highest prevalence from this study.

Moreover, evidence from this study revealed a high seroprevalence of IgG antibody against *P. falciparum* antigen. The majority of these samples were infected with *P. falciparum* infection as

compared to negative cases. This, suggests a strong relationship between production of specific antibodies and *P. falciparum* transmission, rather than protective immunity. Though, associations between measured antibody responses to *P. falciparum* antigen and the risk of malaria have shown to be statistically non-significant. In addition, antibody responses in plasma from children with mild, moderate and high *P. falciparum* malaria were tested. Though, the differences in the seroprevalence of *P. falciparum* antibodies was not significant between mild, moderate and severe *P. falciparum* infection, the seroprevalence was observed at its peak in cases with severe *P. falciparum* infection.

Importantly, findings from this study revealed a fairly similar seroprevalence with age and sex, with no statistical significance between them. However, cases with microscopically detectable high parasite density (severe malaria) observed the highest seroprevalence, and this suggests that immune responses are more stable in this category. Similarly, an evidence (of more than 50%) was found for a boosting of immune responses by sub-microscopic parasite from this study and consequently, sub-microscopic infections will provide an antigenic stimulus to maintain immune responses.

Interestingly, all plasma samples (also microscopically positive for *P. falciparum*) with normal haemoglobin and haematocrit level produced antibody IgG as measured by ELISA against *P. falciparum* antigens, the reason for this remain obscured. In the other way round, though more than half of the population with anaemia also produced IgG antibody against *P. falciparum,* not all plasma samples with anaemia produced antibody IgG as measured by ELISA against *P. falciparum* antigens.

Parasites from blood samples subjected to PCR revealed the predominant allele types for this study as MAD20-MSP1 and FC27-MSP2. Furthermore, it was observed that most patients had both

MSP1-MAD20 and MSP2-FC27 allelic variants and in some cases with three allelic types, MSP1K1, MSP1-MAD20 and MSP2-FC27, this combination was observed most.

When the parasites in cases with low and moderate parasite densities in this study were analyzed, more genotypes were generally found in moderate parasite density with MSP1-MAD20 and MSP2-FC27 having more cases, but moderate parasite density was not significantly higher than cases with low parasite density for all the alleles of MSP1 and MSP2.

Indeed, each of the sample collection sites of *P. falciparum* population were carrying different predominant allele. FC27 and K1 were the predominant allele in samples collected from apparently healthy children from the community and RO33 was not observed from the category. On the other hand, FC27 and MAD20 were predominant for samples collected from the six healthcare facilities. Generally, the determined multiplicity of infection (MOIs) between the different sites was not significant among all the study sites. When the total MOI of the parasites was considered, mild malaria cases with low parasite density had higher numbers of genotypes.

It was generally observed from this study that the malaria transmission level or the intensity of transmission (MOI) was low as compared to other studies, but however, asymptomatic cases revealed a higher intensity of transmission as against the symptomatic cases. This indicates an increased risk of malaria transmission as a result of reservoir infection.

Low level of genetic diversity was observed in the parasite population in Minna. However, upon investigating the MSP1 and MSP2 genes, a clear genetic diversity was observed in the parasite population.

This study showed that both the occurrence of certain allelic variants of parasites and a higher MOI as well as presence of high levels of antibodies could be protective against the development of severe malaria.

Together, these observations suggest that serological analysis could be effective as an adjunct tool used in combination with parasite prevalence for surveillance, control and elimination malaria in highly endemic areas. This study has demonstrated the potential of malarial antibody and gene diversity as important markers for evaluating differences in malaria transmission intensity. The simultaneous measurement of parasite prevalence and serology paved way to compare malariometric indices across various populations and verify the heterogeneity in malaria transmission. The use of serology to evaluate naturally acquired immune responses to malaria can provide promising information for future research, especially in evaluating malaria elimination control interventions in high transmission settings where reductions are most difficult to achieve and sustain.

5.2 Recommendations

Based on the findings from this research, it is therefore recommended thus:

i. Drug-mediated strategies that explicitly target the asymptomatic parasite reservoir should be introduced into the communities.

ii. Scale up of intervention programmes should be introduced such as the promotion of insecticidetreated bed nets (ITNs), the introduction and regulation on the use of artemisinin-based combination therapy (ACT) for malaria chemotherapy which may further decrease the genetic diversity of malaria parasite populations.

iii. Continuous malaria genetic surveillance is important in monitoring the intervention programmes effectiveness. However, monitoring of antimalarial drug efficacy with current genotyping strategies should be enhanced, as well as emphasizing the importance of PCR correction, improved study design and statistical methods.

iv. Greater consideration and attention should be paid to population living in rural areas, towns and villages where there are no secondary or specialize health care facilities. Such areas should be designated as high priority targets for malaria intervention and control programs. These efforts should be doubled during the malaria transmission season which coincides with the rainy season.

Government should also ensure that these communities have access to quality and affordable Artemisinin-based combination therapy (ACTs) in view of being the most powerful weapon against malaria at the moment.

v. The contribution of malaria to the public health problem of anaemia should be interpreted with caution and further research should be carried out in different ecological settings and a larger population.

vi. For future studies, an ELISA is a good start to establish that antibodies are formed against a particular antigen, but in addition, it is important to pick a combination of functional assays, such as invasion inhibition assays (IIAs) and/or Surface Plasmon Resonance (SPR) assays, to aid in understanding how malaria immunity is established and to be able to prioritize antigens for vaccine trials. When merozoite antigens are considered as vaccine candidates, variations between mild and severe malaria should be considered in the evaluations as vaccines that minimize severe disease and mortality would have a significant public health value.

5.3 Contribution to Knowledge

1. This study revealed a high seroprevalence of antibodies in general against *P. falciparum* antigens tested in samples collected from children in Minna and it suggests a strong relationship between production of specific antibodies and *P. falciparum* transmission, rather than protective immunity. Interestingly, all microscopically positive *P. falciparum* plasma samples with normal haemoglobin and haematocrit level produced antibodies IgG as measured by ELISA against *P. falciparum* antigens.

2. The predominant allele observed from this study were MAD20 for MSP1 and FC27 for MSP2 and meanwhile, the predominant allele varies according to the presence or absence of fever: FC27 and K1 were the predominant alleles for asymptomatic cases. On the other hand, FC27 and MAD20 were predominant alleles for symptomatic infections.

3. This study also revealed a high genetic diversity of MSP1 and MSP2 antigenic markers in children in Minna. The *He* indicates the diversity of the gene and it represents the probability of

being infected by two parasites with different alleles at a given locus. However, a low *He* was generally observed in Minna.

REFERENCES

Abd Razak, M. M. R., Sastu, U. R., Norahmad, N. A., Abdul-Karim, A., Muhammad, A. & Muniandy, P. K. (2016). Genetic Diversity of *Plasmodium falciparum* Populations in Malaria Declining Areas of Sabah, East Malaysia. *PLoS ONE*, 11(3), e0152415.

Abdel Hamid, M. M., Mohammed, S. B. & El Hassan, I. M. (2013). Genetic Diversity of *Plasmodium falciparum* Field Isolates in Central Sudan Inferred by PCR Genotyping of Merozoite Surface Protein 1 and 2.*North American Journal of Medical Science*, 5(2), 95–101.

Al-Mekhlafi, A. M., Al-Mekhlafi, H. M., Mahdy, M. A., Azazy, A. A. & Fong, M.Y. (2011). Human malaria in the highlands of Yemen. *Annals of Tropical & Medical Parasitol*ogy, 105, 187–95.

Amodu, O. K Adeyemo, A. A., Ayoola, O. O., Gbadegesin, R. A., Orimadegun, A. E., Akinsola, A. K., Olumese, P. E. & Omotade, O. O. (2005). Genetic diversity of the *msp-1* locus and symptomatic malaria in south-west Nigeria. *Acta Tropica*, 95(3), 226-232.

Anderson, T. J., Haubold, B., Williams, J. T., Estrada-Franco, J. G., Richardson, L. & Mollinedo, R. (2000). Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Molecular Biology & Evolution*, 17(10), 1467–82.

Angeletti, D., Sandalova, T., Wahlgren, M., Achour, A. & Blader, I. J. (2015). "Binding of subdomains 1/2 of PfEMP1-DBL1α to heparan sulfate or heparin mediates *Plasmodium falciparum* rosetting". *PLoS One*, 10(3), e0118898.

Apio, B., Nalunkuma, A., Okello, D., Riley, E. & Egwang, T. G. (2000). Human IgG subclass antibodies to the 19 kilo dalton carboxy terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 (MSP-119) and predominance of the MAD20 allelic type of MSP-1 in Uganda. *East African Medical Journal*, 77, 189–93.

Arnott, A., Barry, A. E. & Reeder, J. C. (2012). Understanding the population genetics of *Plasmodium vivax* is essential for malaria control and elimination. *Malaria Journal*, 11, 14.

Atroosh, W. M., Al-Mekhlafi, H. M., Mahdy, M. A., Saif-Ali, R., Al-Mekhlafi, A. M. & Surin, J. (2011). Genetic diversity of *Plasmodium falciparum* isolates from Pahang, Malaysia based on *MSP-1* and *MSP-2* genes. *Parasite Vectors*, 4, 233.

Avril, M., Bernabeu, M., Benjamin, M., Brazier, A. J. & Smith, J. D. (2016). "Interaction between endothelial protein C receptor and intercellular adhesion molecule 1 to mediate binding of *Plasmodium falciparum*-infected erythrocytes to endothelial cells". *mBio.* 7 (4), e00615– 16.

Avril, M., Brazier, A. J., Melcher, M., Sampath, S., Smith, J. D. & Rogerson, S. J. (2013). "DC8 and DC13 var genes associated with severe malaria bind avidly to diverse endothelial cells". *PLoS Pathogens*, 9, 6.

Avril, M., Tripathi, A. K., Brazier, A. J., Andisi, C., Janes, J. H., Soma, V. L., Sullivan, D. J., Bull, P. C., Stins, M. F. & Smith, J. D. (2012). "A restricted subset of var genes mediates adherence of *Plasmodium falciparum*-infected erythrocytes to brain endothelial cells". *Proceedings of the National Academy of Sciences,* 109 (26), E1782–E1790.

