*PLASMODIUM FALCIPARUM*DRUG RESISTANCE AMONG PATIENTS ATTENDING SOME HOSPITALS IN PARTS OF KADUNA STATE, NIGERIA

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MOLECULAR DETECTION OF GENETIC MARKERS ASSOCIATED WITH *PLASMODIUM FALCIPARUM* DRUG RESISTANCE AMONG PATIENTS ATTENDING SOME HOSPITALS IN PARTS OF KADUNA STATE, NIGERIA

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DECLARATION

I declare that the work in this thesis entitled "Molecular detection of genetic markers associated with *Plasmodium falciparum* drug resistance among patients attending some hospitals in parts of Kaduna state, Nigeria" has been performed by me in the Department of Microbiology, Faculty of Life Sciences, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided.No part of this thesiswas previously presented for another degree or diploma at this or any other institution.

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CERTIFICATION

This thesis entitled "**MOLECULAR DETECTION OF GENETIC MARKERS ASSOCIATED WITH *PLASMODIUM FALCIPARUM*DRUG RESISTANCE AMONG PATIENTS ATTENDING SOME HOSPITALS IN PARTS OF KADUNA STATE, NIGERIA**" by Gideon Yakusak BENJAMIN (P16LSMC9012) meets the regulations governing the award of the Degree of Doctor of Philosophy in Microbiology of the Ahmadu Bello University, and is approved for its' contribution to knowledge and literary presentation.

Prof. H.I. Inabo _____

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Member, Supervisory Committee Signature Date

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DEDICATION

This thesis is dedicated to my loving wife and family who have been a huge source of support and encouragement to me, and to Almighty God for his grace upon my life.

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ABSTRACT

Antimalarial drug resistance is a major obstacle to management and control of malariain Nigeria and sub-Saharan Africa. This research was aimed at carrying out molecular detection of genetic markers associated with *Plasmodium falciparum* specificdrug resistance among patients attending some hospitals in parts of Kaduna state, Nigeria.Three hundred (300) blood samples were collected from consenting individuals attending selected hospitals, in the three senatorial districts of Kaduna State, Nigeria. The samples were screened for malaria parasites by microscopy and CareStart^{TM}malaria rapid diagnostic test kit. Structured questionnaire were used to obtain bio-data and demographic data from the study participants. The data obtained were analysed using Statistical Package for Social Sciences (SPSS) and Chi square to check for association. Out of the 300 blood samples screenedby microscopy and Malaria Rapid Diagnostic Test (MRDT); 71 (23.7%) were positive by microscopy while 65 (21.7%) were positive by MRDT and only *Plasmodium falciparum* was detected. Patients attending General hospital Kafanchan had the highest occurrence (30%) of *Plasmodium falciparum* infection. The age group ≤10 had the highest prevalence of malaria ($p<0.05$). Married participants had higher prevalence (31.8%) of malaria than participants who were single (16%)or divorced (12.5%)($p<0.05$). Those who use insecticide spray at home had lower prevalence (20.0%) of malaria compared to those who do not ($p<0.05$). Of all the malaria positive participants, those with Haemoglobin AA (73%) haemoglobingenotype had the highest percentage followed by Haemoglobin AS (23%), Haemoglobin AC (3%) and HaemoglobinSS (1).*Plasmodiumfalciparum*chloroquineresistance transporter (*pfcrt*), *Plasmodiumfalciparum* multidrug resistance 1 (*pfmdr1*), *Plasmodium falciparum*

dihydrofolatereductase (*pfdhfr*), *Plasmodium falciparum* dihydropteroate synthase (*pfdhps*)genes were detected at expected amplicon sizes from the malaria positive samples in this study. *Pfcrt* (80%) had the highest prevalence, followed by *pfdhfr* (60%), *pfmdr1* (36%) and *pfdhps* (8%).The phylogenetic tree showed that *pfatpase6*sequences in this study were related to published sequences. The findings of the study revealed association between malaria and age, and marital statusof participants. Study also demonstrated the presence of *pfcrt, pfmdr1, pfdhfr, pfdhps*and *pfatpase6*which are associated with antimalarial resistance.DNA sequencing and bioinformatics tools such as sequence alignment and BLASTcontributed to proper identification and confirmation of genes after PCR and gel electrophoresis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is caused by intracellular parasites of the genus *Plasmodium*. It is transmitted through the bite of an infected female *Anopheles* mosquito. Only four *Plasmodium* species infect humans. These include; *Plasmodium falciparum, Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. *Plasmodium falciparum* is the most prevalent malaria parasite in the African continent. It is responsible for most malaria related deaths globally. *Plasmodium vivax* is the dominant malaria parasite in most countries outside of sub-Saharan Africa. (WHO, 2000).

Despite being preventable and treatable, malaria remains one of the major health problems in sub-Saharan Africa (Kebede *et al*., 2014) even though there were encouraging reports that malaria morbidity and mortality were declining (Nyarango *et al*., 2006; Ouji *et al*., 2018). In 2017, it was reported that nearly half of the world's population was at risk of malaria (WHO, 2018a). Nigeria accounted for 93, 446 and 92, 699 of under-five deaths due to malaria in 2015 and 2016 respectively (UNICEF, 2018). Nigeria is currently a malaria endemic country with its entire population at risk of contracting malaria, and a whopping 76% of this population at high risk (WHO, 2015a). In 2015, Nigeria contributed about 29% of the malaria cases and 26% of the malaria deaths worldwide (WHO, 2016). Nigeria represents up to 25% of the malaria burden in Africa. There are an estimated 33,000 malaria cases per 100,000 people, with 110, 000 of these cases resulting in mortality (WHO, 2015b). Countries with higher proportions of their population living in poverty (less than US$ 1.25 per person per day) have higher mortality rates from malaria

(Ananya, 2013). Nigeria suffers the world's greatest malaria burden, with approximately 51 million cases and 207,000 deaths reported annually (Dawaki *et al.*, 2016). Malaria and the costs of treatment trap families in a cycle of illness, suffering and poverty. Since year 2000, malaria has cost sub-Saharan Africa US$ 300 million each year for case management alone and it is estimated to cost up to 1.3 per cent of GDP in Africa (UNICEF, 2018).

Drug resistance is a major obstacle to management and control of malaria and it is progressing at a rapid rate across Africa (Kheir, 2011). The emergence of drug resistance, particularly among *Plasmodium falciparum* strains, has been a major contributor to the global burden of malaria (White, 2004).

Resistance to antimalarial agents arises because of the selection of parasites with genetic mutations that confer reduced susceptibility (WHO, 2010a). Resistance genes continue to be selected in *Plasmodium* thereby causing resurgences in the disease incidence (Hedrick, 2012). One of the main obstacles to malaria control is the ability of the parasites to develop resistance to administered antimalarial drugs. The development of resistance to drugs poses the greatest threats to malaria control and results in increased malaria morbidity and mortality (CDC, 2018). The resistance to different anti-malarial drugs is due to single nucleotide polymorphisms (SNPs) in different *P. falciparum* genes, including *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*), *Plasmodium falciparum* dihydropteroate synthase (*pfdhps*), *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*), *Plasmodium falciparum* adenosine triphosphatase 6 (*pfatpase6*), *Plasmodium falciparum* kelch 13 (*pfk13) and Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*). The

accumulation of SNPs in these parasite genes can produce *in vivo* resistance (Berzosa *et al.*, 2017)**.**

Artemisinin-based combination therapy is the first-line therapy in Nigeria and most malaria endemic countries (Lin and Zaw, 2015; Okoro and Jamiu, 2018). In 2005, Nigeria changed its antimalarial drug policy to artemisinin based combination therapy (ACT) (Okoro and Jamiu, 2018). However, recent gains in reducing the burden of malaria are threatened by the emergence of *Plasmodium falciparum* resistance to artemisinins. The current cornerstones in malaria treatment are artemisinin combination therapy for treatment of uncomplicated *Plasmodium falciparum* malaria (Banek *et al*., 2018) and sulfadoxine-pyrimehamine (SP) for intermittent preventive treatment of pregnant women. While resistance to SP is already established, there are evidence of decreased susceptibility or resistance to the ACT components; artemisinins and the key ACT partner drugs lumefantrine, amodiaquine and mefloquine (WHO, 2010a). The choice of ACT is based on the results of therapeutic efficacy studies against local strains of *Plasmodium falciparum* (WHO, 2018a). Since 2009, there are increasing concerns and reports of delayed parasite clearance to administered artemisinin, especially in parts of Southeast Asia (Dondorp *et al.*, 2009). Resistance to antimalarials used for routine treatment of malaria in Kaduna State has been reported by Aliyu *et al.* (2017); hence, there is need for the study of genetic markers of antimalarial drug resistance which will improve our current understanding of resistance pattern in the State.

Detection of parasite nucleic acid through polymerase-chain reaction (PCR) technique is becoming a more frequently used tool in the diagnosis of malaria, as well as the detection and surveillance of drug resistance in *Plasmodium* species. Specific primers have been developed for each of the four species of human *Plasmodium* parasites (Bloland, 2001).

Continuous monitoring of drug resistance in malaria-endemic countries like Nigeria, along with research into the various contributing factors will enable health authorities and practitioners to effectively prevent drug resistance from spreading. The presence of drug resistance genes bearing Single Nucleotide Polymorphisms (SNPs) associated with resistance to antimalarials in *Plasmodium* sp, can serve as indicators of future therapeutic failure. By measuring the number of SNPs or point mutations among field isolates one can predict the efficacy of a particular drug (Sharma, 2005). Predicting the emergence and the spread of resistance to current antimalarial drugs and newly introduced therapeutic compounds is necessary for planning malaria control, and instituting strategies that might delay the emergence of resistance.

1.2 Statement of the Research Problem

The extent by which antimalarial drug resistance genes are prevalent in Kaduna State is not fully known; Studies are lacking to provide current information necessary to quantify the extent of the problem and then need for prompt intervention.

1.3 Justification for the Study

This study is focused on detection and monitoring of antimalarial drug resistance genes in Kaduna State. The results of this study would provide useful treatment guidance and will form the basis for the improvement of strategies that seek to combine new and old antimalarial drugs in the bid to cure malaria while seeking to reduce the incidence of drug resistance in the study area.

1.4 Aim

The aim of this study was to carry out molecular detection of genetic markers associated with *Plasmodium falciparum* drug resistance in some parts of Kaduna State, Nigeria

1.5 Specific Objectives

The specific objectives of this study were to determine:

1. the prevalence of *Plasmodium falciparum* by microscopy and malaria Rapid Diagnostic Test (RDT) in Kaduna State.
2. some demographic factors associated with *Plasmodium falciparum* infection.
3. the haemoglobin genotypes of malaria positive participants by haemoglobin electrophoresis.
4. some genetic markers associated with *Plasmodium falciparum* drug resistance by multiplex PCR.
5. the sequence of *pfatpase6* gene, and determine its relatedness to published sequences in NCBI GenBank.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Discovery of Malaria Parasites

Charles Louis Alphonse Laveran, a French military physician, discovered the protozoan parasite in 1880, whilst working in Algeria. He was lauded with the Nobel Prize for this in 1907. Grassi and Filetti, Italian researchers named *Plasmodium vivax,* and *Plasmodium malariae* in 1890, and an American, Welch, named *Plasmodium falciparum* in 1897. Stephens named the last of the four, *P. ovale*, in 1922. It was Sir Ronald Ross, an officer in the Indian Medical Service who discovered the transmission of malaria by mosquito from bird to bird in 1897 in Calcutta, India, earning the Nobel Prize in 1902 (Ananya, 2014).

2.2 Malaria Parasites in Humans

Malaria is caused by *Plasmodium* species. The parasites are spread to people through the bites of infected female *Anopheles* mosquitoes, called "malaria vectors", which bite mainly between dusk and down. There are four parasite species that cause malaria in humans: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale*. *Plasmodium falciparum* and *Plasmodium vivax* are the most common. *Plasmodium falciparum* is the most deadly. In recent years, some human cases of malaria have also occurred with *Plasmodium knowlesi* – a species that causes malaria among monkeys and occurs in certain forested areas of South-East Asia (WHO, 2014).

2.2.1 *Plasmodium falciparum*

It is found worldwide in tropical and subtropical areas. It is estimated that every year approximately 1 million people are killed by *P. falciparum*, especially in Africa where this species predominates. *P. falciparum* can cause severe malaria because it multiples rapidly

in the blood, and can thus cause severe blood loss (anaemia). In addition, the infected parasites can clog small blood vessels. When this occurs in the brain, cerebral malaria results, a complication that can be fatal (CDC, 2012).

2.2.2 *Plasmodium ovale*

It 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*. 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, 2012).

2.2.3 *Plasmodium malariae*

It is found worldwide, and it is the only human malaria parasite species that has a quartan cycle (three-day cycle). The three other species have a tertian, two-day cycle. If untreated, *P. malariae* causes a long-lasting, chronic infection that in some cases can last a lifetime. In some chronically infected patients *P. malariae* can cause serious complications such as the nephrotic syndrome (CDC, 2012).

2.2.4 *Plasmodium knowlesi*

It is found throughout Southeast Asia as a natural pathogen of long-tailed and pig-tailed macaques. It has recently been shown to be a significant cause of zoonotic malaria in that region, particularly in Malaysia. *Plasmodium knowlesi* has a 24-hour replication cycle and so can rapidly progress from an uncomplicated to a severe infection; fatal cases have been reported (CDC, 2012). *P. knowlesi* has a daily (quotidian) cycle and, if unchecked, can rapidly reach potentially lethal densities (White, 2008).

Plasmodium knowlesi, considered the fifth human malaria parasite (although human-to-human transmission cycle has not yet been conclusively documented) shares a close phylogenetic relationship with *Plasmodium vivax* (White, 2008) and morphological features that resemble those of both *Plasmodium falciparum* and *Plasmodium malariae*. It differs from *P.vivax* because of the absence of a latent liver stage (hypnozoite) and the length of its asexual cycle. *P. knowlesi* is the only primate malaria species characterized by a quotidian (24h) asexual blood stage development. Similar to *P. vivax*, *P. knowlesi* uses the Duffy blood group antigen as a receptor to invade human erythrocytes (Singh *et al.*, 2003). The genome of *P. knowlesi* is made of 14 chromosomes containing a total of 5188 protein-encoding genes, 80% of which could be identified in both *P. falciparum* and *P. vivax*. The two major variant gene families within *P. knowlesi* (i.e., SICA var and Kir) are randomly located across all the 14 chromosomes a finding that is in contrast with other *Plasmodium* genomes (Pain *et al.*, 2008).

2.2.5 *Plasmodium vivax*

Plasmodium vivax (*P. vivax*) causes approximately between 70 and 80 million cases of malaria per year and is the most amply distributed human malaria in the world (Langhi and Bordin, 2006). *Plasmodium vivax* is the leading cause of human malaria in Asia and Latin America but is absent in most of central Africa due to a near fixation of a mutation that inhibits the expression of its receptor, the Duffy antigen on human erythrocytes (Weimin *et al.*, 2014). It is found mostly in Asia, Latin America, and in some parts of Africa. Due to the population densities especially in Asia it is probably the most prevalent human malaria parasite. *P. vivax* (as well as *P. ovale*) has dormant liver stages (hypnozoites) that can be

activated and invade the blood causing relapse several months or years after the infecting mosquito bite (CDC, 2012).