Babiker, H. A., Abdel-Muhsin, A. A., Hamad, A., Mackinnon, M. J., Hill, W. G. & Walliker, D. (2000). Population dynamics of *Plasmodium falciparum* in an unstable malaria area of eastern Sudan. *Parasitology,* 120:105–11.

Barcus, M. J., Basri, H., Picarima, H., Manyakori, C., Sekartuti, Elyazar, I., Bangs, M. J., Maguire, J. D. & Baird, J. K. (2007). Demographic risk factors for severe and fatal *vivax* and *falciparum* malaria among hospital admissions in northeastern Indonesian Papua. *American Journal of Tropical Medicine and Hygiene*, 77, 984-991.

Barragan, A., Fernandez, V., Chen, Q., von Euler, A., Wahlgren, M. & Spillmann, D. (2000). The duffy-binding-like domain 1 of Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1) is a heparan sulfate ligand that requires 12 mers for binding. *Blood,* 95, 35943599.

Barry, A. E., Schultz, L., Senn, N., Nale, J., Kiniboro, B., Siba, P.M., Mueller, I. & Reeder, J.C. (2013). High levels of genetic diversity of *Plasmodium falciparum* populations in Papua New Guinea despite variable infection prevalence. *American Journal of Tropical Medicine and Hygiene*, 88(4), 718–725.

Baruch, D. I., Gormley, J. A., Ma, C., Howard, R. J. & Pasloske, B. L. (1996). *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proceedings of National Academic Science, U S A*, 93, 3497–3502.

Baruch, D. I., Ma, X. C., Singh, H. B., Bi, X., Pasloske, B. L. & Howard, R. J. (1997). "Identification of a region of PfEMP1 that mediates adherence of *Plasmodium falciparum* infected erythrocytes to CD36: conserved function with variant sequence". *Blood,* 90(9), 3766–3675.

Bashawri, L. A., Mandil, A. A., Bahnassy, A. A. & Ahmed, M. A. (2002). Malaria: hematological aspects. *Annals of Saudi Medicine,* 22, 372-376.

Bates, I., Fenton, C., Gruber, J., Lalloo, D., Medina, L. A.… & Squire, S. B. (2004). Vulnerability to malaria, tuberculosis, and HIV/AIDS infection and disease. Part 1:

determinants operating at individual and household level. *Lancet Infectious Disease*, 4, 267–77.

Baum, J., Richard, D., Healer, J., Rug, M., Krnajski, Z., Gilberger, T. W., Green, J. L., Holder, A. A. & Cowman, A. F. (2006). A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. *Journal of Biological Chemistry*, 281, 5197–208.

Beeson, J. G., Drew, D. R., Boyle, M. J., Feng, G., Fowkes, F. J. I. & Richards, J. S. (2016). Merozoite surface proteins in red blood cell invasion, immunity and vaccines against malaria. *FEMS Microbiology Reviews,* 40(3), 343–372.

Berendt, A. R., Turner, G. D. H. & Newbold, C. I. (1994). Cerebral malaria: the sequestration hypothesis. *Parasitology Today*, 10, 412–414.

Bernabeu, M. & Smith, J. D. (2016). "EPCR and malaria severity: The center of a perfect storm". *Trends in Parasitology,* S1471–4922(16), 30198–2.

Bhatt, S., Weiss, D. J., Cameron, E., Bisanzio, D., Mappin, B., Darlrymple, U., Battle, K… & Gething, P. W. (2016). The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature*, 526, 207–211.

Biemba, G., Dolmans, D., Thuma, P. E., Weiss, G. & Gordeuk, V. R. (2000). Severe anaemia in Zambian children with *Plasmodium falciparum* malaria. *Tropical Medicine and International Health,* 5, 9–16.

Blasco, B., Leroy, D. & Fidock, D. A. (2017). Antimalarial drug resistance: Linking *Plasmodium falciparum* parasite biology to the clinic. *Nature Medicine,* 23, 917.

Bonday, Z. Q. (2002). "Import of host delta-aminolevulinate dehydratase into the malarial parasite: Identification of a new drug target". *Nature Medicine,* 6 (8), 898–903.

Borghino, D. (2015). "Malaria vaccine candidate shown to prevent thousands of cases". *www.gizmag.com*. Retrieved 2016-06-11.

Bourdy, M., Willcox, L., Ginsburg, H., Rasoanaivo, P., Graz, B. & Deharo, E. (2008). Ethnopharmacology and malaria: New hypothetical leads or old efficient antimalarials? *International Journal for Parasitology,* 38, 33–41.

Boyle, M. J., Wilson, D. W. & Beeson, J. G. (2013). New approaches to studying *Plasmodium falciparum* merozoite invasion and insights into invasion biology. *International Journal of Parasitol*ogy, 43, 1–10.

Boyle, M. J., Wilson, D. W., Richards, J. S., Riglar, D. T., Tatteh, K. K., Conway, D. J., Ralph, S. A., Baum, J., Beeson, J. G. (2010). Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development. *Proceedings of National Academic of Science USA*, 107, 14378–83.

Bull, P. C. & Abdi, A. I. (2016). "The role of PfEMP1 as targets of naturally acquired immunity to childhood malaria: prospects for a vaccine". *Parasitology,* 143(02), 171–186.

Bull, P. C., Berriman, M., Kyes, S., Quail, M. A., Hall, N., Kortok, M. M., Marsh K. & Newbold, C. I. (2005). *Plasmodium falciparum* Variant Surface Antigen Expression Patterns during Malaria. *plos.* 1(3), e26.

Bull, P. C., Lowe, B. S., Kortok, M., & Marsh, K. (1999). Antibody Recognition of *Plasmodium falciparum* Erythrocyte Surface Antigens in Kenya: Evidence for Rare and Prevalent Variants. *Infection and Immunity*, 67(2), 733–739. S. H. E. Kaufmann (Ed.).

Bull, P. C., Lowe, B. S., Kortok, M., Molyneux, C. S., Newbold, C. I. & Marsh, K. (1998). Parasite antigens on the infected red cell are targets for naturally acquired immunity to malaria. *Nature Medicine*, 4, 358–360.

Caraballo, H. (2014). "Emergency department management of mosquito-borne illness: Malaria, dengue, and west nile virus". *Emergency Medicine Practice,* 16, 5.

Castro-Gomes, T., Mourão, L. C., Melo, G. C., Monteiro, W. M., Lacerda, M. V. G. & Braga, E. M. (2014). Potential Immune Mechanisms Associated with Anemia in Plasmodium vivax Malaria: a Puzzling Question. *Infection and Immunity,* 82(10), 3990-4000.

CDC. Centre for Disease Control. (2016).

Chaitarra, V., Holm, I., Bentley, G. A., Petres, S. & Longacre, S. (1999). The crystal structure of C-terminal merozoite surface protein 1 at 1.8 resolution, a high protective malaria vaccine candidate. *Molecular Cell,* 3, 457–64.

Chan, J. A., Howell, K., Reiling, L., Ataide, R., Mackintosh, C., Fowkes, F., Petter, M., Chesson, J., Langer, C. & Warimwe, G. (2012). "Targets of antibodies against *Plasmodium falciparum*-infected erythrocytes in malaria immunity".*Journal of Clinical Investigation*, 122, 3227–3238.

Cheesbrough, M. (2009). *District Laboratory Practice in Tropical Countries*. Part 1-2. Edinburg Building, UK: Cambridge University Press.

Cheesbrough, M. (2010). *District Laboratory Practice in Tropical Countries*. Part 1, 2nd edition update. Cambridge University Press, UK. Pp 239-258

Chen, Q., Barragan, A., Fernandez, V., Sundstrom, A., Schlichtherle, M., Sahlen, A., Carlson, J., Datta, S. & Wahlgren, M. (1998). Identification of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) as the rosetting ligand of the malaria parasite *P. falciparum*. *Journal of Experimental Medicine,* 1187, 15-23.

Chen, Q., Schlichtherle, M. & Wahlgren, M. (2000). Molecular aspects of severe malaria. *Clinical Microbiology Reviews*, 13(3), 439-450.

Claessens, A., Adams, Y., Ghumra, A., Lindergard, G., Buchan, C. C., Andisi, C., Bull, P. C… & Mok, S. (2012). "A subset of group A-like var genes encodes the malaria parasite ligands

for binding to human brain endothelial cells". *Proceedings of the National Academy of Sciences*, 109(26), E1772–E1781.

Clifton, N. J. (1984). Methods in Molecular Biology. 1, 349-355. DOI: 10.1385/0-89603-0628:349.

Cowman, A. F. & Crabb, B. S. (2006). "Invasion of red blood cells by malaria parasites". *Cell*, 124(4), 755–766.

Crabb, B. S. & Cowman, A. F. (2002). "*Plasmodium falciparum* virulence determinants unveiled". *Genome Biology*, 3, 11.

Crompton, P. D., Pierce, S. K. & Miller, L. H. (2010). Advances and challenges in malaria vaccine development. *Journal of Clinical Investigations*, 120, 4168–4178.

Crosnier, C., Bustamante, L. Y. & Bartholdson, S. J. (2011). Basigin is a receptor essential for erythrocyte invasion by *Plasmodium falciparum*. *Nature*, 480, 534–7.

Daniyan, M. N. & Muhammed, M. (2018). Analysis of Trend and Dynamics of Urban Sprawl in Minna Niger State Nigeria. *Proceedings of the International Conference on Global and Emerging Trends*, 2645-2405.

Dawaki, S., Al-Mekhlafi, H. M., Ibrahim, J., Atroosh, W. M., Abdulsalam, A. M., Sady, H., Elyana, F. N., Adamu, A. U., Yelwa, S. I., Ahmed, A., Al-Areeqi, M. A., Subramaniam, L. R., Nasr N. A. & Lau, Y. (2016). Is Nigeria winning the battle against malaria? Prevalence, risk factors and KAP assessment among Hausa communities in Kano State. *Malaria Journal*, 15, 351.

Deitsch, K. W. & Chitnis, C. E. (2012). *"Molecular basis of severe malaria"*. *Proceedings of the National Academy of Sciences*, 109 (26), 10130–10131.

Deshmukh, A. S., Srivastava, S. & Dhar, S. K. (2013). *"Plasmodium falciparum:* epigenetic control of var gene regulation and disease". In Kundu, T. K. Epigenetics: Development and Disease. *Dordrecht: Springer*, 659–682.