Plasmodium vivax is a sophisticated and resilient malaria parasite which was once prevalent over much of the inhabited world. It has receded from North America, Europe and Russia, but in the tropics vivax malaria remains a major cause of childhood illness. In most endemic areas, *P. vivax* cohabits with *Plasmodium falciparum*. Mixed infections with the two species are common. *P. vivax* is more difficult to control and eliminate than *P. falciparum* because of its tendency to relapse after resolution of the primary infection. In endemic areas relapse of *vivax* malaria is a major cause of malaria in young children, and an important source of malaria transmission. Relapse also occurs in *Plasmodium ovale* infections and in several of the simian malarias, notably *Plasmodium cynomolgi*, which has often been used as an animal model of *vivax* malaria. The factors which control relapse and determine their remarkable periodicity are not known **(**Nicholas, 2011).

2.3 Epidemiology of Malaria

The first record of treatment for malaria dates as far back as 1600 A.D. in Peru. This involved the utilization of quinine-rich bark of the *Cinchona* tree (Cahill, 2004). The French physician Charles Louis Alphonse Laveran first identified the parasite under the microscope in 1880. Ronald Ross and Giovanni Grassi recognized the mosquito as the malaria vector in 1897 (Good, 2001). However, despite enormous and diverse efforts to control this disease, malaria is among the top three most deadly communicable diseases and the most deadly tropical parasitic disease as at 2002 (Sachs and Malaney, 2002).

The classic example of the strong selective pressure malaria puts on the human population is the high incidence of the potentially fatal sickle cell gene reported in regions with

endemic malaria. People with one allele for sickled blood cells, the sickle cell trait, have a survival advantage in regions with endemic malaria. They are more likely to survive an infection by *P. falciparum.* Therefore, people with the sickle cell trait are more likely to survive to a reproductive age. However, the offspring of two people with sickle cell trait have a 25% chance of bearing offspring with sickle cell anaemia, which is often fatal and greatly reduces a person's expected longevity. The enormous effects that malaria has on countries where it is endemic explains this selection (Cahill, 2004).

Approximately 40% of the world's population lives in regions where malaria transmission is endemic, mainly tropical and sub-tropical regions (Aultman *et al.*, 2002). Malaria has been successfully controlled, in fact effectively eliminated, in temperate regions of the world (Sachs and Malaney, 2002). The control strategies employed in temperate regions included changes in agricultural and construction practices, reducing the availability of standing water, and targeted vector control using insecticides such as dichlorodiphenyltrichloroethane (DDT) (Greenwood and Mutabingwa, 2002). Industrialization and improved housing conditions were instrumental in the elimination of the disease in temperate countries (Budiansky, 2002). Windows and walls reduce the amount of contact people have with mosquitoes. Additionally, the more severe seasons of the temperate regions provide another factor for the success of eradication programs as well. The role of the mosquito in the life cycle of *P. falciparum* requires that the parasite be able to maintain an extended infection in order to ensure transmission ability during the following season (Kyes *et al.*, 2001). Now that the sequences of the three participants in the life cycle of human malaria, *P. falciparum*, *Anopheles gambiae*, and *Homo sapiens*, are all completed and available, perhaps new strategies of disease control will succeed.

According to Filler *et al.* (2003), malaria is transmitted via the bite of a female *Anopheles* spp mosquito, which occurs mainly between dusk and dawn. Other comparatively rare mechanisms for transmission include congenitally acquired disease, blood transfusion, sharing of contaminated needles, and organ transplantation.

Malaria occurs in most parts of the tropical regions of the world, with *P. falciparum* causing the largest burden of disease, followed by *P. vivax* (Guerra *et al.*, 2008). *Plasmodium falciparum* predominates in Africa, New Guinea, and Hispaniola (Haiti and the Dominican Republic); *P. vivax* is more common in the Americas and the western Pacific. The prevalence of these two species is approximately equal in the Indian subcontinent, eastern Asia, and Oceania (Breman, 2009). *Plasmodium malariae* is uncommon and is found in most endemic areas, especially in sub-Saharan Africa. *P. ovale,* even less common, is relatively unusual outside of Africa and, where it is found, comprises <1 percent of isolates. *P. knowlesi,* similar morphologically to *P. malariae*, has been identified by molecular methods in patients in Malaysia, the Philippines, Thailand, and Myanmar (White, 2008); this species has not yet been proven to be transmitted from humans to mosquitoes (i.e., a monkey reservoir may be required to infect mosquitoes).

2.4 Burden of Malaria

Globally, there were 219 million cases of malaria in 2017, up from 217 million cases in 2016. The estimated number of malaria deaths stood at 435 000 in 2017 (WHO, 2019). Five countries; Nigeria (25%), the Democratic Republic of the Congo (11%), Mozambique (5%), India (4%) and Uganda (4%) accounted for nearly half of all malaria cases worldwide in 2017 (WHO, 2018b). The 10 highest burden countries in Africa reported

increases in cases of malaria in 2017 compared with 2016. Of these, Nigeria, Madagascar, and the Democratic Republic of Congo had the highest reported increases, all greater than half a million cases (WHO, 2018b).

Along with direct health cost there is a severe economic burden of the disease in terms of lost days of work. In fact malaria is thought to take off 1.3% from the economic growth of some African countries. In some of the most severely affected countries, it accounts for 40% of public health expenditure, 30-50% of inpatient admissions, and 50% of outpatient visits. It affects developing countries in more ways than one including determent of tourism (Ananya, 2014).The Democratic Republic of the Congo and Nigeria account for over 40% of the estimated total of malaria deaths globally. The Democratic Republic of the Congo, India and Nigeria account for 40% of estimated malaria cases.

Estimated malaria mortality rates are highest in countries with a lower gross national income (GNI) per capita. Countries with higher proportions of their population living in poverty (less than US$ 1.25 per person per day) have higher mortality rates from malaria (Ananya, 2014).

Africa has several factors that make it high risk for malaria. Some of these include very efficient mosquito (*Anopheles gambiae*) responsible for transmission, predominant parasite species (*Plasmodium falciparum)* that leads to more severe malaria, warm and humid climate that allows transmission to occur year round as well as lack of resources and poor socio-economic conditions that prevents malaria control efforts. Other areas that are at risk include some countries in South America and South Asia (Ananya, 2014).

In Nigeria, malaria is holo endemic, with only a small area in the middle belt at a 3% risk of epidemic (Federal Ministry of Health Nigeria, 2006). Available records show that annually, about 50% of the population suffers from at least one episode of malaria while children under 5 have an average of 2–4 attacks of malaria (Adedotun *et al.*, 2010). It has also been found that over 300,000 die each year of the disease and malaria is responsible for 60% outpatient visits to health facilities, 30% childhood death, 25% of death in children under one year, 11% maternal death (Jimoh *et al.*, 2007).

Given this magnitude of disease burden, estimating the economic burden of malaria is necessary to provide a basis or platform for advocacy with Ministries of Finance and donors for increased investments in addressing public health problems such as malaria (Okorosobo *et al.*, 2011). It has been shown that although there are equal exposure and incidence of malaria across the socio-economic groups, the costs of treating malaria cases vary amongst different socio-economic status (SES) groups and geographical locations and that payments for treating malaria cases were uniformly by out-of-pocket payments (Ezeoke *et al.,* 2012). These costs have been shown to be proportionately higher in poor households compared to their income and are catastrophic to poorer households and to rural dwellers (Onwujekwe *et al.*, 2010).

In Nigeria, malaria has been shown to account for over 40% of the total monthly curative healthcare costs incurred by households compared to a combination of other illnesses; the cost of treating malaria and other illnesses depleted 7.03% of the monthly average household income, and treatment of malaria cases alone contributed 2.91% of these costs (Onwujekwe *et al.*, 2000). Household spending on malaria can be classified into expenditure on prevention and expenditure on treatment. Individual or household direct

cost of malaria treatment include direct payment of drugs, consultation, laboratory tests, transportation fees to and from the facility (Asenso-Okyere and Dzatorb,1997) while the indirect cost is the productive time lost due to malaria.

2.4.1 Economic implication of malaria

The costs of malaria are enormous when measured in economic terms. It is estimated to cost Africa $12 billion every year (CDC, 2014). This figure factors in costs of health care, economic losses associated with infant and child mortality and morbidity, lost work time, days lost in education, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism.

African countries south of the Sahara bear the heaviest burden of malaria. These countries have been reported to be among the poorest in the world with widespread poverty which continues to play a role in the burden of the disease. Malaria cases and deaths have been reported to rise steadily in sub-Saharan Africa since the late 1970s, especially in Nigeria. The emergence of resistance to Chloroquine, the cheap anti-malarial agent widely used for clinical management of uncomplicated malaria, has been associated with the major factor in this trend, aided by a general weakening in anti – malaria policy. Malaria occurrence is exacerbated by economic decline in the third world countries, which has direct implications for the people's welfare (Alaba, 2007). For instance, malaria has been reported to be responsible for about a 1.3 % reduction in the average annual rate of economic growth for those countries with the highest malaria burden. In Nigeria, malaria is reported as the major cause of morbidity and mortality, especially among children below age five (Alaba, 2007).

There are several reports on the relationship between malaria and the economic growth in the literatures (WHO, 2000; Alaba and Alaba, 2002; Teklehaimanot and Mejia, 2008). Most of these reports highlight the mathematical significance of malaria to gross national production. The links between malaria and poverty are multiple and complex. Therefore a better understanding of the direction and magnitude of the causal relationship is needed, along with better understanding of the nature of poverty that is related to malaria. Poverty sustains the conditions where malaria thrives, and malaria impedes economic growth and thus keeps communities in poverty. The potential dual relationship between poverty and malaria is amply highlight by poor households which experience high malaria prevalence, that in turn maintains them in poverty thus these households are trapped in perpetual cycle of poverty (WHO, 2006).

There are many pathways through which the relationship between malaria and poverty operates e.g. at the household level, poor housing systems exposes people to contact with myriads of infective mosquitoes. Simple preventive measures such as insecticide-treated bed nets are unaffordable to these people and lack of financial resources prevents people from seeking timely medical health care if available in the vicinity. Often, peak malaria transmission coincides with farming seasons and harvesting times. Many days of work lost to illness can mean food insecurity for the entire family and the nation at large (Jimoh, 2016).

2.5 The Life Cycle of *Plasmodium*

According to Cahil (2004), the life cycle of all species of human malaria parasites is essentially the same. He further described the life cycle of human *Plasmodium* parasites as follows: The life cycle of *Plasmodium* comprises an exogenous sexual phase (sporogony)

with multiplication in certain *Anopheles* mosquitoes and an endogenous asexual phase (schizogony) with multiplication in the vertebrate host. The latter phase includes the development cycle in the red blood cells (erythrocytic schizogony) and the phase taking place in the parenchyma cells in the liver (pre-erythrocytic schizogony).

When a female *Anopheles* mosquito bites an infected person, it ingests blood which may contain the mature sexual cells (male and female gametocytes) which undergo a series of developmental stages in the stomach of the mosquito. Exflagellation (the extrusion of rapidly waving flagellum-like microgametes from microgametocytes) occurs resulting in the production in a number of male and female gametes (CDC, 2020; Cahil, 2004).

Fertilization occurs producing a zygote which matures to an ookinete. This penetrates the stomach wall of the mosquito where it grows into an oocyst and it further matures to become a motile sporozoite. The length of the developmental stage in the mosquito not only depends on the *Plasmodium* species but also the mosquito host and the ambient temperature. This may range from eight days in *Plasmodium vivax* to as long as 30 days in *Plasmodium malariae.* The sporozoites migrate from the body cavity of the mosquito to the salivary glands and then mosquito now becomes infective. Sporozoites enter into the blood stream of a host when the female mosquito obtains a blood meal from a human host. Following the inoculation, the sporozoites leave the blood within 30 minutes and enter the parenchymal cells of the liver. In all five species, asexual development occurs in the liver cells; a process which produces thousands of tiny merozoites which are released into the circulation after about 16 days (Cahil, 2004).

However, in *P. vivax* and *P. ovale* (only), some sporozoites differentiate into hypnozoites which remain dormant in hepatocytes for considerable periods of time. When they are "reactivated" they undergo asexual division and produce a clinical relapse. Once in the circulation, the merozoites invade the red cells and develop into trophozoites. In the course of their development they absorb the haemoglobin of the red cells and leave as the product of digestion a pigment called haemozoin, a combination of haematin and protein. This iron-containing pigment is seen in the body of the parasite in the form of dark granules, which are more obvious in the later stages of development. After a period of growth the trophozoite undergoes an asexual division, erythrocytic schizogony. When the mature trophozoite starts to divide in the red blood cell, separate merozoites are formed resulting in a schizont.

When fully developed, the schizont ruptures the red blood cell containing it, liberating the merozoites into the circulation. These merozoites will then infect new red cells and the process of asexual reproduction in the blood tends to proceed. Some of the merozoites entering red blood cells do not form trophozoites but develop into gametocytes and this process takes place in deep tissue capillaries. This erythrocytic cycle of schizogony is repeated over and over again in the course of infection, leading to a progressive increase of parasitemia (Cahil, 2004).

2.6 Malaria Transmission and Clinical Presentation of Malaria

2.6.1 Malaria transmission

In most cases, malaria is transmitted through the bites of female *Anopheles* mosquitoes. There are more than 400 different species of *Anopheles* mosquito; around 30 are malaria vectors of major importance. All of the important vector species bite more commonly

between dusk and dawn. The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment (WHO, 2018a).

Transmission is more intense in places where the mosquito lifespan is longer (so that the parasite has time to complete its development inside the mosquito) and where it prefers to bite humans rather than other animals. The long lifespan and strong human-biting habit of the African vector species is the main reason why nearly 90% of the world's malaria cases are in Africa (WHO, 2018b).

Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes, such as rainfall patterns, temperature and humidity. In many places, transmission is seasonal, with the peak during and just after the rainy season. Malaria epidemics can occur when climate and other conditions suddenly favour transmission in areas where people have little or no immunity to malaria. They can also occur when people with low immunity move into areas with intense malaria transmission, for instance to find work, or as refugees (WHO, 2018b).

2.6.2 Clinical presentation of malaria

The asexual erythrocytic blood stage parasites leads to massive destruction of erythrocytes associated with accumulation of toxic wastes that result to malaria clinical presentation. These waste products stimulate macrophages and other cells to produce cytokines. Host receptor/parasite ligand interactions and associated pathologies (anaemia, respiratory distress, and acidosis) have also been shown to be responsible for the different clinical presentations. The clinical presentation can be gradual or with a fulminant course having non-specific symptoms and often resembling viral infections. Many factors including

host/parasite genetics, age of the patient and transmission intensity may modulate the clinical presentation (Minja, 2013)

2.6.2.1 Asymptomatic Malaria

Clinical manifestations of *Plasmodium* infection vary from asymptomatic to severe and fatal outcome (Langhorne *et al*., 2008). Asymptomatic malaria cases refers to situations whereby individuals are often infected by malarial parasites but do not develop clinical disease. Continuous exposure to malaria parasites in high transmission areas often results to partial immunity that leads to asymptomatic carriers that can act as potential reservoir of parasites, or gametocyte carriages resulting to persistent transmission (Langhorne *et al*., 2008).

2.6.2.2 Uncomplicated Malaria

In a non-immune individual, symptoms appear seven days or more (usually 10–15 days) after the infective mosquito bite. The first symptoms; fever, headache, chills and vomiting may be mild and difficult to recognize as malaria. If not treated within 24 hours, *P. falciparum* malaria can progress to severe illness often leading to death (WHO, 2014).

Any person with symptomatic malaria with fever and any of the following symptoms; headache, body and joint pains, feeling cold and shivering, loss of appetite and sometimes abdominal pains, diarrhoea, nausea and vomiting and splenomegaly as well as other unspecific symptoms is considered to have uncomplicated malaria. A clinical picture of such infection can mimic/resemble that of many other childhood illnesses (WHO, 2014).

2.6.2.3 Severe Malaria

Plasmodium falciparum is almost exclusively responsible for almost all forms of severe malaria whereas other non-falciparum species are rarely implicated in the most severe forms of the disease. Severe/complicated malaria (SM) is defined as a form of

symptomatic malaria with signs of vital organ disturbance complicated by serious organ failures or abnormalities in the patient's blood or metabolism (Minja, 2013).

The clinical manifestation of SM may include cerebral malaria that is associated with abnormal behaviour, impairment of consciousness, seizures, coma or other neurological abnormalities; severe anaemia due to massive destruction of erythrocytes; haemoglobinuria due to hemolysis; acute respiratory distress syndrome (due to deep breathing as a result of metabolic acidosis); hypotension (low blood pressure) due to cardiovascular collapse; acute kidney failure; hyperparasitaemia (where more than 5% of the RBCs are infected by malarial parasites); metabolic acidosis (excessive acidity in the blood and tissue fluids and hypoglycaemia to mention only a few (Minja, 2013).

2.7 The Mosquito Vector

From over 400 species in the *Anopheles* genus, only 30 to 40 of them transmit malaria in natural condition. The highest reproduction rate of anopheles is observed in tropical regions where the humidity and heat are optimal for the mosquito to live long enough to allow the parasite to complete its life cycle in the *Anopheles* host (female *Anopheles* have a life expectancy of approximately one month). The time required for the mosquito to be infective to a human is 10 to 21 days following its infecting blood meal. This timeline depends on the parasite species and the temperature. In Africa, *Anopheles gambiae and Anopheles funestus* are both strongly anthropophilic, making them the most efficient malaria vectors worldwide (Bloland, 2001).

2.8 Diagnosis of Malaria

Accurate and reliable diagnosis is the key to rational treatment as it will be possible to distinguish malaria from other febrile illnesses such as viral (e.g. dengue fever and

influenza), bacterial (e.g. typhoid, brucellosis, respiratory and urinary tract infections) and other acute septic syndromes. Often, in malaria endemic regions, co-morbidity may occur where malaria parasitaemia is observed in patients with febrile illness due to bacterial or viral infections. The malaria infection may still remain asymptomatic due to development of anti-disease rather than anti-parasite immunity (Pasvol, 2005). These facts underscore the importance of tests that not only can confirm a malaria diagnosis but also tests that can facilitate diagnosis of other causes of febrile illnesses.

Direct microscopic examination of intracellular parasites on stained blood films is the current standard for definitive diagnosis in nearly all settings. However, several other approaches exist or are in development (Bloland, 2001).

2.9 Treatment of Malaria

Early malaria case detection and prompt treatment with safe and effective antimalarial drugs still remains the mainstay of malaria case management (Winstanley, 2000). If not properly managed, either due to missed or delayed diagnoses, malaria may progress from mild through complicated to severe disease. Case management usually depends on the severity of infection, age, therapeutic efficacy of the antimalarial drug as well as their costs and availability (White, 1996). Gestational age is also an important issue to consider prior to prescription of any drugs due to potential risks of harming the embryo/foetus (Nosten *et al*., 2006).

Antimalarial drugs work by disrupting processes or metabolic pathways in different subcellular organelles and most of them target the erythrocytic stages. For effective treatment, antimalarial drugs must be fast acting, highly potent against blood stage

parasites, with minimal toxicity and should be readily available and affordable to residents of endemic regions (Greenwood *et al*., 2008).

Parenteral artesunate is more effective than quinine in resolving fever and parasite clearance for severe malaria cases in children. Quinine is used during pregnancy despite side effects such as quinine-induced hyperinsulinaemic hypoglycaemia that necessitates careful monitoring of glucose level (Pasvol, 2005). As a strategy to prevent adverse pregnancy outcomes due to malaria infection during pregnancy, all pregnant women visiting the antenatal clinics (ANC) are given intermittent preventive treatment using sulfadoxine pyrimethamine (IPTp-SP). This is in accordance to the WHO recommendations on malaria prevention during pregnancy (Pasvol, 2005).

The treatment of malaria is dependent on three main factors, these include: the infecting species of *Plasmodium* parasite population, the clinical situation of the patient (for example, adult, child, or pregnant female) and the drug susceptibility of the infecting parasites. *Plasmodium* species susceptibility to antimalarial drugs varies with geographical locations where the infection was acquired. Different areas of the world have varying types of *Plasmodium* resistance peculiar to that location. The effective antimalarial drugs for different malaria must be prescribed by a doctor who is familiar with malaria treatment protocols (Jimoh, 2016).

The WHO's anti-malarial treatment policy (WHO, 2006) had recommended the treatment of uncomplicated *P. falciparum* malaria with artemisinin-derived combination therapy (ACTs) such as Artesunate-Amodiaquine, Artesunate-Mefloquine, Artesunate-Pyronaridine, Dihydroartemisinin-Piperaquine, and Chlorproguanil-Dapsoneartesunate. This recommendation has been celebrated as being effective in the treatment of

Chloroquine-resistant *P. falciparum*. Unfortunately, in 2009, some reports showed that *P. falciparum*-infected individuals have parasites ACT drugs treatment failure (WHO, 2006).

2.9.1 Treatment failure of malaria

A distinction must be made between a failure to clear malarial parasitaemia or resolve clinical disease following a treatment with an antimalarial drug and true antimalarial drug resistance. While drug resistance can cause treatment failure, not all treatment failure is due to drug resistance (Bloland, 2001).. Many factors can contribute to treatment failure including incorrect dosing, non-compliance with duration of dosing regimen, poor drug quality, drug interactions, poor or erratic absorption, and misdiagnosis. Probably all of these factors, while causing treatment failure (or apparent treatment failure) in the individual, may also contribute to the development and intensification of true drug resistance through increasing the likelihood of exposure of parasites to suboptimal drug levels (Bloland, 2001).

2.10 Control of Malaria

The WHO's global malaria eradication campaign (launched in 1955) formulated a plan to eradicate malaria in 10–15 years with the indoor residual spraying (IRS) of dichlorodiphenyltrichloroethane (DDT) (Loha, 2013). The prevention of breeding of vectors and measures against malaria parasites were also considered in the eradication package, which had taken lessons from earlier control efforts and lasted until 1969. Though it was not highly successful in African countries – due to inability of the health services to manage control programmes, consequently leading to technical difficulties to pursue eradication (Tanner and Savigny, 2008), it resulted in eliminating malaria from

most of Europe and North America (Carter and Mendis, 2002). Malaria control can be defined as reducing the disease burden to a level at which it is no longer a public health problem (WHO, 2008). The main current tools in malaria control are antimalarial drugs, including artemisinin-based combination therapy (ACT) and intermittent preventive treatment (IPT), and vector control measures, including insecticide-treated nets (ITN) and indoor residual spraying with insecticides (IRS) (WHO, 2010a).

2.10.1 Use of artemisinin-based combination therapy (ACT)

Malaria is a preventable and curable disease; the requirement for effective malaria control therefore includes prompt access to affordable and efficacious antimalarial drugs. Artemisinin-based combination therapy is used for treatment of uncomplicated *P. falciparum* malaria with the objective to cure the infection and prevent progression to severe disease (WHO, 2010a).

2.10.2 Preventive chemotherapy

Preventive chemotherapy is recommended for pregnant women and infants in countries with a moderate-to-high/stable transmission of malaria. In addition, seasonal malaria chemoprevention for children 3–59 months old is recommended in areas with highly seasonal malaria transmission. The preventive chemotherapy recommendations are: Intermittent preventive treatment in pregnancy (IPTp) providing Sulfadoxine - Pyrimethamine at each scheduled ante-natal care visit (each dose to be given at least one month apart) starting early in second trimester, Intermittent preventive treatment in infants (IPTi) – providing three doses of Sulfadoxine-Pyrimethamine to infants along with the second and third Diphtheria-Pertussis-Tetanus and Measles vaccines, Seasonal malaria

chemoprevention (SMC) – providing Amodiaquine plus Sulfadoxine-Pyrimethamine, to a maximum of four doses, for children 3–59 months in areas where there is highly seasonal malaria transmission (Loha, 2013).

In Africa intermittent preventive treatment in pregnancy (IPTp) is recommended since pregnant women are especially vulnerable to malaria due to infection of the placenta. In IPTp full treatment doses are administered at least twice during the pregnancy as prophylaxis. Drugs suitable for preventive use must have a long half-life and a very good safety profile, as they are given to healthy subjects, and the only current option fulfilling these criteria is sulfadoxine-pyrimethamine (SP) (WHO, 2008). It has been demonstrated that the use of IPTp reduces the incidence of maternal anaemia and improve infant birth weight, which possibly leads to improved child survival. At the end of 2006 a majority of the countries in Africa had adopted IPTp as national policy (WHO, 2008).

2.10.3 Use of insecticide treated bed nets (ITN)

Untreated bed nets form a protective barrier around persons using them. However, application of an insecticide greatly enhances the protective efficacy of bed nets. If there is high ITN coverage in a community, the numbers and longevity of mosquitoes will be reduced, resulting in protection also of individuals that do not sleep under ITNs. Studies have demonstrated that the use of ITNs can reduce child mortality by one fifth and malaria incidence by half (Lengeler, 2004). Nearly all African countries have adopted the policy of providing ITNs free of charge to children and pregnant women, but the coverage is still not adequate in most countries (WHO, 2008). Insecticide resistance is a threat to the sustainability of ITNs, since most nets are impregnated with pyrethroids, to which vectors are already resistant in some areas of the world (WHO, 2008). More recently, long-lasting

insecticide-treated nets (LLINs) that retain effective concentrations of insecticide for at least three years have been developed.

2.10.4 Indoor residual spraying with insecticides (IRS)

The effectiveness of IRS with insecticides such as DDT for malaria prevention and control in the pre-eradication era paved the way for Indoor Residual Spraying to be the prominent control measure during the 'eradication' era, and in the process also becoming one of the most important tools in recent times (Loha, 2013).

Indoor Residual Spraying is the application of long-acting insecticides on the walls and roofs inside houses to kill the mosquito that land on these surfaces. A number of insecticides are used for IRS, and some can also repel mosquitoes and prevent that they enter in a sprayed room. The use of IRS has declined and is now generally used in foci of high malaria transmission (WHO, 2008). Both ITN and IRS are most effective against mosquitoes that bite and rest indoors, such as *A. gambiae* and *A. funestus*. Rapidly developing resistant vectors to available insecticides jeopardize this strategy, thereby implying the need for the continuous monitoring of insecticide resistance to sustain the benefit of IRS (Ranson *et al*., 2009).

2.10.5 Vaccines against malaria

Mosquirix (RTS,S/AS01 (RTS,S)) is an injectable vaccine that provides partial protection against malaria in young children. The vaccine is being evaluated in sub-Saharan Africa as a complementary malaria control tool that potentially could be added to (and not replace) the core package of WHO-recommended preventive, diagnostic and treatment measures. In July 2015, the vaccine received a positive opinion by the European Medicines Agency, a

stringent medicines regulatory authority. In October 2015, two WHO advisory groups recommended pilot implementation of RTS, S/AS01 in a limited number of African countries. WHO adopted these recommendations and is strongly supportive of the need to proceed with the pilot programme as the next step for the world's first malaria vaccine (WHO, 2018b).

In November 2016, WHO announced that the RTS,S vaccine would be rolled out in pilot projects in selected areas in 3 countries in sub-Saharan Africa: Ghana, Kenya and Malawi. These pilot projects could pave the way for wider deployment of the vaccine if safety and effectiveness are considered acceptable (WHO, 2018b).

2.10.6 Impact and consequences of malaria control

The impacts of the described malaria control tools can be illustrated by the success in specific regions were the application of interventions has been strengthened. In Eritrea, Rwanda, Sao Tomé and Principe, and Zanzibar the malaria burden has been reduced by 50% or more between 2000 and 2007 (WHO, 2008).

2.11 Elimination of Malaria

Malaria elimination is defined as the interruption of local transmission of a specified malaria parasite species in a defined geographical area as a result of deliberate activities. Continued measures are required to prevent re-establishment of transmission (WHO, 2018b).

Malaria eradication is defined as the permanent reduction to zero of the worldwide incidence of malaria infection caused by human malaria parasites as a result of deliberate

activities. Interventions are no longer required once eradication has been achieved (WHO, 2018b).

Countries that have achieved at least 3 consecutive years of zero local cases of malaria are eligible to apply for the WHO certification of malaria elimination. In recent years, 8 countries have been certified by the WHO Director-General as having eliminated malaria: United Arab Emirates (2007), Morocco (2010), Turkmenistan (2010), Armenia (2011), Maldives (2015), Sri Lanka (2016), Kyrgyzstan (2016) and Paraguay (2018). The WHO *Framework for Malaria Elimination* (2017) provides a detailed set of tools and strategies for achieving and maintaining elimination (WHO, 2018a).

2.12 Genome of *Plasmodium falciparum*

The Malaria Genome Project was set up in the year 1995 to sequence the genome of the malaria parasite. Its mitochondrion was sequenced in the same year. In 1996, the plastid (apicoplast) was sequenced. The genome of the nuclear chromosome 2 and chromosome 3 were sequenced in 1998 and 1999 respectively. The entire genome was sequenced on October 3rd, 2002 (Gardner *et al.*, 2002).

The *P. falciparum* 3D7 nuclear genome is composed of 23 megabases (Mb) distributed among 14 chromosomes. The overall (A + T) composition is 80.6% and about 90% in introns and intergenic areas, (the most A + T-rich genome sequenced to date). Approximately 5,300 protein-encoding genes were identified. Genes involved in antigenic variation are concentrated in the subtelomeric regions of the chromosomes (Gardner *et al.*, 2002).

Plasmodium falciparum has a highly conserved genome aside from the highly variable regions clustered close to the telomeres. The three most noted gene families in these

regions, involved in immune evasion, are *var*, *rif*, and *stevor* (Florens *et al.*, 2002). The gene products are *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), repetitive interspersed family (rifin) and sub-telomeric variable ORF (stevor) proteins, respectively (Florens *et al.*, 2002). The PfEMP1 proteins are exported to the surface of the infected erythrocyte where they mediate adherence to host endothelial regions. They give the parasite the ability to sequester large numbers of infected cells into specific organs (Gardner *et al.*, 2002). Almost all *P. falciparum* isolates in red blood cells are capable of cytoadherence, which can result in serious damage of the chosen organ. The damage is believed to be the result of blocked circulation or release of cytokines or nitrous oxide (NO). This can be fatal. Even under ideal medical conditions, 30% of patients with cerebral malaria will die (Kyes *et al.*, 2001). .

2.12.1 Plasticity in *Plasmodium falciparum* genome

Plasmodium falciparum shows plasticity in its genome. For its survival it can delete certain genes (or portions) if not needed for its growth and has the capability to regulate its genes under various stages of its life cycle as well as under unfavourable environmental conditions. The parasite has shown enormous amount of antigenic variation under immune pressure leading to the emergence of vaccine resistant strains (Sharma, 2005). Similarly, under drug pressure, it allows mutations to settle in the target genes. It is becoming clearer that with the continuous exposure to a drug, the parasite accumulates more and more number of mutations in these genes. By measuring the number of these point mutations among field isolates one can predict the efficacy of a particular drug. Therefore, these markers are useful tools at epidemiological level. This molecular surveillance can also help

in slowing down the drug resistance if supported by a careful drug usage policy (Sharma, 2005).