Diop, F., Richard, V., Diouf, B., Sokhna, C., Diagne, N., Trape, J. F., Faye, M. M., Tall, A., Diop, G. & Balde, A. T. (2014). Dramatic declines in seropositivity as determined with crude extracts of *Plasmodium falciparum* schizonts between 2000 and 2010 in Dielmo and Ndiop, Senegal. *Malaria Journal*, 13, 83.

Doolan, D. L., Dobano, C. & Baird, J. K. (2009). *Acquired immunity to malaria*. *Clinical Microbiology Review*, 22, 13–36.

Dorland's Illustrated Medical Dictionary, *Elsevier*. (32nd edition)

Duah, N. O., Matrevi,S. A., Quashie, N. B., Abuaku, B. & Koram, K. A. (2016). Genetic diversity of *Plasmodium falciparum* isolates from uncomplicated malaria cases in Ghana over a decade. *Parasites & Vectors*, 9, 416.

Elliott, S. R.,Fowkes, F. J., Richards, J. S., Reiling, L., Drew, D. R. & Beeson, J. G. (2014). *Research priorities for the development and implementation of serological tools for malaria surveillance,* 6, 100.

English, M., Ahmed, M., Ngando, C., Berkley, J. & Ross, A. (2002). Blood transfusion for severe anaemia in children in a Kenyan hospital. *Lancet,* 359, 494-495.

Epidi, T. T., Nwani, C. D. & Ugorji, N. P. (2008). Prevalence of malaria in blood donors in Abakaliki metropolis, Nigeria. *Scientific Research and Essay,* 3, 162-164.

Erhabor, O., Adias, T. C., & Hart, M. L. (2010). Effects of falciparum malaria on the indices of anaemia among pregnant women in the Niger Delta of Nigeria. *Journal of Clinical Medicine and Research*, 2(3), 035-041.

Escalante, A. A., Lal, A. A. & Ayala, F. J. (1998). Genetic polymorphism and natural selection in the malaria parasite *Plasmodium falciparum. Genetics*, 149, 189-202.

Fairhurst, R. M. & Wellems, T. E. (2010). "Chapter 275. *Plasmodium* species (malaria)". In Mandell, G. L., Bennett, J. E., Dolin, R. Douglas, M. Bennett's Principles and Practice of Infectious Diseases. 2 (7th ed.). Philadelphia, Pennsylvania: Churchill Livingstone/Elsevier. pp. 3437–62. ISBN 978-0-443-06839-3.

Farnert, A., Rooth, I., Svensson, A., Snounou, G. & Bjorkman, A. (1999). Complexity of *Plasmodium falciparum* infections is consistent over time and protects against clinical disease in Tanzanian children. *Journal of Infectious Diseases,* 179989–995.

Färnert, A., Williams, T. N., Mwangi, T. W., Ehlin, A., Fegan, G., Macharia, A., Lowe, B. S., Montgomery, S. M. & Marsh, K. (2009). Transmission-dependent tolerance to multiclonal *Plasmodium falciparum* infection. *Journal of Infectious Disease,* 200, 1166–1175.

Felger, I., Irion, A., Steiger, S. & Beck, H.-P. 2. (1999). Genotypes of Merozoite Surface protein 2 of *Plasmodium falciparum* in Tanzania. *Transaction of the Royal Society of Tropical Medicine & Hygiene,* 93, 3–9.

Ferreira, M. U. & Hart, D. L. (2007). *Plasmodium falciparum*: worldwide sequence diversity and evolution of the malaria vaccine candidate merozoite surface protein-2 (MSP-2). *Experimental Parasitology*, 115, 32–40.

Foquet, L., Hermsen, C., van Gemert, G-J., Van Braeckel, E., Weening, K., Sauerwein, R., Meuleman, P. & Leroux-Roels, G. (2014). "Vaccine-induced monoclonal antibodies targeting circumsporozoite protein prevent Plasmodium falciparum infection". *Journal of Clinical Investigation,* 124 (1), 140–4.

Forsyth, K. P., Philip, G., Smith, T., Kum, E., Southwell, B. & Brown, G. V. (1989). Diversity of antigens expressed on the surface of erythrocytes infected with mature *Plasmodium falciparum* parasites in Papua New Guinea. *American Journal of Tropical Medicine & Hygiene*, 41, 259–265.

Fowkes, F. J., Boeuf, P. & Beeson, J. G. (2016). Immunity to malaria in an era of declining malaria transmission. *Parasitology,* 143, 139–153.

Fowkes, F. J., Richards, J. S., Simpson, J. A. & Beeson, J. G. (2010). The relationship between anti-merozoite antibodies and incidence of *Plasmodium falciparum* malaria: A systematic review and meta-analysis. *PLoS medicine,* **7:**e1000218.

Frech, C. & Chen, N. (2013). Variant surface antigens of malaria parasites: functional and evolutionary insights from comparative gene family classification and analysis. *BMC Genomics*, 14, 427.

Fried, M. & Duffy, P. E. (2015). "Designing a VAR2CSA-based vaccine to prevent placental malaria". *Vaccine*, 33(52), 7483–7488.

Friedman, J. F., Kwena, A. M., Mirel, L. B., Kariuki, S. K., Terlouw, D. J., Phillips-Howard, P. A., Hawley, W. A., Nahlen, B. L., Shi, Y. P. & ter Kuile, F. O. (2005). Malaria and nutritional status among pre-school children: results from cross-sectional surveys in western Kenya. *American Journal of Tropical Medicine and Hygiene.* 73: 698-704.

Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E… & Bowman, S. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature*, 419(6906), 498-511.

Ghanchi, N. K., Mårtensson, A., Ursing, J., Jafri, S., Bereczky, S., Hussain, R. & Beg, M. A. (2010). Genetic diversity among *Plasmodium falciparum* field isolates in Pakistan measured with PCR genotyping of the merozoite surface protein 1 and 2. *Malaria Journal*, 9, 1.

Giha, H. A., Rosthoj, S., Dodoo, D., Hviid, L., Satti, G. M. H., Scheike, T., Arnot, D. E. & Theander, T. G. (2000). The epidemiology of febrile malaria episodes in an area of unstable and seasonal transmission. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 94, 645-6.

Gilson, P. R. & Crabb, B. S. (2009). Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. *International Journal of Parasitology,* 39, 91–6.

Gosi, P., Lanteri C. A., Tyner, S. D., Se, Y., Lon, C… & Spring, M. (2013). Evaluation of parasite subpopulations and genetic diversity of the msp1, msp2 and glurp genes during and following artesunate monotherapy treatment of *Plasmodium falciparum* malaria in Western Cambodia. *Malaria J*ournal, 12, 403.

Greenwood, B. M., Bojang, K., Whitty, C. J. & Targett, G. A. (2005). "Malaria". *Lancet*, 365(9469), 1487–98.

Guinovart, C. & Alonso, P. L. (2007). Defining and Defeating the Intolerable Burden of Malaria III: Progress and Perspectives. *American Journal of Tropical Medicine and Hygiene*, 77(6).

Haldar, K. & Mohandas, N. (2009). Malaria, erythrocytic infection, and anemia. *Hematology American Society of Hematology Education Program,* 87-93.

Healer, J., Murphy, V., Hodder, A. N., Masciantonio, R., Gemmill, A. W., Anders, R. F., Cowmn, F. & Batchelor, A. (2004). Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in *Plasmodium falciparum*. *Molecular Microbiology*, 52:159–68.

Holder, A. A., Blackman, M. J., Burghaus, P. A., Chappel, J. A., Ling, I. T., McCallum-Deighton, N. & Shai, S. (1992). A malaria merozoite surface protein (MSP1)-structure, processing and function. *Memeriasdo Instituto Oswaldo Cruz,* 87(3), 37–42.

Howell, D. P. G., Samudrala, R. & Smith, J. D. (2006). "Disguising itself—insights into Plasmodium falciparum binding and immune evasion from the DBL crystal structure". *Molecular and Biochemical Parasitology,* 148(1), 1–9.

Howell, D. P.-G., Levin, E. A. Springer, A. L., Kraemer, S. M., Phippard, D. J., Schief, W. R. & Joseph D. (2007). "Mapping a common interaction site used by Plasmodium falciparum Duffy binding-like domains to bind diverse host receptors". *Molecular Microbiology,* 67 (1), 78–87.

Hsieh, F., Turner, L., Bolla, J. R., Robinson, C. V., Lavstsen, T. & Higgins, M. K. (2016). "The structural basis for CD36 binding by the malaria parasite". *Nature Communications*, 7, 12837.

Hughes, A. L. & Verra, F. (2002). Extensive polymorphism and ancient origin of *Plasmodium falciparum*. *Trends of Parasitology*, 18, 348-351.

Hussain, M. M., Kumar, S. R., Branch, O. H., Adak, T. & Raziuddin, M. (2011). Genetic diversity in merozoite surface protein-1 and 2 among *Plasmodium falciparum* isolates from malarious districts of tribal dominant state of Jharkhand, India. *Annals of Tropical Medicine and Parasitology*, 105(8), 579–592.

Hviid, L. & Jensen, A. T. R. (2015). "PfEMP1 – A Parasite Protein Family of Key Importance in *Plasmodium falciparum* Malaria Immunity and Pathogenesis". In Rollinson, D., Stothard, J.R. *Advances in Parasitology, Elsevier,* 51–84.

Idris, Z. M. D., Chan, C. W., Kongere, J., Hall, T., Logedi, J., Gitaka, J., Drakeley, C.& Kaneko, A. (2017). Naturally acquired antibody response to *Plasmodium falciparum* describes heterogeneity in transmission on islands in Lake Victoria. *Nature Research Journal*, 7, 9123.

Idro, R., Aloyo, J., Mayende, L., Bitarakwate, E., John, C. C. & Kivumbi, G. W. (2006). Severe malaria in children in areas with low, moderate and high transmission intensity in Uganda.
*Tropical Medicine and International Health.*11: 115-124.

Iqbal, J., Perlmann, P. & Berzins, K. (1993) Serological diversity of antigens expressed on the surface of erythrocytes infected with *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 87, 583–588.

Ismail, H. A., Ribacke, U., Reiling, L., Normark, J., Egwang, T., Kironde, F., Beeson, J. G., Wahlgren, M. & Persson, K. E. M. (2017). Acquired Antibodies to Merozoite Antigens in Children from Uganda with Uncomplicated or Severe *Plasmodium falciparum* Malaria. *Clinical and vaccine immunology*, 156.

Jakeman, G. N., Saul, A., Hogarth, W. L. & Collins, W. E. (1999). Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. *Parasitology*, 119, 127-133.

Jakobsen, P. H., Bate, C. A., Taverne, J. & Playfair, J. H. (1995). Malaria: toxins, cytokines and disease. *Parasite Immunology*, 17, 223-231.

Jamal, M., Hameed, A., & Imtiaz, F. (2015). Burden of Anemia in Malarial Parasite Infection. *Journal of Infectious Diseases & Therapy.* 3:228.

Jespersen, J. S., Wang, C. W., Mkumbaye, S. I., Minja, D. T. R., Petersen, B., Turner, L., Petersen, J. E. V., Lusingu, J. P. A., Theander, T. G. & Lavstsen, T. (2016). "*Plasmodium falciparum* var genes expressed in children with severe malaria encode CIDRα1 domains". *EMBO Molecular Medicine*, 8(8), 39–850..

Jootar, S., Chaisiripoomkere, W., Pholvicha, P., Leelasiri, A., Prayoonwiwat, W., Mongkonsvitragoon, W. & Srichaikul, T. (1993). Suppression of erythroid progenitor cells during malarial infection in Thai adults caused by serum inhibitor. *Clinical Laboratory Haematology*, 15, 87-92.

Kadekoppala, M. & Holder, A. A. (2010). "Merozoite surface proteins of the malaria parasite: The MSP1 complex and the MSP7 family". *International Journal for Parasitology*, 40(10), 1155–1161.

Kilama, W. & Ntoumi, F. (2009). Malaria: A research agenda for the eradication era. *Lancet*, 374:1480–2.

Khaireh, B. A., Assefa, A., Guessod, H. H., Basco, L. K., Khaireh, M. A. & Pascual, A. (2013). Population genetics analysis during the elimination process of *Plasmodium falciparum* in Djibouti. *Malaria Journal*, 12, 201.

Khunrae, P., Dahlbäck, M., Nielsen, M. A., Andersen, G., Ditlev, S. B., Resende, M., Pinto, V. V., Theander, T. G., Higgins, M. K. & Salanti, A. (2010). "Full-length recombinant Plasmodium falciparum VAR2CSA binds specifically to CSPG and induces potent parasite adhesion-blocking antibodies". *Journal of Molecular Biology*, 397(3), 826–834.

Koita, O. A., Sangaré, L., Sango, H. A., Dao, S., Keita, N., Maiga, M., Mounkoro, M., Fané, Z., Maiga, A. S., Traoré, K., Diallo, A. & Krogstad, D. J. (2012). Effect of Seasonality and Ecological Factors on the Prevalence of the Four Malaria Parasite Species in Northern Mali. *Journal of Tropical Medicine*, 367160, 9.

Kraemer, S. M. & Smith, J. D. (2006). "A family affair: var genes, PfEMP1 binding, and malaria disease". *Current Opinion in Microbiology*, 9(4), 374–380.

Krausel, D. R., Gatton, M. L., Frankland, S., Eisen, D. P., Good, M. F., Tilley, L. & Cheng,Q. (2007). Characterization of the Antibody Response against *Plasmodium falciparum* Erythrocyte Membrane Protein 1 in Human Volunteers. *Infectious Immunology*, 75(12), 5967-597.

Kurtzhals, J. A., Akanmori, B. D., Goka, B. Q., Adabayeri, V., Nkrumah, F. K., Behr, C. & Hviid, L. (1999). The cytokine balance in severe malarial anemia. *Journal of Infectious Disease*, 180, 1753-1755.

Kurtzhals, J. A., Rodrigues, O., Addae, M., Commey, J. O., Nkrumah, F. K. & Hviid, L. (1997). Reversible suppression of bone marrow response to erythropoietin in *Plasmodium falcipa*rum malaria. *British Journal of Haematology*, 97, 169-174.

Ladda, R., Aikawa, M. & Sprinz, H. (1969). Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites. *Journal of Parasitology*, 55:633–44.

Lalchhandama, K. (2017). "*Plasmodium falciparum* erythrocyte membrane protein 1". *Wiki Journal of Medicine*, 4(1).

Lamarque, M., Besteiro, S., Papoin, J. Roques, M., Vulliez-Le Normand, B., Morlon-Guyot, J… & Lebrun, M. (2011). The RON2-AMA1 interaction is a critical step in moving junctiondependent invasion by apicomplexan parasites. *PLoS Pathogen*, 7:e1001276.

Langhorne J., Ndungu, F. M., Sponaas, A. M. & Marsh, K. (2008). Immunity to malaria: more questions than answers. *Nature Immunology*, 9, 725-732.

Langreth, S. G., Nguyen-Dinh, P. & Trager, W. (1978). *Plasmodium falciparum*: merozoite invasion in vitro in the presence of chloroquine. *Experimental Parasitology*, 46, 235–8.

Lau, C. K.Y., Turner, L., Jespersen, J. S., Lowe, E. D., Petersen, B., Wang, C. W., Petersen, J. E.V., Lusingu, J… & Higgins, M. K. (2015). "Structural conservation despite huge sequence diversity allows EPCR binding by the PfEMP1 family implicated in severe childhood malaria". *Cell Host & Microbe*, 17(1), 118–129.

Levin, K. A. (2006). Study design III: Cross-sectional studies. *Evidence-Based Dentistry*, 7, 24–25.

Lindblade, K. A., Steinhardt, L., Samuels, A., Kachur, S. P. & Slutsker, L. (2014). The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Review of Anti-infective Therapy*, 11(6), 623-639.

Loha, E. & Lindtjørn, B. (2012). Predictors of *Plasmodium falciparum* malaria incidence in Chano Mille, South Ethiopia: a longitudinal study. *American Journal of Tropical Medicine and Hygiene*, 87, 450–9.

Looareesuwan, S., Davis, T. M., Pukrittayakamee, S., Supanaranond, W., Desakorn, V., Silamut, K., Krishna, S., Boonamrung, S. & White, N. J. (1991). Erythrocyte survival in severe *falciparum* malaria. *Acta Tropica*, 48, 263-270.

MacPherson, G. G., Warrell, M. J., White, N. J., Looareesuwan, W. & Warrell, D. A. (1995). Human cerebral malaria: Quantitative ultrastructural analysis of parasitized erythrocyte sequestration. *American Journal of Pathology*, 119, 385–401.

Maier, A. G., Duraisingh, M. T., Reeder, J. C., Patel, S. S., Kazura, J. W., Zimmerman, P. A. & Cowman, A. F. (2003). *Plasmodium falciparum* erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. *Nature Medicine*. 9:87–92.

Malaguarnera, L. & Musumeci, S. (2002). The immune response to Plasmodium falciparum malaria. *The Lancet Infectious Diseases*, 2(8), 472-478.

Malaria Vaccine Initiative Path. (2013). "RTS,S malaria candidate vaccine reduces malaria by approximately one-third in African infants". malariavaccine.org.

Marsh, K. & Howard, R. J. (1986). Antigens induced on erythrocytes by *P. falciparum*: expression of diverse and conserved determinants. *Science*, 231, 150–153.

Marsh, K. & Kinyanjui, S. (2006) Immune effector mechanisma in malaria. *Parasite Immunol*ogy, 28, 51-60

Mats, W., Suchi, G. & Reetesh, R. A. (2017). Variant surface antigens of *Plasmodium falciparum* and their roles in severe malaria. *Nature Reviews Microbiology*, 15, 479–491.

Mayengue, P. L., Ndounga, M., Malonga, F. V., Bitemo, M. & Ntoumi, F. (2011). Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in *Plasmodium falciparum* isolates from Brazzaville, Republic of Congo. *Malaria Journal*, 10, 276.

Mayxay, M., Chotivanich, K., Pukrittayakamee, S., Newton, P…. & Looareesuwan, S. (2001). Contribution of humoral immunity to the therapeutic response in *falciparum* malaria. *American Journal of Tropical Medical Hygiene*, 65, 918–923.

McCallum, F. J., Persson, K. E. M., Mugyenyi, C. K., Fowkes, F. J., Simpson, J. A., Richards, J. S., Williams, T. N., Marsh, K. & Beeson, J. G. (2008). Acquisition of growth-inhibitory antibodies against blood-stage *Plasmodium falciparum. PLoS One,* 3, e3571.

McCuskee, S., Brickley, E. B., Wood, A. & Mossialos, E. (2014). Malaria and Macronutrient Deficiency as Correlates of Anemia in Young Children: A Systematic Review of Observational Studies. *Annals of Global Health,* 80(6), 458-465.

Miller, K. L., Silverman, P. H., Kullgren, B. & Mahlmann, L. J. (1989). Tumor necrosis factor alpha and the anemia associated with murine malaria. *Infection and Immunity,* 57, 15421546.

Miller, L. H., Rowe, J. A., Moulds, J. M. & Newbold, C. I. (1997). *"P. falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1". *Nature,* 388(6639), 292–295.

Mishra, S. & Mohanty, S. (2002). Problems in Management of Severe Malaria. *The Internet Journal of Tropical Medicine,* 1, 1.

Mkumbaye, S. I., Wang, C. W., Lyimo, E., Jespersen, J. S., Manjurano, A., Mosha, J., Kavishe, R. A., Mwakalinga, S. B., Minja, D. T. R., Lusingu, J. P., Theander, T. G. & Lavstsen, T. (2017). "The severity of *Plasmodium falciparum* infection is associated with transcript levels of var genes encoding EPCR-binding PfEMP1". *Infection and Immunity,* IAI.00841–16.

Mockenhaupt, F. P., Bienzle, U., May, J., Falusi, A. G., Ademowo,O. G., Olumese, P. E. & Meyer

C. G. (1999). *Plasmodium falciparum* Infection: Influence on Hemoglobin Levels in αThalassemia and Microcytosis. *The Journal of Infectious Diseases,* 180(3), 925–928.

Mohammed, H., Mindaye, T., Belayneh, M., Kassa, M., Assefa, A., Tadesse, M., Woyessa, A., Mengesha, T. & Kebede, A. (2015). Genetic diversity of *Plasmodium falciparum* isolates based on MSP-1 and MSP-2 genes from Kolla-Shele area, Arbaminch Zuria District, southwest Ethiopia, *Malaria Journal,* 14, 73.