Malaria parasite contains three genomes: the nuclear genome, the plastid genome and the mitochondrial genome. The latter two are extrachromosomal elements of sizes 35 and 6kb, respectively. The nuclear genome is the largest of the three. This genome consists of 14 chromosomes and shows maximum fluidity. The fluidity in the *Plasmodium falciparum* genome is well documented where it can delete large portions of the DNA. According to Sharma (2005) the deletion of subtelomeric ends of the chromosomes including some coding regions among the field isolates. It is also known that the parasite can regulate the expression of its genes differently in *in vitro* culture conditions than the *in vivo* conditions. For example, the knob-associated histidine-rich protein (KAHRP) gene transcription may not take place if the cultures were running continuously for a long period of time. Not only the transcription is controlled by the parasite under such culture conditions, but it can also delete the gene if it is not required for its survival. Again the best example is the deletion of KAHRP gene in certain culture adapted *Plasmodium falciparum* lines which do not produce knobs. The parasite thus adapts to the adverse environment by regulating its gene expression as well as permitting the gene alterations (Sharma, 2005).

2.13 Haemoglobin Disorders and Malaria

During the six decades since Haldane first proposed the malaria hypothesis (Thomas and David, 2012), evidence has steadily grown to confirm that malaria is indeed the primary force behind the high frequency of inherited haemoglobin disorders that is seen in many tropical and subtropical populations. The evidence supporting this assertion comes from four main sources: the similarity between the distributions of malaria and specific

haemoglobin disorders at local, regional, and global scales; from population genetic predictions of their historic age; from clinical studies conducted in malaria-endemic areas; and from mechanistic studies conducted both *in-vitro* and *ex-vivo* (in living tissue outside an organism). Although such evidence is strongest for Haemoglobin S (HbS) and for the α thalassemias, which have been the focus of most researches, there can now be little doubt that malaria is responsible for the current distributions of all the major haemoglobin disorders (Thomas and David, 2012).

2.13.1 Haemoglobin S

The heterozygous state for haemoglobin S; haemoglobin AS (HbAS), is the best described of all malaria-protective traits and is used as the classic example of balanced polymorphism in schools and colleges throughout the world. The close resemblance between the geographic distribution of HbAS and that of malaria, documented by a number of different investigators more than 60 years ago (Thomas and David, 2012), gave the first clues. But it was Allison who first articulated the malaria hypothesis with regard to HbS most definitively (Allison 1954a,b,c). Not only, as others before him, did he note that "sicklers" were significantly less likely than "non-sicklers" to carry malaria parasites under conditions of natural exposure but he also showed that, when inoculated intravenously with large volumes of parasite-infected blood, sicklers were less likely to develop a clinical malaria infection. Many studies have since been conducted that put the malaria hypothesis with regard to HbS beyond reasonable doubt. First, the close correspondence between the frequencies of the beta S (β^s) allele and the historic prevalence of malaria has been confirmed through a growing number of surveys at both the local and global scales (Enevold *et al.,* 2007). This relationship has been quantified statistically, an analysis that

found strong geographical support for the malaria hypothesis in Africa but not in either the Americas or in Asia (Piel *et al.,* 2010). Haemoglobin S is absent from indigenous populations in the Americas, probably because malaria did not reach the continent until recently, whereas in Asia it follows a rather restricted distribution, being confined to a small number of tribal populations in India (Piel *et al.,* 2010).

Direct clinical support for a protective effect of HbAS has been provided by case control, cohort, and family-based association studies conducted in multiple populations over the last 30 years (Jallow *et al.,* 2009). In general, such studies have shown that HbAS is approximately 50% protective against uncomplicated and >80% protective against severe *P. falciparum* malaria. Although few individual studies have been powered to detect an effect on malaria-specific mortality, HbAS was associated with 55% protection against all-cause mortality in children 6–16 months old in a single cohort study conducted in Western Kenya (Aidoo *et al.,* 2002). Although HbAS protects against clinical malaria infections, parasite-infected blood from HbAS subjects is several times more infectious to the *Anopheles* vector than that of normal subjects (Gouagna *et al.,* 2010). This observation, which is echoed by a similar observation from Senegal (Lawaly *et al.,* 2010), suggests that the personal advantage of HbAS is not only balanced by the cost of potential homozygosity in offspring but also by that of increased malaria transmission to the general population.

Whereas the malaria-protective effect of HbAS is clear, the effect of the homozygous state, HbSS, on malaria risk is more controversial. Biologically, there are hypothetical reasons why subjects with HbSS might either enjoy a greater degree of protection than those with HbAS or, conversely, might be at more increased risk than normal subjects (Williams and

Obaro 2011). Few controlled studies of this question have been reported but the balance of evidence suggests that both scenarios might be true. Subjects with HbSS appear to be less susceptible than normals to developing malaria infections (Makani *et al.,* 2010; McAuley *et al.,* 2010) but are highly susceptible to the catastrophic consequences of malaria, particularly severe anaemia, if they do become infected with the disease (Makani *et al.,* 2010; Williams and Obaro 2011). The net result is that malaria is almost certainly a major cause of premature mortality in children born with HbSS in malaria-endemic areas and that the early detection of HbSS in association with active malaria prevention could have a major impact on survival (Serjeant, 2005).

2.13.1.1 The mechanism of protection against malaria afforded by HbAS

Although the fact that HbAS confers malaria protection is now well established, the mechanism by which it does so remains a matter of some speculation. Through early studies, conducted soon after it became possible to grow malaria parasites in culture, investigators converged on the general hypothesis that protection resulted from impairment in the invasion and growth of *P. falciparum* parasites into HbAS red cells under conditions of low oxygen tension that were physiologically representative of *in vivo* conditions (Friedman 1978; Friedman *et al.,* 1979).

However, a number of alternative hypotheses have subsequently developed. Several investigators have suggested that the mechanism may relate to the enhanced removal of parasite-infected HbAS red blood cells. Some have proposed that this may relate to the fact that such cells sickle under low oxygen tension (Luzzatto *et al.,* 1970; Friedman 1978) and that this leads to their premature destruction in the spleen (Friedman 1978; Shear *et al.,* 1993), whereas Ayi *et al*. (2004) had proposed a mechanism that is generic to a number of

red cell disorders and involves the enhanced opsonization of red cells infected with ring-stage parasites through a process that involves increased oxidative stress.

An alternative hypothesis by Cholera *et al*. (2008) had shown that *in vitro*, relative to parasitized normal haemglobin AA (HbAA) red blood cells, the binding of parasitized HbAS red blood cells to both microvascular endothelial cells and to blood monocytes was significantly reduced and that this correlated with altered surface display of the parasite-encoded protein *P. falciparum* Erythrocyte Protein-1(PfEMP1). Because the PfEMP1-mediated sequestration of mature parasites in the post-capillary venules of critical tissues such as the brain have been implicated in both the pathogenesis of severe malaria and the evasion of parasite-infected red cells from immune clearance by the spleen, this provides an attractive explanation for the protective effect of HbAS. Cyrklaff and colleagues have elaborated on this hypothesis by showing, again *in vitro*, that HbAS affects the trafficking system that directs PfEMP1 to the surface of infected erythrocytes. Using cryo-electron tomography they showed that within the cytoplasm of normal red blood cells the parasite proteins are transported to the surface via a parasite-generated host-derived actin cytoskeleton but that haemoglobin oxidation products disrupted this process in HbAS red cells (Cyrklaff *et al.*, 2011).

Whereas all the hypotheses discussed so far imply that HbAS protects against malaria entirely through innate mechanisms, data from both epidemiological and clinical studies suggest that this may not be the entire story. The protective effect of HbAS in naturally exposed populations' increases with age, indicating that the mechanism might not be entirely innate but might also include an acquired, immunological component.This hypothesis is supported by a number of studies that have reported enhanced malaria-

specific immune responses in HbAS individuals (Cabrera *et al.*, 2005; Verra *et al.*, 2007), and potentially by recent studies using a mouse model that suggest an immuno-modulatory mechanism mediated through hemoxygenase-1(Ferreira *et al.*, 2011). However, the mouse model of the sickling disorders is metabolically very different to the human sickle-cell traits. Furthermore, whereas such studies appear to support an immune component to the malaria protective effects of HbAS they are balanced by others that show no apparent differences between HbAS and normal subjects with regard to a range of immunological responses (Tan *et al.*,, 2011), leaving the balance between innate and acquired mechanisms unresolved.

2.13.2 Haemoglobin C (HbC)

Fewer studies have focused on the malaria-protective effects of HbC than for HbS. The geographic range of HbC is considerably more limited than of HbS, being centered on West and North-West Africa with the exception of a low-frequency corridor between West Africa and Egypt that appears to reflect patterns of human migration. Although historically HbC has been restricted to malaria-endemic communities, to the best of our knowledge the relationship between the population frequency of HbC and the endemicity of malaria has not been formally tested. Early studies investigating the association between HbC and clinical protection were either inconclusive (Guinet *et al.*, 1997) or suggested only a marginal effect of HbC in comparison to the marked protection afforded by HbS (Ringelhann *et al.*, 1976). This was confirmed in a large cross-sectional survey (Danquah *et al.*, 2010). This may well be explained by more recent studies suggesting that protection might be specific to particular categories of strictly defined severe malaria (May *et al.*, 2007) and that it is greater in homozygotes (with HbCC) than in heterozygotes (with

HbAC) (Mockenhaupt *et al.,* 2004a). The strongest evidence for a homozygous advantage of HbC comes from a large case-control study conducted by Modiano and colleagues in Burkina Faso, in which they found a 29% reduction in the risk of clinical malaria among children with HbAC compared to a 93% reduction among children with HbCC (Modiano *et al.,* 2001). Finally, like HbS, it appears that HbC has a marked effect on the transmissibility of malaria by the *Anopheles* vector (Gouagna *et al.,* 2010).

Mechanistically, similar hypotheses have been pursued for HbC as for HbS. As for HbAS, early studies suggested that the protective effect of HbC might result from a reduced ability of *P. falciparum* parasites to grow and multiply in red blood cells containing HbC (Friedman *et al.,* 1979; Olson and Nagel 1986; Fairhurst *et al.,* 2003). Subsequently, however, it has been suggested that the mechanism might involve an immunological component. This is supported by the finding of higher immune responses to PfEMP1 and various malaria antigens among children expressing the HbC allele in a low transmission urban area of Burkina Faso (Verra *et al.,* 2007), although no differences were found in a higher transmission rural population in the same study or in a more recent study conducted in Mali (Tan *et al.,* 2011). An alternative hypothesis has recently been developed: that HbC exerts its protection through a specific effect on cytoadherence, mediated by the altered display of surface-expressed parasite proteins (Fairhurst *et al.,* 2005; Cyrklaff *et al.,* 2011).

2.13.3 Haemoglobin E (HbE)

Much less is known about the effects of HbE on malaria than for the other common structural variants of haemoglobin. Like most of the other disorders, HbE is restricted to populations that have traditionally been endemic for malaria and there is some evidence for

a correlation between the prevalence of the two conditions (Win *et al.*, 2005). Moreover, in one Thai population, it has recently been estimated on the basis of genetic linkage analysis that the most frequent variant, HbE β26 Glu → lys, has reached its current frequency in <5000 years, a period compatible with the malaria hypothesis (Ohashi *et al.*, 2004). Nevertheless, few clinical studies have reported the relative risk of clinical malaria in subjects with HbE. In one such study conducted in adults with malaria who were admitted to the hospital in Thailand, the manifestations of malaria were less severe in patients with HbE trait (HbAE) (Chotivanich *et al.*, 2002). However, in a similar study, Oo and colleagues found no significant relationship between either HbAE or HbEE and the severity of malaria in adults admitted to the hospital in Burma (Oo *et al.*, 1995). Similarly, a small recent study found no relationship between HbE and the risk of cerebral malaria in Thai adults (Naka *et al.*, 2008).

2.13.4 The thalassemias

There is strong evidence from population data that malaria selection explains the current distribution of the thalassemias. First, at a global scale, both α and β thalassemia follow remarkably similar distribution to that of malaria, an observation that also holds true at a micro-epidemiological scale. For example, in early studies, Siniscalco and colleagues noted a strong correlation between the population prevalence of β thalassemia and the historic incidence of malaria among villages in Sardinia in southern Italy (Thomas and David, 2012). Similar observations have subsequently been made regarding the distribution of α thalassemia in South Asia (Modiano *et al.*, 1991), Tanzania (Enevold *et al.*, 2007), and the Pacific, where in some populations these conditions have virtually reached fixation. Second, unlike many of the other disorders of haemoglobin, the

molecular defects that result in the thalassemias are extremely diverse (Higgs, 2013), having arisen separately and been locally amplified, in multiple populations throughout the malaria-endemic regions of the world.

Despite the overwhelming epidemiological evidence for malaria selection, the protective effect of β thalassemia has been the subject of few clinical studies. Nevertheless, in one case-control study conducted in northern Liberia, Willcox and colleagues estimated a protective effect of approximately 50% (Thomas and David, 2012). Whereas many more studies have recorded, the clinical relationship between α thalassemia and malaria, the results have not been entirely consistent. The incidence of uncomplicated clinical malaria has been lower in α thalassemic than normal subjects in some cohort studies (Wambua *et al.*, 2006), whereas in others the incidence has either been equal (Lin *et al.*, 2010) or even higher (Veenemans *et al.*, 2011). Conversely, studies focused on severe malaria have shown consistent evidence for a strongly protective effect that is generally more marked in homozygous than heterozygous subjects (Mockenhaupt *et al.*, 2004b; Williams *et al.*, 2005b; May *et al.*, 2007). Available data, therefore, provide good evidence for a protective effect of α thalassemia against severe and fatal malaria, but the data on uncomplicated malaria are somewhat perplexing. It seems likely that these observations are pointing to important clues about the mechanism by which α thalassemia protects against severe malaria.

2.13.5 Epistatic interactions between the haemoglobin disorders

If, as discussed above, malaria is responsible for the Mendelian selection of many of the inherited disorders of haemoglobin, a question remains about why they have not all become common throughout the whole of the malaria-endemic world. This is a question

that has been asked particularly from the perspective of pairs of haemoglobin disorders by a number of different investigators. Studies conducted both in Kenya and in Ghana have recently shown that when inherited alone, both HbAS and α thalassemia are strongly protective against *P. falciparum* malaria but that the protective effects of each are lost when both conditions are inherited together (Williams *et al.*, 2005a; May *et al.*, 2007). From the perspective of HbAS, this may be explained by the changes in the intracellular concentration of HbAS that accompany the coinheritance of α thalassemia (Thomas and David, 2012). This negative interaction between the two malaria-protective phenotypes could well explain why both are held at intermediate frequencies in populations where both occur together (Williams *et al.*, 2005a; Hedrick 2011; Penman *et al.*, 2011). A similar hypothesis has more recently been advanced to explain the relative frequencies of HbAS and the α and β thalassaemias within the Mediterranean region (Penman *et al.*, 2009).

2.14 Antimalarial Drugs

There are only a limited number of drugs which can be used to treat or prevent malaria (Bloland, 2001).The most widely used are quinine and its derivatives and antifolate combination drugs.

2.14.1 Quinine and related compounds

Quinine, along with its dextroisomer quinidine, has been the drug of last resort for the treatment of malaria, especially severe disease. Chloroquine is a 4-aminoquinoline derivative of quinine first synthesized in 1934 and has since been the most widely used antimalarial drug. Historically, it has been the drug of choice for the treatment of non-severe or uncomplicated malaria and for chemoprophylaxis, although drug resistance has

dramatically reduced its usefulness. Amodiaquine is a relatively widely available compound closely related to chloroquine. Other quinine-related compounds in common use include primaquine (specifically used for eliminating the exoerythrocytic forms of *P. vivax* and *P. ovale* that cause relapses), and mefloquine (a quinoline- methanol derivative of quinine) (Bloland, 2001).