Mohan, K., Dubey, M. L., Ganguly, N. K. & Mahajan, R. C. (1995). *Plasmodium falciparum*: role of activated blood monocytes in erythrocyte membrane damage and red cell loss during malaria. *Experimental Parasitology,* 80, 54-63.

Mwingira, F., Nkwengulila, G., Schoepflin, S., Sumari, D., Beck, H. P., Snounou, G., Felger, I., Olliaro, P. & Mugittu, K. (2011). *Plasmodium falciparum msp1, msp2* and *glurp* allele frequency and diversity in sub-Saharan Africa. *Malaria J*ournal, 10, 79.

Nabet, C., Doumbo, S., Jeddi, F., Konaté, S., Manciulli, T., Fofana, B., L'Ollivier, C., Camara, A., Moore, S. & Ranque, S. (2016). Genetic diversity of Plasmodium falciparum in human malaria cases in Mali. *Malaria Journal*, 15, 353.

Nakazawa, S., Looareesuwan, S., Fujioka, H., Pongponratn, E., Luc, K. D., Rabbege, J. & Aikawa, M. (1995). A correlation between sequestered parasitized erythrocytes in subcutaneous tissue and cerebral malaria. *American Journal of Tropical Medicine and Hygiene*, 53, 544-
546.

National Oceanic and Atmospheric Administration. "Minna Climate Normals 1961–1990"Retrieved 22 July 2016.

National Population Commission of Nigeria, 2017.

Nazareth R., Horumpende, P., Sonda, T., Ndaro, A., Mollel, E., Paul, E., Athanase, E. & Chilongola, J. (2017). Naturally Acquired Antibody Responses to a Synthetic Malaria Antigen AS202.11. *Journal of Tropical Medicine,* 6843701, 5.

Newbold, C. I., Pinches, R., Roberts, D. J. & Marsh, K. (1992). *Plasmodium falciparum*: the human agglutinating antibody response to the infected red cell surface is predominantly variant specific. *Experimental Parasitology,* 75, 281–292.

Ngnie-Teta, I., Receveur, O. & Kuate-Defo B. (2007). Risk factors for moderate to severe anemia among children in Benin and Mali: insights from a multilevel analysis. *Food and Nutrition Bulletin*, 28(1), 76–89.

Niang, M., Niass, O., Diagne, N., Sarr, F. D., Faye, M, M., Diop, F., Diouf, B., Faye, J., Badiane, A., Perraut, R., Sokhna, C., Trape, J. F., Tall A. & Toure-Balde, A. (2017). Temporal analysis of IgG antibody responses to *Plasmodium falciparum* antigens in relation to changing malaria epidemiology in a West African setting. *Malaria Journal*, 16, 283.

Nkhoma S. C., Nair S., Al-Saai, S., Ashley, E., McGready, R., Phyo, A. P., Nosten, F. & Anderson, T. J. C. (2013). Population genetic correlates of declining transmission in a human pathogen. *Molecular Ecology*. 22(2), 273–85.

Noelle, Y. J., Makhtar, N. & Peter, R. P. (2010). Small variant surface antigens and *Plasmodium* evasion of immunity. *Future Microbiology*, 5(4), 663-682.

Nsobya, S. L., Parikh, S., Kironde, F., Lubega, G., Kamya, M. R., Rosenthal, P. J. & Dorsey G. (2004). Molecular Evaluation of the Natural History of Asymptomatic Parasitemia in Ugandan Children. *The Journal of Infectious Diseases*, 189(12), 2220–2226.

Ogouye'mi-Hounto, A., Gazard, D. K., Ndam, N., Topanou, E., Garba, O., Elegbe, P., Hountohotegbe, T. & Massougbodji, A. (2013). Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in *Plasmodium falciparum* isolates from children in South of Benin. *Parasite,* 20, 37.

Olasehinde, G. I., Yah, C. S., Singh, R., Ojuronbge, O., Ajayi, A. A., Valecha, N., Abolaji, A. O. & Adeyeba, A. O. (2012). Genetic diversity of *Plasmodium falciparum* field isolates from south western Nigeria. *Africa Health Sci*ences, 12(3), 355–361.

Olasehinde, G. I., Ojurongbe, D. O., Akinjogunla, O. J., Egwari, L. O. & Adeyeba, A. O. (2015). Prevalence of Malaria and Predisposing Factors to Antimalarial Drug Resistance in Southwestern Nigeria. *Research Journal of Parasitology,* 10, 92-101.

Omalu, I. C. J., Mgbemena, C., Mgbemena, A., Ayanwale, V., Olayemi, I. K., Lateef, A., & Chukwuemeka, V. I. (2012). Prevalence of Congenital Malaria in Minna, North Central Nigeria. *Journal of Tropical Medicine,* 2012, 274142.

Omalu, I.C. J., Olayemi, I., K., Otuu,I., A. Hassan, C., C. Eke, S., Paul, S., & Uzoaga, G. O. (2014). Entomological and Parasitological Indices of Malaria Transmission in Minna, Niger State, North Central Nigeria. *Advances in Research*, *3*(2), 181-188.

Onyiri, N. (2015). Estimating malaria burden in Nigeria: a geostatistical modelling approach. *Geospat Health*, 10, 306.

Osier, F. H., Fegan G., Polley, S.D., Murungi, L., Verra, F., Tetteh, K.K., Lowe, B., Mwangi, T., Bull, P.C., Thomas, A.W., Cavanagh, D. R., McBride, J.S., Lanar, D.E., Mackinnon, M. J., Conway, D. J. & Marsh, K. (2008). Breadth and magnitude of antibody responses to multiple *Plasmodium falciparum* merozoite antigens are associated with protection from clinical malaria. *Infectious Immunology*, 76, 2240–2248.

Oski, F. A. (2003). Nathan and Oski's Hematology of Infancy and Childhood. A diagnostic approach to the anemic patient. Philadelphia, Pa.: Saunders. 409–418.

Oyebola, M. K., Idowu, E. T., Olukosi, Y. A., Iwalokun, B. A., Agomo, C. O., Ajibaye, O. O., Tola, M. & Otubanjo, A. O. (2014). Genetic diversity and complexity of *Plasmodium falciparum* infections in Lagos, Nigeria. *Asian Pacific Journal of Tropical Biomedicine.* 4, 1, S87-S91.

Pain, A., Ferguson, D. J., Kai, O., Urban, B. C., Lowe, B., Marsh, K. & Roberts D. J. (2001). Platelet-mediated clumping of *Plasmodium falciparum*-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. *Proceedings of National Academic Science U S A*, 98, 1805–1810.

Parish, T. (2012). "Lifting malaria's deadly veil: Mystery solved in quest for vaccine". Burnet Institute.

Pasternak, N. D. & Dzikowski, R. (2009). "PfEMP1: An antigen that plays a key role in the pathogenicity and immune evasion of the malaria parasite *Plasmodium falciparum"*. *The International Journal of Biochemistry & Cell Biology*, 41(7), 1463–1466.

Persson, K. E., McCallum, F. J., Reiling, L., Lister, N. A., Stubbs J., Cowman, A. F., Marsh, K. & Beeson, J. G. (2008). Variation in use of erythrocyte invasion pathways by *Plasmodium falciparum* mediates evasion of human inhibitory antibodies. *Journal of Clinical Investigation,* 118, 342–51.

Phillips, R. E. & Pasvol, G. (1992). Anaemia of *Plasmodium falciparum* malaria. *Baillieres Clinical Haemato*logy, 5(2), 315-30.

Phillips, R. E., Looareesuwan, S., Warrell, D. A., Lee, S. H., Karbwang, J., Warrell, M. J., White, N. J., Swasdichai, C. & Weatherall, D. J. (1986). The importance of anaemia in cerebral and uncomplicated *falciparum* malaria: role of complications, dyserythropoiesis and iron sequestration. *Quarterly Journal of Medicine,* 58: 305-323.

Pongponratn, E., Riganti, M., Punpoowong, B., & Aikawa, M. (1991). Microvascular sequestration of parasitized erythrocytes in human falciparum malaria: a pathological study. *American Journal of Tropical Medical Hygiene*, 44, 168–175.

Pongponratn, E., Turner, G. D., Day, N. P., Phu, N. H., Simpson, J. A., Stepniewska, K., Mai, N.

T. H… & White, N. J. (2003). An ultrastructural study of the brain in fatal *Plasmodium falciparum* malaria. *American Journal of Tropical Medicine & Hygiene,* 69, 345–359.

Price, R. N., Simpson, J. A., Nosten, F., Luxemburger, C., Hkirjaroen, L., ter Kuile, F., Chongsuphajaisiddhi, T. & White, N. J. (2001). Factors contributing to anemia after uncomplicated *falciparum* malaria. *American Journal of Tropical Medicine and Hygiene,* 65, 614-622.

Proietti, C., Pettinato, D. D., Kanoi, B. N., Ntege, E., Crisanti, A., Riley, E. M., Egwang, T. G., Drakeley, C. & Bousema, T. (2011). Continuing intense malaria transmission in northern Uganda. *The American journal of tropical medicine and hygiene,* 84, 830–837.

Quintero, J. P., Siqueira, A. M., Tobón, A., Blair, S., Moreno, A., Arévalo-Herrera M., Lacerda, M. V. G. & Sócrates H. V. (2011). Malaria-related anaemia: a Latin American perspective. *Memórias do Instituto Oswaldo Cruz*. 106(1).

Raj, D. K., Das, R. B., Dash, A. P. & Supakar, P. C. (2004). Genetic diversity in the merozoite surface protein 1 gene of *Plasmodium falciparum* in different malaria-endemic localities. *American Journal of Tropical Medicine and Hygiene*, 71(3), 285–289.

Rayner, J. C., Tran, T. M., Corredor, V., Huber C. S., Barnwell, J. W. & Galinski, M. R. (2005). Dramatic difference in diversity between *Plasmodium falciparum* and *Plasmodium vivax* reticulocyte binding-like genes. *American Journal of Tropical Medicine and Hygiene,* 72, 666-674.

Razak, M. R. M. A., Sastu, U. R., Norahmad, N. A., Abdul-Karim, A., Muhammad, A., Muniandy, P. K., Jelip, J., Rundi, C., Imwong, M. & Mudin, R. N. (2016). Genetic diversity of *Plasmodium falciparum* populations in malaria declining areas of Sabah, East Malaysia. *PLoS ONE*, 11, e0152415.

Reddy, S. B., Anders, R. F., Beeson, J. G., Färnert, A., Kironde, F., Berenzon, S. K., Wahlgren, M., Linse, S. & Persson, K. E. M. (2012). High Affinity Antibodies to *Plasmodium falciparum* Merozoite Antigens Are Associated with Protection from Malaria. *PLoS journal,* 7(2).

Reddy, S. B., Anders, R. F., Beeson, J. G., Färnert, A., Kironde, F., Berenzon, S. K., Wahlgren, M., Linse, S. & Persson, K. E. M. (2012). High Affinity Antibodies to *Plasmodium falciparum* Merozoite Antigens Are Associated with Protection from Malaria. *PLoS journal,* 7(2).

Reeder, J. C., Cowman, A. F., Davern, K. M., Beeson, J. G., Thompson, J. K., Rogerson, S. J. & Brown, G. V. (1999). The adhesion of Plasmodium falciparum-infected erythrocytes to choandroitin sulfate A is mediated by *P. falciparum* erythrocyte membrane protein 1. *Proceedings of National Academic Science*, USA, 96, 5198-5202.

Reeder, J. C., Rogerson, S. J., Al-Yaman, F., Anders, R. F., Coppel, R. L., Novakovic, S., Alpers, M. P. & Brown, G. V. (1994). Diversity of agglutinating phenotype, cytoadherence, and

rosette-forming characteristics of *Plasmodium falciparum* isolates from Papua New Guinean children. *American Journal of Tropical Medicine & Hygiene*, 51, 45–55.

Richards S. & Beeson, J. G. (2009). The future for blood-stage vaccines against malaria. *Immunology and Cell Biology*, 87(5), 377–390.

Richards, J. S. Arumugam, T. U., Reiling, L., Healer, J., Hodder, A. N., Fowkes, F. J., Cross, N., Langer, C., Takeo, S., Uboldi, A. D., Thompson, J. K., Gilson, P. R., Coppel, R. L., Siba, P. M., King, C. L., Torii, M., Chitnis, C. E., Narum, D. L., Mueller, I., Crabb, B. S., Cowman, A. F., Tsuboi, T. & 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*, 191, 795–809.

Ridzuan, M. M. A., Sastu, U. R., Norahmad, N. A., Abdul-Karim, A., Muhammad, A., Muniandy,
P. K., Jelip, J., Rundi, C., Imwong, M., Mudin, R. N. & Abdullah, N. R. (2016). Genetic Diversity of *Plasmodium falciparum* Populations in Malaria Declining Areas of Sabah, East Malaysia. *PLos Journal*, 11(3), e0152415.

Ritter, K., Kuhlencord, A., Thomssen, R. & Bommer, W. (1993). Prolonged haemolytic anaemia in malaria and autoantibodies against triosephosphate isomerase. *Lancet*, 342, 1333-1334.

Roberts, D. J., Craig, A. G., Berendt, A. R., Pinches, R., Nash, G., Marsh, K. & Newbold, C. I. (1992). Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature*, 357, 689–692.

Robinson, B. A., Welch, T. L. & Smith, J. D. (2003). Widespread functional specialization of *Plasmodium falciparum* erythrocyte membrane protein 1 family members to bind CD36 analysed across a parasite genome. *Molecular Microbiology*, 47, 1265–1278.

Rono, J., Osier, F. H., Olsson, D., Montgomery, S., Mhoja, L., Rooth, I., Marsh, K. & Färnert, A. (2013). Breadth of anti-merozoite antibody responses is associated with the genetic diversity of asymptomatic Plasmodium falciparum infections and protection against clinical malaria. *Clinical Infectious Disease*, 57, 1409–1416.

Rowe, J. A., Claessens, A., Corrigan, R. A. & Arman, M. (2009). "Adhesion of Plasmodium falciparum-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications". *Expert Reviews in Molecular Medicine*, 11.

Rowe, J. A., Moulds, J. M., Newbold, C. I. & Miller, L. H. (1997). *Plasmodium falciparum* rosetting is mediated by a parasite-variant erythrocyte membrane protein and complementreceptor 1. *Nature*, 388, 292–295.

Salanti, A., Dahlbäck, M., Turner, L., Nielsen, M. A., Barfod, L., Magistrado, P., Jensen, A. T.R.,
Lavstsen, T…& Theander, T. G. (2004). "Evidence for the involvement of VAR2CSA in pregnancy-associated malaria". *The Journal of Experimental Medicine,* 200(9), 1197–1203.

Sarr, J. B., Remoue, F., Samb, B., Dia, I., Guindo, S., Sow, C., Maiga, S., Tine, S., Thiam, C., Schacht, A. M., Simondon, F., Konate L. & Riveau, G. (2007). Evaluation of antibody response to *Plasmodiumfalciparum* in children according to exposure of *Anophelesgambiae* s.l or *Anophelesfunestus* vectors. *Malaria Journal*, 6, 117.

Schwartz, L., Brown, G. V., Genton, B. & Moorthy, V. S. (2012). A review of malaria vaccine clinical projects based on the WHO rainbow table. *Malaria Journal*, 11(11).

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*, 15:98(10), 3132-3135.

Serghides, L., Smith, T. G., Patel, S. N. & Kain, K. C. (2003). CD36 and malaria: friends or foes? *Trends in Parasitology*, 19, 461-469.

Shekalaghe, S., Alifrangis, M., Mwanziva, C., Enevold, A., Mwakalinga, S., Mkali, H., Kavishe, R., Manjurano, A., Sauerwein, R., Drakeley, C. & Bousema, T. (2009). Low density parasitaemia, red blood cell polymorphisms and *Plasmodium falciparum* specific immune responses in a low endemic area in northern Tanzania. *BMC infectious diseases*, 9:69.

Silue, K. D., Felger I, Utzinger, J., Beck, H. P., Smith, T. A., Tanner, M. & N'goran, E. K. (2006). Prevalence, genetic diversity and multiplicity of *plasmodium falciparum* infection in school children in central Cote d'Ivoire. *Medecine Tropicale* (*Mars*), 66, 149–56.

Silverman, P. H., Schooley, J. C. & Mahlmann, L. J. (1987). Murine malaria decreases hemopoietic stem cells. *Blood*, 69, 408-413.

Simpson, J. A., Silamut, K., Chotivanich, K., Pukrittayakamee, S. & White, N. J. (1999). Red cell selectivity in malaria: a study of multiple-infected erythrocytes. *Transactions of Royal Society of Tropical Medicine and Hygiene*, 93, 165-168.

Smith, J. D. (2014). "The role of PfEMP1 adhesion domain classification in *Plasmodium falciparum* pathogenesis research". *Molecular and Biochemical Parasitology*, 195(2), 82–87.

Smith, J. D., Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S., Pinches, R., Newbold, C. I. & Miller, L. H. (1995). Switches in expression of *Plasmodium falciparum* var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell*, 82, 101-110.

Smith, J. D., Craig, A. G., Kriek, N., Hudson-Taylor, D., Kyes, S., Fagan, T., Pinches, R., Baruch, D. I., Newbold, C. I. & Miller, L. H. (2000). "Identification of a *Plasmodium*

falciparum intercellular adhesion molecule-1 binding domain: A parasite adhesion trait implicated in cerebral malaria". *Proceedings of the National Academy of Sciences,* 97(4), 1766–1771.

Smith, T., Felger, I., Tanner, M. & Beck, H. P. (1999). Premunition in *Plasmodium falciparum* infection: insights from the epidemiology of multiple infections. *Transactions of Royal Society of Tropical Medicine and Hygiene,* 93(1), 59–64.

Soe, T. N., Wu, Y., Tun, M. W., Xu, X., Hu, Y., Ruan, Y., Win, A. Y. N., Nyunt, M. H., Nwe Mon, N. C., Han, K. T., Aye, K. M., Morris, J., Su, P., Yang, Z., Kyaw, M. P. & Cui, L. (2017). Genetic diversity of *Plasmodium falciparum* populations in southeast and western Myanmar. *Parasites & Vectors,* 10, 322.

Solomon, A. & Weiss, D.T. (1995) Structural and functional properties of human lambda-lightchain variable-region subgroups. *Clinical and Diagnostic Laboratory Immunology,* 2, 387394.

Srinivasan, P., Beatty, W. L., Diouf, A., Herrera, R., Ambroggio, X., Moch, J. K., Tyler, J. S… & Miller, L. H. (2011). Binding of Plasmodium merozoite proteins RON2 and AMA1 triggers commitment to invasion. *Proceedings of National Academic of Science USA,* 108, 13275–80.

Starzengruber, P., Fuehrer, H., Ley, B., Thriemer, K., Swoboda, P., Habler, V. E., Jung, M., Graninger, W., Khan, W. A., Haque R. & Noed, H. (2014). High prevalence of asymptomatic malaria in south-eastern Bangladesh. *Malaria Journal,* 13, 16.

Stewart, L., Gosling, R., Griffin, J., Gesase, S., Campo, J., Hashim, R., Masika, P., Mosha, J., Bousema, T., Shekalaghe, S., Cook, J., Corran, P., Ghani, A., Riley, E. M. & Drakeley, C. (2009). Rapid assessment of malaria transmission using age-specific sero-conversion rates. *PloS one,* 4, e6083.

Stoute, J. A. (2011). "Complement receptor 1 and malaria". *Cellular Microbiology,* 13(10), 1441– 1450.

Stoute, J. A., Odindo, A. O., Owuor, B. O., Mibei, E. K., Opollo, M. O. & Waitumbi, J. N. (2003). Loss of red blood cell-complement regulatory proteins and increased levels of circulating immune complexes are associated with severe malarial anemia. *Journal of Infectious Disease,* 187, 522-525.

Su, X. Z., Heatwole, V. M., Wertheimer, S. P., Guinet, F., Herrfeldt, J. A., Peterson, D. S., Ravetch, J. A. & Wellems, T. E. (1995). The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. *Cell,* 82, 89-100.

Sumbele I. U. N., Nkuo-Akenji T., Samje M., Ndzeidze T., Ngwa E. M. & Titanji V. P. K. (2010). Haematological changes and recovery associated with treated and untreated

*Plasmodium falciparum*infection in children in the Mount Cameroon Region. *Journal of Clinical Medicine and Research*, 2(9), 143–151.