2.14.2 Antifolate combination drugs

These drugs are various combinations of dihydrofolate-reductase inhibitors (proguanil, chlorproguanil, pyrimethamine, and trimethoprim) and sulfa drugs (dapsone, sulfalene, sulfamethoxazole, sulfadoxine, and others). Although these drugs have antimalarial activity when used alone, parasitological resistance can develop rapidly. When used in combination, they produce a synergistic effect on the parasite and can be effective even in the presence of resistance to the individual components. Typical combinations include sulfadoxine/ pyrimethamine (SP or Fansidar1), sulfalene- pyrimethamine (metakelfin), and sulfametho- xazole-trimethoprim (co-trimoxazole). A new antifolate combination drug is currently being tested in Africa. This drug, a combination of chlorproguanil and dapsone, also known as Lap-Dap, has a much more potent synergistic effect on malaria than existing drugs such as SP. Benefits of this combination include; a greater cure rate, even in areas currently experiencing some level of SP resistance, a lower likelihood of resistance developing because of a more advantageous pharmacokinetic and pharmacodynamic profile, and probable low cost (Bloland, 2001).

2.14.3 Use of antibiotics as antimalarial agents

Tetracycline and derivatives such as doxycycline are very potent antimalarials and are used for both treatment and prophylaxis. In areas where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates. Clindamycin has been used but offers only limited advantage when compared to other available anti-malarial drugs. Parasitological response is slow to clindamycin and recrudescence rates are high. Its efficacy among non-immune individuals has not been fully established (Bloland, 2001).

2.14.4 Artemisinin compounds

A number of sesquiterpine lactone compounds have been synthesized from the plant *Artemisia annua* (artesunate, artemether, arteether). These compounds are used for treatment of severe malaria and have shown very rapid parasite clearance times and faster fever resolution than occurs with quinine. In some areas of South-East Asia, combinations of artemisinins and mefloquine offer the only reliable treatment for even uncomplicated malaria, due to the development and prevalence of multidrug- resistant falciparum malaria. Combination therapy (an artemisinin compound given in combination with another antimalarial, typically a long half-life drug like mefloquine) has reportedly been responsible for inhibiting intensification of drug resistance and for decreased malaria transmission levels in South-East Asia (Bloland, 2001).

2.14.5 Other compounds

Halofantrine is a phenanthrene-methanol compound with activity against the erythrocytic stages of the malaria parasite. Its use has been especially recommended in areas with

multiple drug-resistant falciparum. Studies have indicated, however, that the drug can produce potentially fatal cardiac conduction abnormalities (specifically, prolongation of the PR and QT interval), limiting its usefulness. Atovaquone is a hydroxynaptho-quinone that is currently being used most widely for the treatment of opportunistic infections in immunosuppressed patients. It is effective against chloroquine-resistant *P. falciparum*, but because, when used alone, resistance develops rapidly, atovaquone is usually given in combination with proguanil. A new fixed dose antimalarial combination of 250 mg atovaquone and 100 mg proguanil (MalaroneTM) is being brought to market worldwide and is additionally being distributed through a donation programme. Two drugs originally synthesized in China are currently undergoing field trials. Pyronaridine was reportedly 100% effective in one trial in Cameroon; however, it was only between 63% and 88% effective in Thailand. Lumefantrinel, a fluoro-methanol compound, is being produced as a fixed combination tablet with artemether (Bloland, 2001).

2.15 Antimalarial Drug Resistance

Antimalarial drug resistance has been defined by the World Health Organisation as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject". This definition was later modified to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bloland, 2001). Most researchers interpret this as referring only to persistence of parasites after treatment doses of an antimalarial rather than prophylaxis failure, although the latter is a useful tool for early warning of the presence of drug resistance. This definition of resistance requires demonstration of malaria

parasitaemia in a patient who has received an observed treatment dose of an antimalarial drug and simultaneous demonstration of adequate blood drug and metabolite concentrations using established laboratory methods (such as high performance liquid chromatography) or *in vitro* tests. In practice, this is rarely done with *In vivo* studies. *In vivo* studies of drugs for which true resistance is well known (such as chloroquine) infrequently include confirmation of drug absorption and metabolism; demonstration of persistence of parasites in a patient receiving directly observed therapy is usually considered sufficient. Some drugs, such as mefloquine, are known to produce widely varying blood levels after appropriate dosing and apparent resistance can often be explained by inadequate blood levels (Bloland, 2001).

Parasite survival is achieved by developing mutations in enzymes related to drug targets to encode a protein that enables it to skip an anti- malarial drug; this then is followed by selection of parasites with genetic changes that confer reduced susceptibility (Sharma, 2005).

Resistance is the most likely explanation for the doubling of malaria-attributable child mortality in eastern and southern Africa (Korenromp *et al.*, 2003; Hedrick, 2012; Okoro and Jamiu, 2018). There is a continuous increase in resistance to anti-malarial drugs as a result of indiscriminate use, which in turn drives a strong selective force in favour of the resistant parasites. This facilitates global spread of drug resistance and hence worsening the status of management and control of the disease, escalating morbidity and mortality (WHO, 2006). Resistance to all known anti-malarial drugs, including the newly introduced artemisinin based combination therapy (Carrara, *et al.*, 2009), has developed to various degrees in several countries.

2.15.1 Epidemiology of antimalarial drug resistance

Malaria epidemiology varies in the tropical zones, as the prevalence of parasite and the disease varies significantly from one area to another. One limiting factor to differences in magnitude of malaria is the abundance of the mosquito vector. The first reports of *P.falciparum* chloroquine (CQ) resistance came from southeastern Asia and south America in the late 1950s, the earliest report of antimalarial resistance was that of *P. falciparum* to quinine reported from Brazil in 1910 (Spencer, 1985). The resistant parasite then has spread out to all known malaria-endemic areas except the countries located north of the Panama Canal and Haiti Island. Increasing CQ resistance has driven those countries in which resistance has developed to switch their first line treatment from CQ to SP, which is inexpensive, relatively safe, and has simple dosing. By the late 1980s, resistance to SP became prevalent on the Thai-Cambodian and Thai-Myanmar (Thai-Burmese) borders which later became a multi-drug resistance (MDR) area. Only in the 1980s severe resistance started to emerge in east Africa and spread across the continent. As a consequence most African countries have switched their first-line drug to SP but unfortunately the efficacy of this drug in Africa is progressively deteriorating (Bjorkman and Phillips-Howard, 1990). In west or central Africa, and in Madagascar, around 40% of *P. falciparum* isolates remained sensitive to CQ. It is important to note that SP efficacy life span was relatively short compared to CQ because *P. falciparum* had developed resistance to SP within only five years of introducing the drug while CQ had been in use for several decades before the emergence of CQ resistance in Asia. Difference in the rate of development of resistance towards SP and CQ could be attributed to their mode of action and the target molecules involved. CQ resistance could involve multiple genes whereas the

target molecules for SP are fairly well established (Wongsrichanalai *et al*., 2002; Sharma, 2005).

2.15.2 Origin and evolution of antimalarial drug resistance

Two processes are necessary for evolution of drug resistance. First, a resistant genotype is generated by mutation; second, the spread of this mutation within and between parasite populations takes place (Anderson *et al*., 2005). Recent molecular surveys have shown that resistance to commonly used anti-malarial drugs has rather few independent genotypes. This suggests that *de novo* mutations, that confer resistance to these drugs, do not occur frequently. However, limited number of drug resistant genotypes is spreading across different parasite populations and then increase in frequency to hinder management and control measures. The spread of limited drug resistant genotype(s) can lead to removal of genetic variation from the chromosomal regions surrounding the selected site, such as flanking neutral polymorphisms which are carried along with a mutation site resulting in what can be described as hitchhiking. The spread of the selected allele also results in increased linkage disequilibrium (LD) with flanking markers and skews the allele frequency spectra at loci nearby on the chromosome. The size of genomic regions affected is influenced by the strength of selection, as well as the rates of recombination and mutation. These characteristic patterns of variation are tools for identifying regions of the genome that are under selection (Anderson *et al*., 2005).

2.16 Mechanisms of Antimalarial Resistance

Mechanisms of drug resistance can be summarised as follows: altered transport of the drug, enzymatic inactivation of the drug, decreased conversion of the drug to the active

compound, increased amount of a metabolite antagonizing the drug action, altered amount of the drug target, decreased affinity of the drug target (Dahlström, 2009).

In *P. falciparum* the most well studied mechanisms of resistance include decreased affinity for the drug of the target (e.g. SP resistance through mutations in DHFR and DHPS) and altered drug transport (e.g. chloroquine resistance through mutations in the *P. falciparum* Chloroquine Resistance Transporter (PfCRT). Genetic alterations include gene amplification, Single Nucleotide Polymorphisms (SNPs) as well as insertions or deletions (Dahlström, 2009).

In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs, only a single point mutation is required to confer resistance, while for other drugs, multiple mutations appear to be required. Provided the mutations are not deleterious to the survival or reproduction of the parasite, drug pressure will remove susceptible parasites while resistant parasites survive. Single malaria isolates have been found to be made up of heterogeneous populations of parasites that can have widely varying drug response characteristics, from highly resistant to completely sensitive. Similarly, within a geographical area, malaria infections demonstrate a range of drug susceptibility. Over time, resistance becomes established in the population and can be very stable, persisting long after specific drug pressure is removed. The biochemical mechanism of resistance has been well described for chloroquine, the antifolate combination drugs, and atovaquone (Bloland, 2001).

2.16.1 Chloroquine resistance

As the malaria parasite digests haemoglobin, large amounts of a toxic by-product are formed. The parasite polymerizes this by-product in its food vacuole, producing non-toxic haemozoin (malaria pigment). It is believed that resistance of *P. falciparum* to chloroquine is related to an increased capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of haem polymerization (Bloland, 2001). This chloroquine efflux occurs at a rate of 40 to 50 times faster among resistant parasites than sensitive ones. Further evidence supporting this mechanism is provided by the fact that chloroquine resistance can be reversed by drugs which interfere with this efflux system (Bloland, 2001). The spread of resistance to chloroquine (CQ) led to its withdrawal from use in most countries in sub-Saharan Africa in the 1990s (Mwai *et al*., 2009). In the presence of the CQ selection pressure, parasites with the resistant alleles predominated the natural populations. However, with the discontinuations of the drug to counteract resistance to the drug, parasites with allele for susceptibility to CQ are expected to proliferate and replace those with alleles for resistance, since adaptations conferring resistance to the drug are expected to be costly to parasite in absence of CQ pressure (Mwai *et al*., 2009).

2.16.2 Antifolate combination drugs

Antifolate combination drugs, such as sulfadoxine and pyrimethamine, act through sequential and synergistic blockade of two key enzymes involved with folate synthesis. Pyrimethamine and related compounds inhibit the step mediated by dihydrofolate reductase (DHFR) while sulfones and sulfonamides inhibit the step mediated by dihydropteroate synthase (DHPS). Specific gene mutations encoding for resistance to both

DHPS and DHFR have been identified. Specific combinations of these mutations have been associated with varying degrees of resistance to antifolate combination drugs (Bloland, 2001).

2.16.3 Atovaquone in malaria treatment

Atovaquone acts through inhibition of electron transport at the cytochrome bc1 complex. Although resistance to atovaquone develops very rapidly when used alone, when combined with a second drug, such as proguanil (the combination used in MalaroneTM) or tetracycline, resistance develops more slowly. Resistance is conferred by single-point mutations in the cytochrome-b gene (Price *et al.*, 2004).

2.17 Artemisinin-based Combination Therapy (ACT)

The operational concept of ACTs is dependent on the extremely fast pharmacodynamics action of its artemisinin derivative partners (ARTs). With a characteristic parasite reduction ratio (PRR) of 1/10.000 in the first 48 hours of treatment, its action in the combination allows a fast reduction in the patient parasite load in the first hours of treatment. The remaining parasites are then eliminated by the long half-life partner drug - typically an antimalarial of the quinoline structural class - now left to handle a parasite population thousands-fold smaller than at clinical presentation. Seen in function of time, if the slow acting long standing drug, with a characteristic PRR of 1:100 (data available for mefloquine), would act alone in monotherapy, by the time that would reach the same low levels of parasitaemia (if reaching them at all) its own concentration would be significantly lower, as compared with the situation aided by ART.

It is likely that the remaining parasites would then be exposed to sub-therapeutic levels of the drug. This not only would be nonefficacious to eliminate this parasite population, but would also set the conditions for promoting the development of resistance (Price *et al*., 2004).

The ACT partners are hoped to be associated to different mechanisms of action, promoting possible situations of synergy and an expected reduction in probability of resistance emergence. As of now, two major ACTs represent the backbone of most African national malaria control programs: artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ). To this adds the pioneering use of artesunate-mefloquine in Thailand, which represents the first employed ACT in a national malaria program. Two other formulations, representing second generation ACTs, are also emerging: dihydroartemisininpiperaquine (Zani *et al*., 2014) and artesunate-pyronaridine (Bukirwa *et al*., 2014). The first generation ACTs have been notoriously successful, being associated with very significant decreases in malaria incidence worldwide (WHO, 2010b). Such success has prompted the reemergence of the national malaria elimination plans in the last years, which include large mass ACT administration plans (Song *et al.,* 2010). Unfortunately this drug strategy has not proved to be totally resistance proof, as signs of *in vivo* decreased sensitivity to its components - including the artemisinin derivatives - are emerging, as first noticed for artesunate-mefloquine, in Thailand (Price *et al*., 2004; Carrara *et al.*, 2009).

In this region, ACT came as a response to the fast declining efficacy of mefloquine monotherapy, its introduction leading to a remarkable decrease in clinical failure. But the parasite kept developing eventually becoming less sensitive even to the combination. Although the regimen has been modified and upgraded throughout the years since its

introduction (Carrara *et al.*, 2009), resistance to the artesunate-mefloquine combination has been recognized since the late 1990s. Partly this evolution can be considered as a consequence of the fact that this ACT was designed in order to increase the useful life span of a failing drug (mefloquine), a strategy no longer supported by the WHO. *In vivo* and *in vitro* resistance to mefloquine has been strongly associated with the presence of increased *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*) copy number in the multidrug resistance gene (Price *et al*., 2004), a frequent occurrence in South East Asia and in some regions of South America, but a relatively rare event in the African continent (Ursing *et al*., 2006)

Soon after its first implementations in Africa, Artemether Lumefantrine (AL) was shown to drive the post-treatment selection of the *pfmdr1* amino acid 86N-coding allele in clinical efficacy trials performed in Zanzibar (Sisowath *et al.*, 2007). Soon this observation was confirmed in most settings where AL has been trialed. Besides mefloquine and lumefantrine, *in vivo* resistance to amodiaquine has been observed (Holmgren *et al*., 2006), even in the context of ACT (Holmgren *et al.*, 2007). Finally, concerning piperaquine, part of a second generation ACT planned to be implemented in Africa, it has been long known that the parasite is able to develop resistance against these drug, at least when it was employed as a monotherapy in Southern China 25 years ago (Davis *et al*., 2005).

2.18 Genes and Mutations Associated with Antimalarial Drug Resistance

Many *Plasmodium* genes have been associated with the development of resistance to previous and currently used antmalarial drugs.

2.18.1 Genes associated with sulphadoxine-pyrimethamine resistance

The second line of treatment for uncomplicated chloroquine resistant *P. falciparum* malaria cases is the sulfadoxine-pyrimethamine (SP) combination of drugs. In many parts of the world where chloroquine resistance is very high, the SP combination is prescribed as first-line of drug because of its low cost, simple dosing and relative safety (Driessen *et al*., 2002). High therapeutic efficacy for SP is being reported from several countries (Nwanyanwu *et al*., 2000; Basco *et al*., 2002). However, some countries in Africa, Asia and South-America have already started reporting resistance to this drug combination. Use of SP is also limited for pregnant women during the early trimester and its efficacy against *P. vivax* is not very high (Korsinczky *et al*., 2004).