Sumbele, I. U. N., Sama, S. D., Kimbi, H. K. & Taiwe, G. S. (2016). Malaria, Moderate to Severe Anaemia, and Malarial Anaemia in Children at Presentation to Hospital in the Mount Cameroon Area: A Cross-Sectional Study. *Anemia*, 5725634.

Takala, S. L., Escalante, A. A., Branch, O. H., Kariuki, S., Biswas, S., Chaiyaroj, S. C. & Lal, A. A. (2006). Genetic diversity in the Block 2 region of the merozoite surface protein 1 (MSP1) of *Plasmodium falciparum:* additional complexity and selection and convergence in fragment size polymorphism. *Infection, Genetics and Evolution*, 6, 417–24.

Takala, S., Branch, O., Escalante, A. A., Kariuki, S., Wootton, J. & Lal, A. A. (2002). Evidence for intragenic recombination in *Plasmodium falciparum*: identification of a novel allele family in block 2 of merozoite surface protein-1: Asembo Bay Area Cohort Project XIV. *Molecular and Biochemical Parasitology*, 125, 163–71.

Tham, W. H., Healer, J. & Cowman, A. F. (2012). Erythrocyte and reticulocyte binding-like proteins of *Plasmodium falciparum*. *Trends in Parasitology*, 28, 23–30.

Todd C. & Lorenz, T. C. (2012). Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. *Journal of Visualized Experiment*, 63, 3998.

Tongren, J. E., Zavala, F., Roos, D. S. & Riley, E. M. (2004). Malaria vaccines: if at first you don't succeed. *Trends of Parasitololgy*, 20, 604-610.

Trape, J. F. (1985). Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 79(2), 181–184.

Turner, G. D. G., Morrison, H., Jones, M., Davis, T. M. E., Looareesuwan, S., Buley, I. D., Gatter, K. C., Newbold, C. I., Pukritayakamee, S., Nagachinta, B., White, N. J. & Berendt, A. R. (1994). An immunohistochemical study of the pathology of fatal malaria: evidence for widespread endothelial activation and a potential role of intercellular adhesion molecule1. *American Journal of Pathology*, 145, 1057–1069.

Turner, L., Lavstsen, T., Berger, S. S., Wang, C. W., Petersen, J. E. V., Avril, M., Brazier, A. J., Freeth, J… & Theander, T. G. (2013). "Severe malaria is associated with parasite binding to endothelial protein C receptor". *Nature*, 498(7455), 502–505.

Umaru, M. L., & Gabriel, U. N. (2015). "Prevalence of Malaria in Patients Attending the General Hospital Makarfi, Makarfi Kaduna – State, North-Western Nigeria".*American Journal of Infectious Diseases and Microbiology*, 3(1), 1-5.

Urban, B. C., Ferguson, D. J., Pain, A., Willcox, N., Plebanski, M., Austyn, J. M. & Roberts, D. J. (1999). *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature*, 400, 73–77.

Vigan-Womas, I., Guillotte, M., Juillerat, A., Hessel, A., Raynal, B., England, P., Cohen, J. H., Bertrand, O., Peyrard, T., Bentley, G. A., Lewit-Bentley, A., Mercereau-Puijalon, O. & Rowe, J. A. (2012). "Structural basis for the ABO blood-group dependence of *Plasmodium falciparum* rosetting". *PLoS Pathogens,* 8(7).

Viriyakosol, S., Siripoon N., Petcharapirat C., Petcharapirat P., Jarra W,, Thaithong S., Brown, K. N. & Snounou, G. (1995). Genotyping of *Plasmodium falciparum* isolates by the polymerase chain reaction and potential uses in epidemiological studies. *Bulletin of the World Health Organization*, 73(1), 85–95.

Waitumbi, J. N., Opollo, M. O., Muga, R. O., Misore, A. O. & Stoute, J. A. (2000). Red cell surface changes and erythrophagocytosis in children with severe *Plasmodium falciparum* anemia. *Blood,* 95, 1481-1486.

Weiss, G. E., Gilson, P. R., Taechalertpaisarn, T., Tham, W., de Jong, N. W. M., Harvey K. L. Fowkes, F. J. I… & Crabb, B. S. (2015). Revealing the sequence and resulting cellular morphology of receptor-ligand interactions during *Plasmodium falciparum* invasion of erythrocytes. *PLoS Pathog*en, 11, e1004670

WHO World Health Organization. (2012).

Wickramasinghe, S. N. & Abdalla, S. H. (2000). Blood and bone marrow changes in malaria. *Baillieres Best Practice and Research Clinical Haematology*, 13, 277-299.

Wilson, D. W., Goodman, C. D., Sleebs, B. E., Weiss, G. E., de Jong, N. W. M., Angrisano, F., Langer, C… & Beeson, J. G. (2015). Macrolides rapidly inhibit red blood cell invasion by the human malaria parasite, *Plasmodium falciparum*. *BMC Biology*, 13:52.

Winskill, P., Rowland, M., Mtove, G., Malima, R. C. & Kirby, M. J. (2011). Malaria risk factors in north-east Tanzania. *Malaria J*ournal, 10:98.

WHO, World Health Organization. (2000). Severe *falciparum* malaria. World Health Organization, Communicable Diseases Cluster. *Transactions of Royal Society of Tropical Medicine and Hygiene.* 94(1), S1-90.

WHO, World Health Organization. (2003).The Africa Malaria Report

WHO, World Health Organization. (2010). WHO media center – Malaria.

WHO, World Health Organization. (2014)."Malaria Fact sheet N°94".

WHO, World Health Organization. (2015). Malaria fact sheet. http://www.who.int/mediacentre/factsheets/fs094/en

WHO, World Health Organization. (2015a). Statistics profile, Nigeria. http://www.who.int/gho/countries/nga.pdf

WHO, World Health Organization. (2015b). World malaria report. (WHO, Geneva, Switzerland, 2016).

WHO, World Health Organization. (2016). *Malaria vaccine. Weekly epidemiological record,* 91(4), 33–52.

WHO, World Health Organization. (2017). Key points: World malaria report 2017.

WHO, World Health Organization. Nigeria malaria profile. 2015. http://www.who.int/malaria/publicztions/country-profiles/profile_nga_en.pdf.

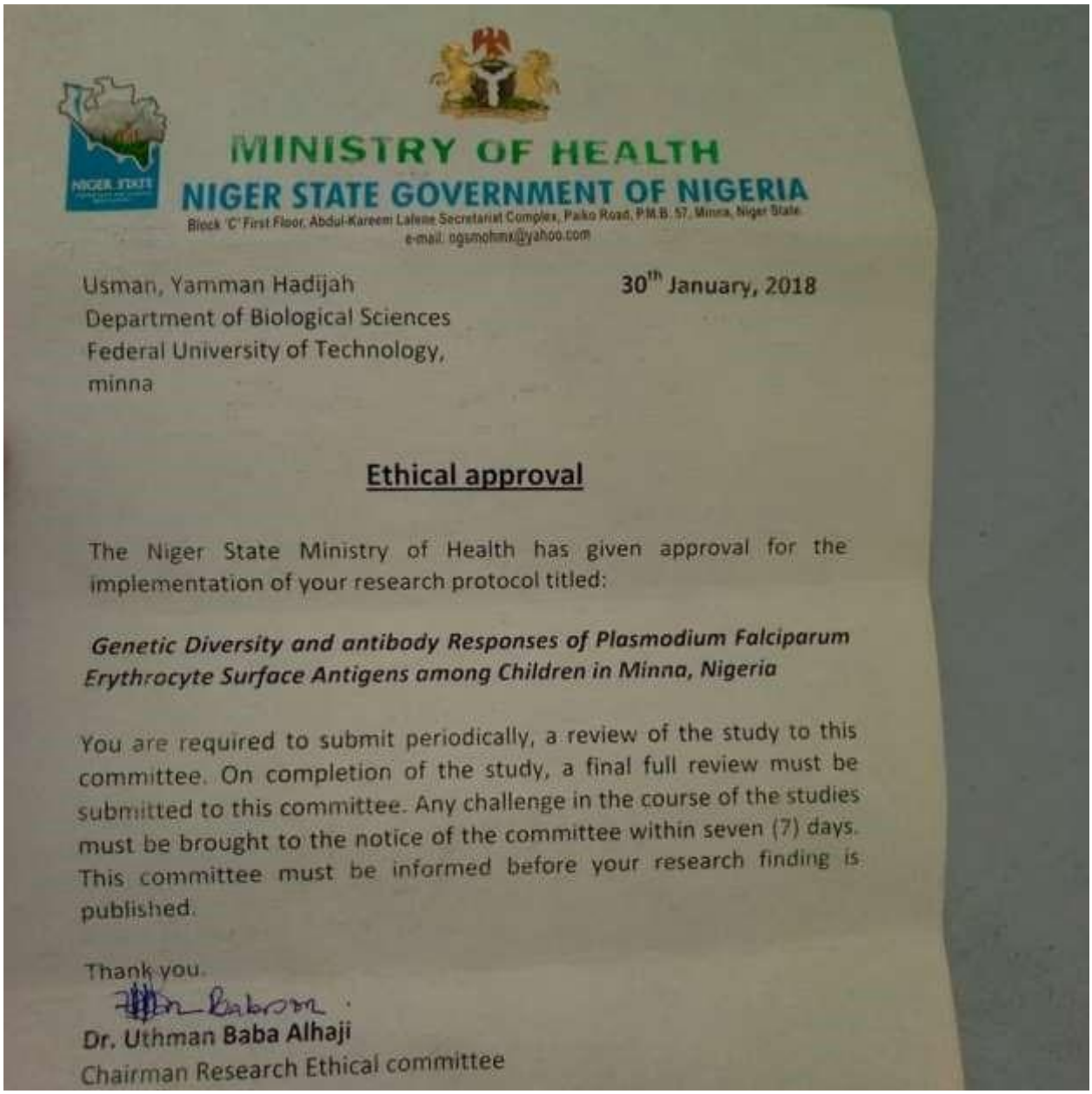
Zakeri, S., Bereczky, S., Naimi, P., Pedro Gil, J., Djadid, N. D… & Farnert, A. (2005). Multiple genotypes of the merozoite surface proteins 1 and 2 in *Plasmodium falciparum* infections in a hypoendemic area in Iran. *Tropical Medicine & International Health,* 10:1060–4.

Zhang, V. M., Chavchich, M. & Waters, N. C. (2012). "Targeting protein kinases in the malaria parasite: update of an antimalarial drug target". *Current Topics in Medicinal Chemistry,* 12(5), 456–72.

Zwetyenga, J., Rogier, C., Tall, A., Fontenille, D., Snounou, G., Trape, J.F. & Puijalon, O. M. (1998). No influence of age on infection complexity and allelic distribution in *Plasmodium falciparum* infections in Ndiop, a Senegalese village with seasonal, mesoendemic malaria. *American Journal of Tropical Medicine and Hygiene,* 59, 726–35.

APPENDICES

Appendix A: Ethical Approval Letter from Niger State Ministry of Health



MINISTRY OF HEALTH
NIGER STATE GOVERNMENT OF NIGERIA
Block 'C' First Floor, Abdul-Kareem Lafene Secretariat Complex, Paiko Road, P.M.B. 57, Minna, Niger State.
e-mail: ogumohmx@yahoo.com

Usman, Yamman Hadijah
Department of Biological Sciences
Federal University of Technology,
minna

30th January, 2018

Ethical approval

The Niger State Ministry of Health has given approval for the implementation of your research protocol titled:

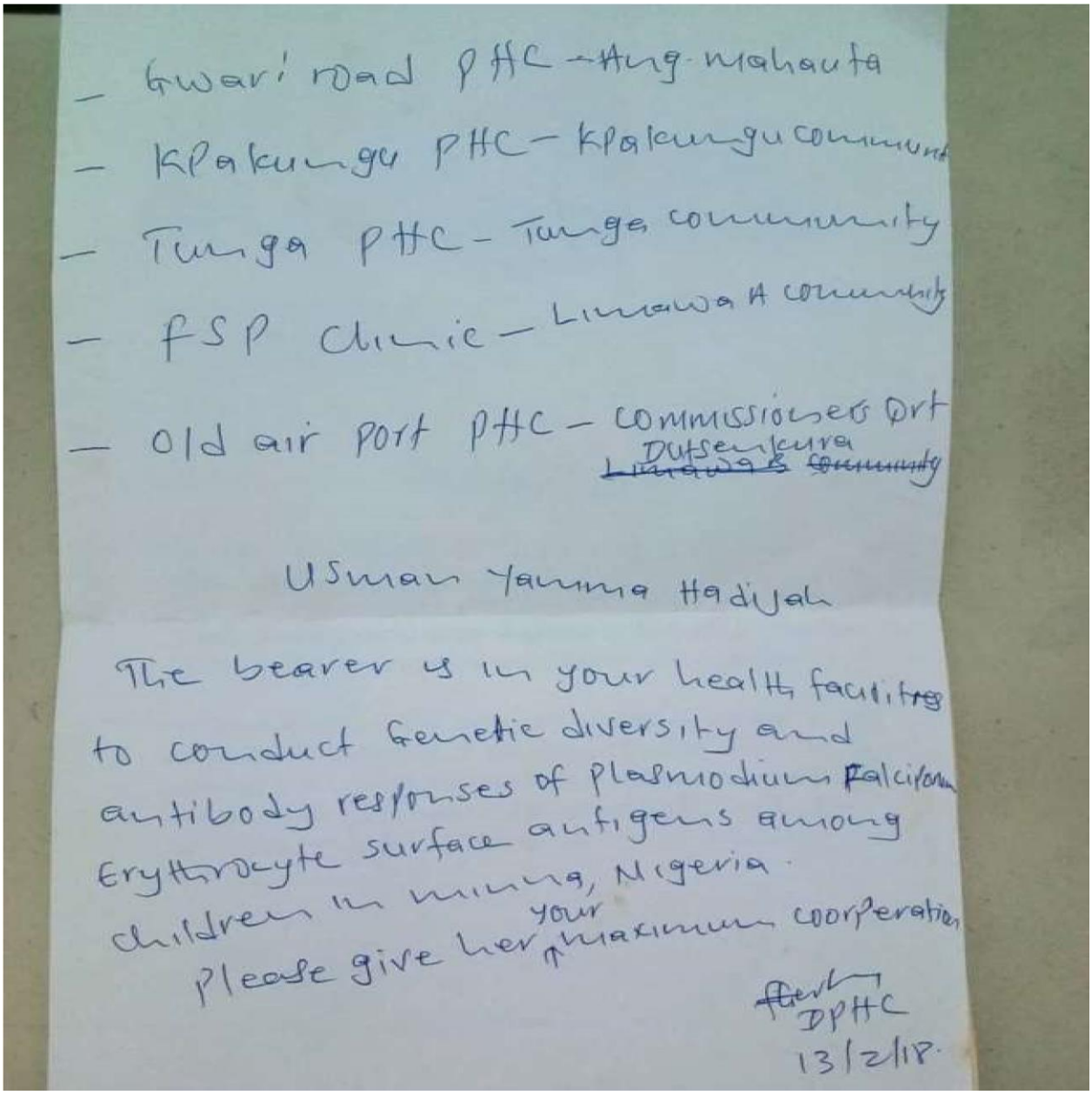
Genetic Diversity and antibody Responses of Plasmodium Falciparum Erythrocyte Surface Antigens among Children in Minna, Nigeria

You are required to submit periodically, a review of the study to this committee. On completion of the study, a final full review must be submitted to this committee. Any challenge in the course of the studies must be brought to the notice of the committee within seven (7) days. This committee must be informed before your research finding is published.

Thank you.

Dr. Uthman Baba Alhaji
Chairman Research Ethical committee

Appendix B: Letter of Permission from the Director, Primary Healthcare, Chanchaga LGA



- Gwari road PHC – Ang. Mahauta
- Kpakungu PHC – Kpakungu community
- Tunga PHC – Tunga community
- FSP Clinic – Limawa A community
- Old air port PHC – Commissioners Qrt Dutsenkura ~~Limawa B community~~

Usman Yamma Hadijah

The bearer is in your health facilities to conduct Genetic diversity and antibody responses of Plasmodium Falciparum Erythrocyte surface antigens among children in Minna, Nigeria.

Please give her your maximum cooperation

DPHC
13/2/18.

Appendix C: Multinomial logistic regression analysis examining anaemia with parasite density

Parameter Estimates			
Parasite density		***P* value**	**95% (Confidence Interval)**
Negative		Intercept	.000
	Haemoglobin	0.599	(0.130-34.252)
	Haematocrit	0.257	(0.310-80.101)
<1000 low parasitemia		Intercept	.000
	Haemoglobin	0.543	(0.145-39.506)
	Haematocrit	0.323	(0.250-67.438)
1001-10000 moderate parasitemia		Intercept	.000
	Haemoglobin	0.646	(0.122-29.746)
	Haematocrit	0.370	(0.226-54.468)

The reference category is: >10,001 high parasite density.

Appendix D: Multinomial logistic regression analysis examining anaemia subtypes with parasite density

Parameter Estimates			
Parasite Density		***P* value**	**95% (Confidence Interval)**
Negative	Intercept	0.028	

Nmt hypo	.723	(0.144-16.308)
Nmt nmc	.595	(0.039-6.439)
Macro hypo	.485	(.030-5.248)
Macro hyper	.	(0.755-0.755)
Micro hypo	.365	(0.022-4.060)
<1000 low parasitemia	Intercept	0.069
Nmt hypo	.751	(0.133-16.390)
Nmt nmc	.529	(0.031-5.985)
Macro hypo	.955	(0.071-12.136)
Macro nmc	.272	(0.004-4.612)
Micro hypo	.604	(0.036-6.862)
1001-10000 moderate parasitemia	Intercept	0.003
Nmt hypo	.853	(0.079-8.148)
Nmt nmc	.453	(0.032-4.625)
Macro hypo	.558	(0.040-5.664)
Macro nmc	.261	(0.009-3.542)
Micro hypo	.583	(0.042-5.924)

a The reference category is: >10,001 high parasitemia.

Keys

Nmt hypo = Normocytic hypochromic Macro hypo = Macrocytic hypochromic	Micro hypo = Microcytic hypochromic
Nmt hyper = Normocytic hyperchromic Macro hyper = Macrocytic hyperchromic	Micro hyper = Microcytic hyperchromic
Nmt nmc = Normocytic normochromic Macro nmc = Macrocytic normochromic	Micro nmc = Microcytic normochromic

Appendix E: Mann-Whitney Test for IgG against *P. falciparum* for positive and negative blood samples

Test Statistics[a]

	IgG absorbance value
Mann-Whitney U	304.000
Wilcoxon W	3544.000
Z	-.232
Asymp. Sig. (2-tailed)	.816

a. Grouping Variable: Parasite

Appendix F: Mann-Whitney Test for IgG against *P. falciparum* for male and female

Test Statistics[a]

	IgG absorbance value
Mann-Whitney U	741.500
Wilcoxon W	1731.500
Z	-1.891
Asymp. Sig. (2-tailed)	.059

a. Grouping Variable: Sex

Appendix G: Heterozygosity index (*He*) for the determination of genetic diversity

$He = \frac{n - \sum(1 - p)^2}{n - 1}$

Where n is the number of isolates analyzed and, p

represents the frequency of each different allele at a locus

For overall Genetic diversity n=111

$(1-p)^2 = (1-3)^2 + (1-3)^2 + (1-2)^2 + (1-4)^2 + (1-2)^2 + (1-2)^2 + (1-2)^2 + (1-3)^2 + (1-3)^2 + (1-2)^2 + (1-3)^2 + (1-2)^2 + (1-2)^2 + (1-2)^2 + (1-2)^2 + (1-1)^2 + (1-2)^2 + (1-0)^2 + (1-3)^2 + (1-0)^2 + (1-3)^2 + (1-3)^2 + (1-3)^2 + (1-0)^2 + (1-1)^2 + (1-3)^2 + (1-3)^2 + (1-1)^2 + (1-2)^2 + (1-1)^2 + (1-1)^2 + (1-0)^2 + (1-3)^2 + (1-3)^2 + (1-4)^2 + (12)^2 + (1-2)^2 + (1-3)^2 + (1-2)^2 + (1-3)^2 + (1-3)^2 + (1-3)^2 + (1-4)^2 + (1-2)^2 + (1-2)^2 + (1-3)^2 + (1-2)^2 + (1-3)^2 + (1-2)^2 = 118$

$He = \frac{111-118}{110} = \frac{7}{110} = 0.063$