Malaria parasite seems to develop resistance to SP faster than to chloroquine. For example, with its use as a first line therapy in Thailand, the parasite developed resistance within five years. On the contrary, chloroquine had been in use for several decades in the field. This difference in the resistance development towards SP and chloroquine could be related to their mode of action and the target molecules involved therein. As stated earlier, the chloroquine resistance could involve multiple genes whereas the target molecules for sulphadoxine and pyrimethamine are fairly well established. Sulphadoxine and pyrimethamine inhibit the enzymes dihydropteroate synthase and dihydrofolate reductase, respectively, although additional target molecules cannot be ruled out (Nirmalan *et al*., 2004a). Indeed, sulpha-drugs are the oldest antimicrobial agents used widely even today to treat various bacterial, fungal and parasitic infections. Certain point mutations in these enzymes reduce their binding capacity to the drug thus allowing the resistance to develop.

These mutations and their impact on the epidemiology of malaria are described below (Gatton *et al*., 2004).

2.18.1.1 Plasmodium falciparum Dihydropteroate Synthase (pfdhps)

By mimicking p-aminobenzoic acid, sulphadoxine acts as a competitive inhibitor in folate biosynthetic pathway of the parasite. This drug acts by inhibiting the enzyme dihydropteroate synthase (DHPS) thus interfering in the step of conversion of dihydropteridine pyrophosphate to dihydropteroate (Sharma, 2005). However, the parasite has developed resistance towards sulphadoxine and this resistance arises due to alterations in the parasite enzyme DHPS17 (Sharma, 2005). Several key point mutations have been identified in this parasite enzyme which can reduce its binding affinity to the drug. Most of these alterations are at codons 436 (Serine to Alanine/Phenylalanine), 437 (Alanine to Glycine), 540 (Lysine to Glutamate), 581 (Alanine to Glycine) and 613 (Alanine to Serine/ Threonine). Molecular methods have been developed to monitor these mutations in the field isolates so as to evaluate the efficacy of sulpha drugs to treat malaria. Similar to dihydrofolate reductase (DHFR), the increased level of sulphadoxine drug resistance has been shown to be associated with the higher number of mutations in dihydropteroate synthase (DHPS). Also, similar to S108N mutation in DHFR, the A437G is the key point mutation in DHPS which allows the parasite to reduce its susceptibility towards sulphadoxine. Other mutations are mostly found to be associated with this mutation (Ahmed *et al*., 2004).

2.18.1.2 Plasmodium falciparum Dihydrofolate Reductase (pfdhfr)

Early studies had demonstrated that pyrimethamine resistance in *P. falciparum* was caused by decreased affinity of the drug to a structurally modified dihydrofolate reductase (DHFR). Subsequent sequencing of *pfdhfr* in parasite isolates with different pyrimethamine susceptibilities identified the following alterations as associated with resistance: S108N, N51I, C59R, and I164L (Dahlström, 2009). Further experiments confirmed that mutations in these positions modified the structure of DHFR so that pyrimethamine binding was impaired (Sirawaraporn *et al.*, 1997).

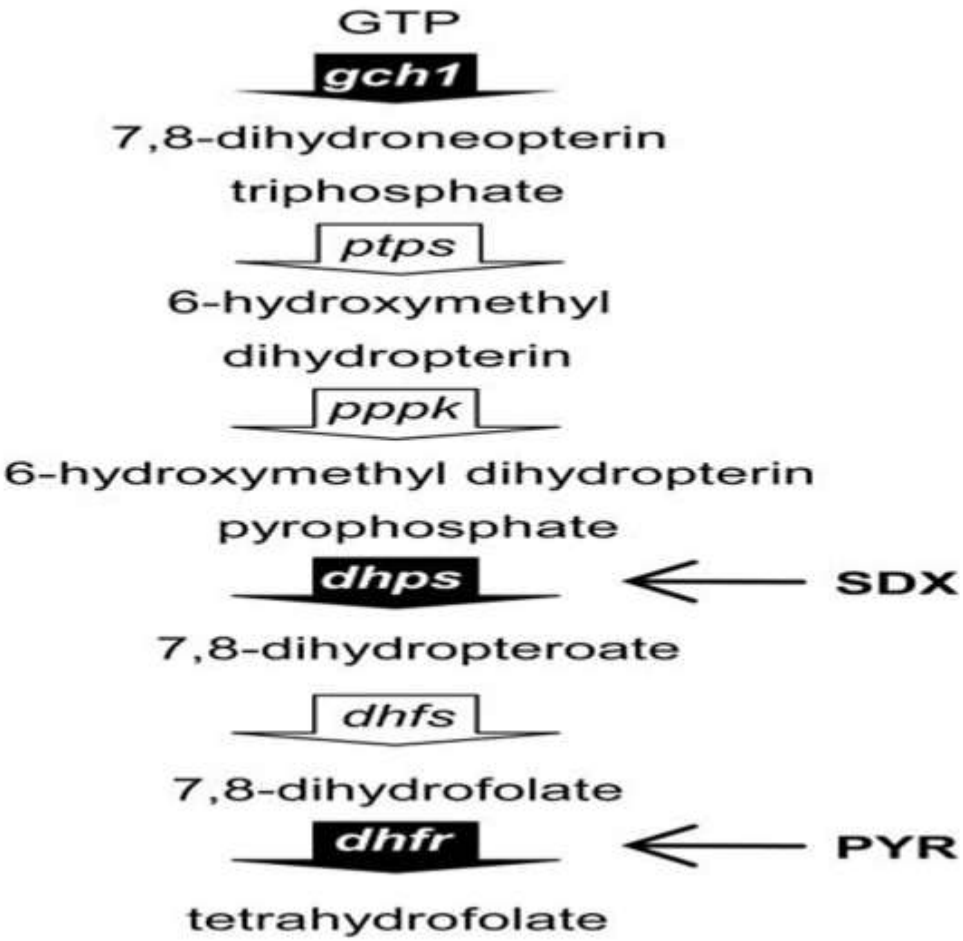
Pyrimethamine inhibits the dihydrofolate reductase (DHFR) enzyme of *P. falciparum* and thus its folate biosynthesis pathway. However, the parasites can upregulate the translation, not the transcription, of DHFR under the influence of pyrimethamine to counter its effect (Nirmalan *et al.*, 2004a). The crystal structure of *P. falciparum* DHFR is known and pyrimethamine binding sites elucidated (Sirawaraporn *et al*., 2002). Mutations in some of the key amino acids of this enzyme however lead to its reduced binding affinity towards the drug (Le Bras and Durand, 2003).

Indeed, almost all the parasite isolates showing pyrimethamine resistance were found to contain this mutation. Any other mutation (at codons 51 (N51I), 59 (C59R) and 164 (I164L)) was associated with S108N mutation. The sequence analysis of the isolates of known drug susceptibility profile revealed that a single DHFR mutation or double DHFR mutations alone will not cause SP treatment failure but triple DHFR mutations or quadruple DHFR mutations will certainly provide a higher level of drug resistance. Among these mutations, the I164L was found to play a critical role as its association with other DHFR mutations always resulted in the higher level of drug resistance. Countries where

high level of SP resistance is reported were also found to contain quadruple mutations in DHFR unlike in India where a maximum of triple DHFR mutations was reported (Ahmed *et al*., 2004). Another drug cycloguanil also inhibits this enzyme and resistant *P. falciparum* parasites have shown mutation in codon 16 (A16V) which is again associated with codon 108 but with different mutation (S108T). These mutations are common in parasite isolates from those countries where this drug is being commonly used, but not in India (Ahmed *et al*., 2004). The quantitative proteomics data on *P. falciparum* under the influence of pyrimethamine had shown decreased synthesis for certain proteins viz., heat shock protein 72 (HSP72), enolase, actin-1, phosphoethanolamine N-methyltransferase (PMT) (Nirmalan *et al.*, 2004b).

2.18.1.3 Synergistic effect of Sufadoxine-pyrimethamine on Folate Synthesis in Plasmodium sp

The synergistic effect of suphadoxine-pyrimethamine on folate synthesis in *Plasmodium* sp is shown in the folate biosynthesis pathway below (figure 2.1). The steps catalysed by guanosine triphosphate-cyclohydrolase 1, dihydrofolate reductase and dihydropteroate synthase are highlighted, and the positons at which antifolate drugs (pyrimethamine (PYR) and sulfadoxine (SDX) target the pathway are marked.



Nair *et al.* (2008)

Figure 2.1. The Folate Biosynthesis Pathway of *Plasmodium* sp

Key: Guanosine triphosphate (GTP), GTP-cyclohydrolase 1 (gch 1), pyruvoyltetrahydropterin synthase (ptps), hydroxymethyldihydropterin pyrophosphokinase (pppk), dihydropteroate synthase (dhps), dihydrofolate synthase (dhfs), dihydrofolate reductase (dhfr), sulfadoxine (SDX), pyrimethamine (PYR).

2.18.1.4 Combined Sulphadoxine-pyrimethamine induced mutations in DHFR and DHPS enzymes

Since SP is given as a combined dose to malaria patients, its resistance is measured by detecting mutations in both DHFR and DHPS enzymes. The higher the number of combined DHFR-DHPS mutations, the higher was the SP resistance shown by the parasite. Kublin *et al*. (2002) have found that quintuple DHFR-DHPS mutations (a triple DHFR mutation and a double DHPS mutations) caused SP treatment failure. Only C59R mutation in DHFR and K540E mutation in DHPS can be used as an indicator of the quintuple mutations and predictor of SP resistance (Kublin *et al*., 2002). However, these findings need to be confirmed with larger sample size covering different geographical areas of variable malaria endemicity. This is because Indian isolates were found to contain quintuple mutations but no SP resistance.

Based on sequence analysis (Sharma, 2005) had earlier suggested that a single DHFR mutation or double DHFR mutations alone will not cause SP treatment failure but that double DHFR mutations plus a single DHPS mutation or triple DHFR mutations alone can cause higher level of SP resistance. The number of combined two-locus mutations is high among isolates of the countries where SP resistance is high because of its first line use for treatment. In India, this combined two-locus mutation rate is still lower, except north-eastern States (Ahmed *et al*., 2004). However, we have noticed that there is a temporal increase in these mutations which should serve us a warning signal for prescribing the SP treatment.

In vivo combinations of mutations in *pfdhfr* and *pfdhps* had been shown to be correlated with SP treatment failure (Wongsrichanalai *et al*., 2002). In particular a quintuple mutant

(*pfdhfr*: S108N, N51I, C59R + *pfdhps*: A437G, K540E) has been strongly associated with SP treatment failure in the African continent (Kublin *et al.*, 2002; Happi *et al.*, 2005).

2.18.2 *Plasmodium falciparum* multidrug resistance 1 (*pfmdr1*)

Plasmodium falciparum multidrug resistance represents an intronless gene located at chromosome 5. It codes for the P-glycoprotein homologues (Pgh), a protein of 1419 amino acids, dependent on the extension of a central polymorphic asparagine based repeat segment. Pgh is essentially located in the food vacuole membrane, with a small fraction present in the plasma membrane. It is probably oriented towards the lúmen of the vacuole (Rohrbach *et al*., 2006).

Recent observations suggest that pfmdr1 is an importer of antimalarial drugs and other substrates into the food vacuole (Rohrbach *et al.*, 2006) and that SNPs in *pfmdr1* can alter the transport by affecting substrate specificity (Sanchez *et al.*, 2008).

Gene amplification of *pfmdr1* has been seen to be associated with increased *in vitro* susceptibility to mefloquine and artesunate in field isolates. In accordance, parasites with decreased *pfmdr1* copy number have been shown to have decreased *in vitro* susceptibility to mefloquine, lumefantrine and artemisinin (Rohrbach *et al.*, 2006). *In vivo*, *pfmdr1* amplification has been associated with treatment failures after mefloquine and artesunate-mefloquine treatment in Asia (Lim *et al.*, 2009).

Single Nucleotide Polymorphisms in *pfmdr1* may also alter drug response. In transfection based experiments, leading to specific allele changes, and by analyzing field isolates, *pfmdr1* SNPs N86Y, Y184F, S1034C, N1042D and D1246Y have been shown to be associated with alterations of *in vitro* susceptibility to mefloquine, lumefantrine,

artemisinin, artesunate and dihydroartemisinin (Anderson *et al.*, 2005). *In vivo pfmdr1* N86, 184F and D1246 alleles have been selected in recurrent infections after treatment with artemether-lumefantrine (Dokomajilar *et al.*, 2006, Sisowath *et al.*, 2009) while the opposite alleles *pfmdr1* 86Y, Y184 and 1246Y have been associated with recurrent infections after amodiaquine or artesunate-amodiaquine treatment (Holmgren *et al.*, 2006; Holmgren *et al.*, 2007). Among these the mutation from asparagine to tyrosine at codon 86 has been used widely. Polymerase chain reaction (PCR) based molecular methods have been used to detect this mutation in the *in vitro* and *in vivo* tested parasites for chloroquine sensitivity as well as in the field isolates of different countries with variable range of chloroquine resistance.

Therefore, polymorphisms in *pfmdr1*, including increased copy number and sequence variation (especially N86Y, 1034, 1042 and 19 D1246Y) have been reported to modulate the parasite susceptibility to Mefloquine (Lopes *et al*., 2002; Dondorp *et al*., 2010), Halofantrine (Reed *et al*., 2000; Sidhu *et al.*, 2006) Lumefantrine (Sisowath *et al.*, 2007; Sisowath *et al.*, 2009; Sanchez *et al*., 2011), Quinine (Reed *et al*., 2000), Dihydroartemisinin (Price *et al.*, 2004), Artemisinin (Reed *et al*., 2000), Chloroquine (Barnes *et al*., 1992), Amodiaquine (Holmgren *et al*., 2006; Holmgren *et al*., 2007) and Piperaquine. *In vitro* approches have supported the view that Pgh functions as a drug transporter (Sanchez *et al*., 2008; Sanchez *et al*., 2011).

2.18.3 *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*)

This 13 exon gene located in chromosome 7, codes for a 424 amino acid, transmembrane domain protein localized on the parasite food vacuole membrane (Fidock *et al*., 2000b). It has been described to play a key role in *P. falciparum* resistance to chloroquine. The

encoded protein PfCRT is proposed to be a member of drug metabolites transporter superfamily (Martin *et al*., 2009). The presence of single nucleotide mutations (SNPs) in *pfcrt* can confer to its coded protein the capacity to transport the chloroquine out of the digestive vacuole (Cabrera *et al.,* 2009; Martin *et al.,* 2009).

PfCRT is located in the membrane of the food vacuole where chloroquine has been suggested to act by binding to hematin, a toxic byproduct from the digestion of haemoglobin, thereby preventing synthesis of non toxic hemozoin (Bray *et al.*, 1998). The K76T mutation in *pfcrt* has been demonstrated to be the main determinant for chloroquine resistance (Djimde *et al.*, 2001; Sidhu *et al.,* 2002, Lakshmanan *et al.*, 2005). It has been suggested that mutant *pfcrt* can transport chloroquine out of the food vacuole (Valderramos and Fidock, 2006; Sanchez *et al.*, 2007).

PfCRT has also been shown to influence the effect of both ART and the partner drugs. Transfection of mutant *pfcrt* resulted in increased susceptibility to artemisinin, dihydroartenisinin and mefloquine, as well as some decrease in amodiaquine susceptibility (Sidhu *et al.*, 2002). *In vivo* studies have shown that artemether-lumefantrine treatment selects for K76 (Sisowath *et al.*, 2009) whereas amodiaquine and artesunate-amodiaquine selects for 76T (Happi *et al.*, 2005, Dokomajilar *et al.*, 2006; Holmgren *et al.*, 2006).The selection pressure in these studies are thought to be derived from the partner drug.

2.18.3.1 K76T mutation in pfcrt

Several point mutations in the coding region of this gene were reported to be associated with chloroquine resistance (Fidock *et al*., 2000a; Sidhu *et al.,* 2002). However, mutation at codon 76 (Lys to Thr) has been found in almost all the chloroquine resistant parasite

lines and clinical isolates (Fidock *et al*., 2000a; Bosco and Ringwald, 2001). Therefore, it has been proposed as a molecular marker to monitor the chloroquine resistance in field isolates. While it is true that K76T mutation is associated with chloroquine resistance, this mutation is not absolute. Because large number of chloroquine responders are also found to harbour this mutation and it is highly prevalent in Indian isolates (Vathsala *et al*., 2004). This raises several issues like the involvement of host response such as the status of immune system which can clear the parasite irrespective of its being chloroquine resistant or not (Djimde *et al*., 2003). Similarly, the drug absorption and metabolic rate of individuals will also affect the outcome of chloroquine treatment. There is yet another possibility that other mutations in *pfcrt* are also involved to give rise to this resistance. Else, more than one gene is involved in making the parasite to become chloroquine resistant.

Other mutations in *pfcrt:* Besides K76T mutation in *pfcrt*, mutations at codon 72, 74, 75, 97, 220, 271, 326, 356 and 371 have also been found to be associated with chloroquine resistance (Fidock *et al*., 2000a). There are reports that mutation in codon 220 (Ala to Ser) in *pfcrt* is associated with chloroquine resistance in African countries but not in the Philippines (Chen *et al*., 2003). Also, the Philippines isolates were found to have two novel mutations (A144T and L160Y) in chloroquine resistant parasites not found elsewhere. Therefore, more elaborate studies need to be carried out in the South-East Asian countries on this and other *pfcrt* codons. It seems that host genetic factors and several other epidemiological factors may be involved in influencing mutations in the *pfcrt* gene. Otherwise how would one explain the discrepancy of a particular mutation being associated with chloroquine resistance in African countries and not in South-East Asia and

vice versa? These mutations need to be monitored over the period of time to record temporal changes. This is to ensure that the efficacy of the drug is being maintained, otherwise make a policy change for the usage of other alternative drugs.

Pfmdr1 and *pfcrt* combined mutations: Although involvement of *pfmdr1* in chloroquine resistance is not very clear, several workers have preferred to monitor the mutations in *pfmdr1* and *pfcrt* genes together (Djimde *et al*., 2001). This has yielded variable results in the field studies. Some workers found these two markers to yield better results than a single marker while others did not find any role for *pfmdr1* mutations. It is yet to be seen if the increased rate of mutations in the *pfmdr1* is due to drug pressure in the field.

Pfcrt SNPs have also been found to be associated with the *in vitro* and/or *in vivo* parasite response to lumefantrine, mefloquine, quinine, artemisinin (Sidhu *et al*., 2002; Cooper *et al*., 2005), and possibly piperaquine (Eastman *et al*., 2009).

2.18.4 *Plasmodium falciparum* multidrug resistance associated protein *(pfmrp1)*

The *pfmrp1* gene codes for a large 12 transmembrane domain ABC-transporter, PfMRP1, located in the parasite plasma membrane (Klokouzas *et al*., 2003). It has been proposed to act as a GSH/GSSG pump (Bozdech *et al*., 2004) involved in the REDOX stress management of the parasite. It is also expected to be able to transport a large range of drugs. These functional assumptions are supported by studies based on the targeted disruption of *pfmrp1* in the W2 clone. The resulting genetically modified parasites showed not only an accumulation of oxidized glutathione (GSSG), but also an increase in the sensitivity to a number of drugs, including chloroquine, quinine, piperaquine and – most importantly - artemisinin. This effect was shown for some of the tested antimalarials

(chloroquine and quinine) as the result of a decreased accumulation in the parasite, indicating an efflux activity of pfMRP1.

Pfmrp1 SNPs have been linked with the *In vivo* parasite response to ACT. This was concluded from the observation of significant selection patterns of the I876V amino acid position upon artemether-lumefantrine treatment and K1466R with sulfadoxine-Pyrimethamine (Dahlstrom *et al*., 2009). *In vitro* based reports, have also provided evidence for the potential importance of *pfmrp1* SNPs in modulating *P. falciparum* drug sensitivity, namely the I876V and H191Y with chloroquine, as well as F1390I with quinine (Phompradit *et al*., 2014).

2.18.5 *Plasmodium falciparum Kelch13* propeller gene

This gene has been described in *P. faciparum* in homology to the human Kelch-like ECH-associated Protein 1 (KEAP1) gene. The 726 amino acids protein contains an N terminal containing a *Plasmodium* specific sequence, followed by a BTB/POZ domain, and finally by the kelch propeller domain towards the C terminal. The Kelch13 (K13) propeller has been so far studied in *Plasmodium falciparum* in *in vitro* adapted parasites that underwent several years of exposure to increasing doses of artemisinin. Throughout the process, the exposed parasites gradually accumulated a number of SNPs into the C terminal Kelch propeller domain. Some of these SNPs have showed to be correlated with the rate of survival rings after the RSA (Ring Stage Survival Assay) (Ariey *et al*., 2014). This association was confirmed in a number of field isolates from Cambodia. Finally, these mutations were also observed to be associated with Day 3 positivity upon ACT treatment. As a result, a set of K13 propeller domain mutations has been proposed in Cambodian

Plasmodium isolates to be associated to the *in vitro* and *in vivo* resistance to ART. Four main alleles were observed as significantly involved: C580Y, R539T, I543T, and Y493H.

Importantly, the gene seems to be able to accommodate significant polymorphism, with 17 non-synonymous SNPs, having been found in Cambodia ((Ariey *et al*., 2014), showing that probably there is considerable room for structural changes in this protein. As in many Kelch proteins, mutations in the kelch domain are predicted to alter the protein structure or modify the charge altering in the same way the protein biological function. Such changes could eventually allow the emergence of a protein better suited to deal with the specific stresses associated with ART exposure (Kone, 2014).

It became as such important to better understand the origin and distribution of this biodiversity in several different settings. ACT treatment efficacy studies were assessed in different settings in Africa, India and South-East Asia. The K13 propeller mutations appeared to be significantly associated to a mean increase in parasites half-life in South-East Asia, but not in Africa and India. In both India and Africa, the K13 propeller SNPs, when present, were different from the one previously described as artemisinin resistance potential markers (Ashley *et al*., 2014). Further investigations aimed to find a common genetic origin to this polymorphism (mutants K13 propeller) has showed different strains background for the South African parasites and the Asian ones which also has emerged and spread independently throughout South-East Asia.

The function of the encoded protein is still under speculation. Its human homolog KEAP1 has been described in lung cancer cells as interacting with the Nrf2 by sequestration of this protein in the cytosol. Under oxidative stress, Nfr2 is liberated from the complex Nfr2/KEAP1 and induces a cytoprotective response. These models have been extrapolated

to the *P. falciparum* K13 propeller for whom antioxidant response is high in late trophozoite stages, where the haemoglobin digestion is considerable. The propeller could serve the KEAP1 functions in the parasite, albeit no *P. falciparum* Nrf2 homologue has been identified yet (Kone, 2014).

2.19 Sarco/Endoplasmic Reticulum CA^{2+}-ATPase (SERCA)

2.19.1 *Plasmodium falciparum* SERCA

Calcium has been shown to regulate several processes in apicomplexan parasites including host cell invasion and motility (Nagamune *et al.*, 2008). In *Plasmodium* it has been suggested that similar mechanisms may be involved in host cell invasion (Billker *et al.*, 2004). Calcium may also be important for *Plasmodium* gametocyte differentiation (Billker *et al.*, 2004) and for synchronization of the parasite life cycle in response to the host melatonin production (Garcia *et al.*, 2008). The calcium concentration in the cytosol of *Plasmodium* parasites is likely to be low and the uninfected RBCs also have low calcium concentration (Nagamune *et al.*, 2008). To explain how the parasite can overcome the absence of the essential extracellular calcium it has been suggested that the intraerythrocytic calcium concentration increases during malaria infections since the RBCs become more permeable and reduce export (Garcia, 1999) and/or that there is a relatively high calcium concentration in the parasitophorous vacuole, in which *Plasmodium* reside (Gazarini *et al.*, 2003). Calcium storage in a compartment similar to the mammalian ER has been demonstrated in *Plasmodium* (Garcia *et al.*, 2008). Only one *P. falciparum* SERCA orthologue, PfATPase6, has been identified. The overall homology between mammalian SERCA and PfATPase6 is relatively low. However, all key residues previously shown to be related with calcium transport, e.g. calcium binding and ATP

binding, in rabbit SERCA, were conserved in PfATPase6. Further, it was demonstrated that the SERCA inhibitor thapsigargin could induce calcium release into the cytosol from intracellular stores, probably ER, by inhibition of the PfATPase6, suggesting that PfATPase6 is essential for *P. falciparum* calcium homeostasis and that PfATPase6 is functionally related with higher mammal homologues (Varotti *et al*., 2003).

2.19.2 *Plasmodium falciparum* Adenosine Triphosphatase 6 (*pfatpase6*)

Several studies have associated mutations in the *PfATPase6* gene to artemisinin resistance, associated mutations in this gene act as biomarkers to measure artemisinin efficacy (Price *et al.*, 2004; Cojean *et al.*, 2006; Nagasundaram *et al*., 2016).

The SERCA inhibitor thapsigargin is a sesquiterpene lactone, as are ART. From these structural similarities the hypothesis emerged that ART act by inhibiting PfATPase6. This was supported by the demonstration that artemisinin specifically inhibited PfATPase6 expressed in *Xenopus laevis*, as thapsigargin. The two drugs showed an antagonistic interaction in *P. falciparum* cultures and similar localization in the parasite. Hence PfATPase6 was suggested to be a target of ART (Eckstein-Ludwig *et al.,* 2003). In human SERCA thapsigargin binding is determined by residues in the transmembrane domains M3, M5 and M7. Mutations within these domains can reduce thapsigargin affinity and/or inhibitory effect (Xu *et al.,* 2004). Results from homology modeling of PfATPase6 and docking simulation artemisinin to PfATPase6 suggest that residues in M3, M5 and M7 are important also for artemisinin binding (Jung *et al.,* 2005). Investigations of the differences in the thapsigargin-binding cleft of mammalian and Plasmodia SERCA revealed that mutations introduced in residue 263 in PfATPase6 reduced artemisinin inhibition, suggesting that this amino acid is involved in artemisinin binding to PfATPase6

(Uhlemann *et al.*, 2005). However natural variation in this amino acid has not been found (Cojean *et al.*, 2006; Price *et al.*, 2004), which might indicate a much conserved and hence functionally important region of the protein. Natural variation in PfATPase6 and association with artemisinin susceptibility has recently started to be investigated. In French Guiana an S769N SNP was associated with decreased *in vitro* susceptibility to artemether in fresh isolates (Jambou *et al.*, 2005). This challenging initial report could not be confirmed since the S769N SNP has not been found in subsequent studies (Price *et al.*, 2004; Sisowath *et al.*, 2009), with the exception of one sample that was fully sensitive to DHA *in vitro* (Cojean *et al.*, 2006). Variant *Pfatpase6* including S769N have been linked to increased 50% inhibitory concentrations (IC_{50}) of artemether against *Plasmodium falciparum* growth in culture. Maslachah *et al.* (2017) reported in their study that all the resistant *Plasmodium falciparum* isolates contained *pfatpase6* S769N mutation. PfATPase6 is the only SERCA-type Ca^{2+}-ATPase enzyme present in the malaria parasite and it is considered to be the suitable molecular target for artemisinin (Nagasundaram *et al.*, 2016).

The combination of two additional SNPs, E431K and A623E, was identified in a fresh isolate from Senegal with increased Inhibitory Concentration 50 (IC50) to artemether (Jambou *et al.*, 2005).Through partial or full sequencing of PfATPase6, only three additional SNPs have been identified in field samples: I89T in Thailand (Price *et al.*, 2004), H243Y in Africa (Cojean *et al.*, 2006) and a synonymous SNP in nucleotide position T2694A in São Tomé and Príncipe. In only two studies PfATPase6 has been comprehensively sequenced in a significant number of clinical samples, i.e. by Jambou and colleagues (Jambou *et al.*, 2005) that fully sequenced the gene in 60 samples, but did not

report the location of further variations, and Cojean and colleagues (Cojean *et al.*, 2006) that partially sequenced the gene in 154 samples. Further studies were needed to describe the biodiversity of PfATPase6 (Dahlström, 2009).

2.20 Factors Contributing to the Spread of Resistance

Numerous factors contributing to the advent, spread, and intensification of drug resistance exist, although their relative contribution to resistance is unknown. Factors that have been associated with antimalarial drug resistance include such disparate issues as human behaviour, vector and parasite biology, pharmacokinetics, and economics. As mentioned previously, conditions leading to malaria treatment failure may also contribute to the development of resistance (Bloland, 2001).

2.20.1 Biological influences on resistance

Based on data on the response of sensitive parasites to antimalarial drugs *in vitro* and the pharmacokinetic profiles of common antimalarial drugs, there is thought to always be a residuum of parasites that are able to survive treatment (Wernsdorfer, 1991). Under normal circumstances, these parasites are removed by the immune system (non-specifically in the case of non-immune individuals). Factors that decrease the effectiveness of the immune system in clearing parasite residuum after treatment also appear to increase survivorship of parasites and facilitate development and intensification of resistance. This mechanism has been suggested as a significant contributor to resistance in South-East Asia, where parasites are repeatedly cycled through populations of non-immune individuals (Verdrager , 1995); the non-specific immune response of non-immune individuals is less

effective at clearing parasite residuum than the specific immune response of semi-immune individuals.

The same mechanism may also explain poorer treatment response among young children and pregnant women. The contribution to development and intensification of resistance of other prevalent immuno-suppressive states has not been evaluated. Among refugee children in the former Zaire, those who were malnourished (low weight for height) had significantly poorer parasitological response to both chloroquine and SP treatment (Wolday *et al.*, 1995). Similarly, evidence from prevention of malaria during pregnancy suggests that parasitological response to treatment among individuals infected with the human immunodeficiency virus (HIV) may also be poor.

There is some evidence that certain combinations of drug-resistant parasites and vector species enhance transmission of drug resistance, while other combinations inhibit transmission of resistant parasites. In South-East Asia, two important vectors, *Anopheles stephensi* and *A. dirus*, appear to be more susceptible to drug-resistant malaria than to drug- sensitive malaria ((Bloland, 2001). In Sri Lanka, researchers found that patients with chloroquine-resistant malaria infections were more likely to have gametocytaemia than those with sensitive infections and that the gametocytes from resistant infections were more infective to mosquitos. The reverse is also true; some malaria vectors may be some-what refractory to drug-resistant malaria, which may partially explain the pockets of chloroquine sensitivity that remain in the world in spite of very similar human populations and drug pressure (e.g. Haiti). Many antimalarial drugs in current usage are closely related chemically and development of resistance to one can facilitate development of resistance to others. Chloroquine and amodiaquine are both 4-aminoquinolines and cross-resistance

between these two drugs is well known. Development of resistance to mefloquine may also lead to resistance to halofantrine and quinine. Antifolate combination drugs have similar action and widespread use of sulfadoxine/ pyrimethamine for the treatment of malaria may lead to increased parasitological resistance to other antifolate combination drugs. Development of high levels of SP resistance through continued accumulation of DHFR mutations may compromise the useful life span of newer antifolate combination drugs such as chlorproguanil/dapsone (LapDap) even before they are brought into use. This increased risk of resistance due to SP use may even affect non- malarial pathogens; use of SP for treatment of malaria increased resistance to trimethoprim/sufa- methoxazole among respiratory pathogens. There is an interesting theory that development of resistance to a number of antimalarial drugs among some falciparum parasites produces a level of genetic plasticity that allows the parasite to rapidly adapt to a new drug, even when the new drug is not chemically related to drugs previously experienced (Rathod *et al.*, 1997). This capacity may help explain the rapidity with which South-East Asian strains of falciparum develop resistance to new antimalarial drugs.

The choice of using a long half-life drug; Sufadoxine-Pyrimethamine, Mefloquine, in preference to one with a short half-life; Chloroquine, Chlorproguanil-dapsone (LapDap), Quinine has the benefit of simpler, single- dose regimens which can greatly improve compliance or make directly observed therapy feasible. Unfortunately, that same property may increase the likelihood of resistance developing due to prolonged elimination periods.

The relative contribution of low compliance versus use of long half-life drugs to development of resistance is not known. Parasites from new infections or recrudescent parasites from infections that did not fully clear will be exposed to drug blood levels that

are high enough to exert selective pressure but are insufficient to provide prophylactic or suppressive protection.

When blood levels drop below the minimum inhibitory concentration (the level of drug that fully inhibits parasite growth), but remain above the EC5 (the concentration of drug that produces 5% inhibition of parasite growth), selection of resistant parasites occurs. In areas of high malaria transmission, the probability of exposure of parasites to drug during this period of selective pressure is high. In Africa, for instance, people can be exposed to as many as 300 infective bites per year (in rare cases, even as much as 1000 infective bites per year), and during peak transmission, as many as five infective bites per night (Bloland, 2001).

2.20.2 Programmatic influences on resistance

Programmatic influences on development of anti-malarial drug resistance include overall drug pressure, inadequate drug intake (poor compliance or inappropriate dosing regimens), pharmacokinetic and pharmacodynamic properties of the drug or drug combination, and drug interactions. Additionally, reliance on presumptive treatment can facilitate the development of antimalarial drug resistance. Overall drug pressure, especially that exerted by programmes utilizing mass drug administration, probably has the greatest impact on development of resistance (Wernsdorfer, 1994).

Studies have suggested that resistance rates are higher in urban and periurban areas than rural communities, where access to and use of drug is greater. Confusion over proper dosing regimen has been described. The use of presumptive treatment for malaria has the potential for facilitating resistance by greatly increasing the number of people who are

treated unnecessarily but will still be exerting selective pressure on the circulating parasite population. In some areas and at some times of the year, the number of patients being treated unnecessarily for malaria can be very large (Wernsdorfer, 1994).

2.21 Detection of Resistance

In general, four basic methods have been routinely used to study or measure antimalarial drug resistance: *in vivo, in vitro,* animal model studies, and molecular characterization. Additionally, less rigorous methods have been used, such as case reports, case series, or passive surveillance. Much discussion has occurred regarding the relative merits of one test over another, with the implication always being that one type of test should be used preferentially (Bloland, 2001).

2.21.1 Use of *in vivo* test for detection of drug resistance

An *in vivo* test consists of the treatment of a group of symptomatic and parasitaemic individuals with known doses of drug and the subsequent monitoring of the parasitological and/or clinical response over time. One of the key characteristics of *in vivo* tests is the interplay between host and parasite. Diminished therapeutic efficacy of a drug can be masked by immune clearance of parasites among patients with a high degree of acquired immunity (White, 1997).

Of the available tests, *in vivo* tests most closely reflect actual clinical or epidemiological situations (i.e. the therapeutic response of currently circulating parasites infecting the actual population in which the drug will be used). Because of the influence of external factors (host immunity, variations of drug absorption and metabolism, and potential misclassification of reinfections as recrudescences), the results of *in vivo* tests do not

necessarily reflect the true level of pure antimalarial drug resistance. However, this test offers the best information on the efficacy of antimalarial treatment under close to actual operational conditions; what can be expected to occur among clinic patients if provider and patient compliance is high (Bloland, 2001).

2.21.2 Use of *in vitro* tests for detection of drug resistance

From the point of view of a researcher interested in pure drug resistance, *in vitro* tests avoid many of the confounding factors which influence *in vivo* tests by removing parasites from the host and placing them into a controlled experimental environment. In the most frequently used procedure, the micro-technique, parasites obtained from a finger-prick blood sample are exposed in microtitre plates to precisely known quantities of drug and observed for inhibition of maturation into schizonts (Bloland, 2001).

This test more accurately reflects "pure" anti- malarial drug resistance. Multiple tests can be performed on isolates, several drugs can be assessed simultaneously, and experimental drugs can be tested. However, the test has certain significant dis- advantages. The correlation of *in vitro* response with clinical response in patients is neither clear nor consistent, and the correlation appears to depend on the level of acquired immunity within the population being tested. Prodrugs, such as proguanil, which require host conversion into active metabolites cannot be tested. Neither can drugs that require some level of synergism with the host's immune system. *In vitro* tests are technologically more demanding and relatively expensive, which makes them potentially more difficult to adapt successfully to routine work in the field (Bloland, 2001).

2.21.3 Use of Animal model studies for the detection of drug resistance

This type of test is, in essence, an *in vivo* test conducted in a non-human animal model and, therefore, is influenced by many of the same extrinsic factors as *in vivo* tests. The influence of host immunity is minimized by using lab-reared animals or animal-parasite combinations unlikely to occur in nature, although other host factors would still be present. These tests allow for the testing of parasites which cannot be adapted to *in vitro* environments (provided a suitable animal host is available) and the testing of experimental drugs not yet approved for use in humans. A significant disadvantage is that only parasites that can grow in, or are adaptable to, non-human primates can be investigated (Bloland, 2001).

2.21.4 Use of molecular techniques for detection of drug resistance

These tests offer promising advantages to the methods described above. Molecular tests use Polymerase Chain Reaction (PCR) to indicate the presence of mutations encoding biological resistance to antimalarial drugs. Theoretically, the frequency of occurrence of specific gene mutations within a sample of parasites obtained from patients from a given area could provide an indication of the frequency of drug resistance in that area analogous to information derived from *in vitro* methods. Advantages include the need for only small amounts of genetic material as opposed to live parasites, independence from host and environmental factors, and the ability to conduct large numbers of tests in a relatively short period of time. Disadvantages include the obvious need for sophisticated equipment and training, and the fact that gene mutations that confer antimalarial drug resistance are currently known or debated for only a limited number of drugs (Bloland, 2001).

2.22 Fitness Cost of Drug Resistance in *Plasmodium* sp

Anti-pathogenic drugs target metabolic and structural proteins, which are essential for growth and multiplication. Therefore, genetic changes (mutations) in genes whose products are associated with these proteins/processes can encode resistance but often have deleterious effects (Abdel-Muhsin *et al.,* 2003). Consequently, development of drug resistance may incur a fitness cost in the absence of drug selection. This has been demonstrated in many pathogens, including parasites and bacteria (Maiga *et al.*, 2007).

Advances in molecular typing have led to identification of a number of genetic events involved in or responsible for parasite resistance to antimalarial drugs. Among these are single nucleotide polymorphisms (SNP) or gene amplifications in genes encoding drug targets. Polymorphism in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) is central to CQ resistance (Sidhu *et al.,* 2002) with the mutation K76T playing a major role in determining the outcome of CQ treatment (Babiker *et al.,* 2001; Djimde *et al.,* 2001). The role of *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) in drug resistance is controversial. Some authors found an association of certain SNPs, in particular the mutation N86Y, with chloroquine resistance (e.g. (Djimde *et al.,* 2001) whereas others did not (Dorsey *et al.*, 2001).

Transfection experiments (Reed *et al.*, 2000) showed that SNPs in the *pfmdr1* gene were involved in modulating the susceptibility to CQ and a joined action of the two genes *pfcrt* and *pfmdr1* was suggested to be involved in high level CQ resistance (Babiker *et al.,* 2001). No conclusive evidence was found so far for the importance of variations in copy number of the *pfmdr1* gene on treatment outcome. Resistance to SP was found to be associated with polymorphism in the *P. falciparum* dihydrofolate reductase gene (*Pfdhfr*)

(pyrimethamine resistance) and the dihydropteroate synthase gene (*Pfdhps*) (sulfadoxine resistance).

The mutations S108N in *pfdhfr* and A437G in *pfdhps* are considered the key mutations that confer resistance against SP, whereas the accumulation of additional mutations in these genes increases the level of resistance (Basco and Ringwald, 2000). The calcium dependent ATPase6 is considered a target of Artemisinin drug action (Jambou *et al.*, 2005), however, field data on the involvement of ATPase6 mutations in drug resistance to Artemisinin are controversial.

Mutations are thought to be costly for parasites in absence of drug pressure. Before the widespread use of antimalarial drugs, the frequency of resistant parasites was generally low, and only rose in response to drug pressure. The frequency at which drug resistance alleles occur in a population is determined by a balance between the selective advantage conferred by the mutated alleles in the presence of the drug and natural purifying selection eliminating mutations that incur fitness cost in absence of the drug. There are a number of observations indicating that mutations associated with drug resistance reduce the parasites' fitness in absence of drug pressure. Experimental support comes from an *in vitro* competition experiment showing a reduced survival of drug resistant compared to sensitive parasites, when drug pressure was removed (Hayward *et al.*, 2005). Similar results were observed in competition experiments in mice where a pyrimethamine resistant *Toxoplasma gondii* strain was found to have a fitness defect over a sensitive strain (Fohl and Roos, 2003) or where a drug resistant *P. chabaudi* clone was completely suppressed by a sensitive clone in absence of drug pressure (de Roode *et al.*, 2004).

Evidence from field studies comes from longitudinal surveys reporting temporal fluctuations in the frequency of mutations in the genes *pfcrt* and *pfmdr1* (Abdel-Muhsin *et al.,* 2004; Ord *et al.,* 2007).

A study in Hainan, China, found the prevalence of chloroquine-resistant *P. falciparum* strains to decrease after abolishment of CQ as first-line treatment (Wang *et al.,* 2005). The frequency of the resistance marker *pfcrt* 76T decreased from 90% in 1978 to 54% in 2001. Similarly, re-emergence of sensitive parasites after suspension of CQ as first line treatment has also been reported from Malawi **(**Kublin *et al.,* 2003**)**, where a progressive decline in the prevalence of *pfcrt* 76T was observed from 85% to 13% between 1992 and 2000.

Studies have reported a selection of the *pfcrt* K76 wild type allele (Sisowath *et al.,* 2009) and the *pfmdr1* wild type alleles N86, F184 and D1246 following treatment with the artemisinin combination therapy Coartem ® (artemether-lumefantrine) (Sisowath *et al.,* 2009; Sisowath *et al.,* 2007). Therefore, Coartem ® seems to be an ideal replacement for CQ as a first line-treatment in areas with high levels of CQ resistance. It's likely that a faster reversal of drug resistance against CQ is achieved through Coartem ® treatment compared to the situation in absence of treatment.

Estimating the reduction in parasite fitness is desirable as it remains a crucial parameter for epidemiological models that aim at predicting the transmission dynamics of malaria and the spread of drug resistance. Gaining further knowledge on the extent of fitness loss in drug resistant parasites will increase the prediction power of mathematical models (Laxminarayan, 2004).

The fitness of parasites determines their survival in the host or vector and their reproductive success. One key factor of fitness is thus the transmission rate to a new host, which is represented by the basic reproductive number (R 0). In the case of malaria, several parameters need to be measured in order to determine R 0. Among these is for example the infectiousness from humans to mosquitoes, the duration of infection in the human host, the probability that the mosquito survives the development of the parasite, the infectiousness from mosquitoes to humans (Koella and Antia, 2003). However, measuring the actual transmission from one host to another is experimentally very difficult.

2.23 Risk Factors for Malaria

2.23.1 Immunity to malaria

Human populations could develop immunity to malaria following continued exposure to malaria infection (Rono *et al*., 2015; Wanjala and Kweka, 2016). In areas with a high malaria transmission, newborn babies are protected through the immunity they acquire from their mothers for the first few months after birth. This immunity will gradually decrease, and children are at an increased risk of malaria as they become older. A study from Ethiopia has shown a high malaria incidence among children between the age of 1 to 9 years in lowland areas (areas below 1,500 metres above sea level), although the malaria incidence was similar for all age-groups in the highland area (Woyessa *et al*., 2012). This could indicate that people living in highland areas are less exposed to malaria infection, and hence all age groups lack immunity to malaria. On the other hand, in lowland areas where the risk of malaria infection is higher, adults, but not young children, could develop some protection to malaria infections through previous exposure to malaria (Gari, 2018).

2.23.2 Socio-demographic factors

Studies have reported that people from poorer families (Coleman *et al*., 2010; Jaleta *et al*., 2013), housing conditions such as the presence of open eaves (Animut *et al*., 2013) and having less educated parents (Roberts and Matthews, 2016) are at increased risk of malaria infection. Moreover, evidence also shows that improved housing (Kirby *et al*., 2012) and knowledge on the proper use of Long Lasting Insecticidal Nets (LLINs) (Deribew *et al*., 2012) could be effective intervention to prevent malaria. Climate temperature (Loha and Lindtjørn, 2010; Yewhalaw *et al*., 2009), rainfall and altitude (Loha and Lindtjørn, 2010) and relative humidity (Li *et al*., 2013) are reported to be predictors of malaria transmission. The development and survival of malaria parasite in the *Anopheles* mosquito is mostly determined by the mean annual temperature (Lunde *et al*., 2013). For example, climate change influences the El Niño cycle; and during the El Niño season the rainfall decreases and the temperature increase, which could result in a low malaria incidence (Gao *et al*., 2012). On the other hand, following the El Niño season, heavy rainfall could create many water bodies that favour mosquito breeding, and result in increased malaria transmission. As a result of climate change or global warming, previously malaria-free highland (with lower temperatures) areas are now becoming at risk of malaria transmission (Ngarakana-Gwasira *et al*., 2016). A recent study from the Oromia Region in Ethiopia showed an association between malaria and an increase in sea surface temperature (Bouma *et al*., 2016). Consequently, wormer highland areas favour malaria transmission (Ngarakana-Gwasira *et al*., 2016). This poses a challenge for the future malaria control and prevention programme.

2.23.3 Human activities

In Africa, a high population growth rate has led to an increased demand for food and energy. In order to meet the population demand, many countries have been forced to initiate a large-scale dam and irrigation projects to help cultivate crops (Ijumba and Lindsay, 2001). In low malaria transmission areas like Ethiopia, such irrigation scheme creates favourable environments for malaria vector breeding (Jaleta *et al*., 2013). Studies have shown that people living close to irrigation dams are at increased risk of malaria infection (Yewhalaw *et al*., 2009; Ijumba and Lindsay, 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was conducted using samples from selected general hospitals located within the three Senatorial Districts of Kaduna State, Nigeria. These hospitals were; Hajiya Gambo Sawaba General Hospital Zaria, Barau Diko Teaching Hospital and General Hospital Kafanchan. Hajiya Gambo Sawaba General Hospital is located within Zaria Local Government Council in Kaduna North Senatorial District. Barau Diko Teaching Hospital is located within Kaduna North Local Government Council, in Kaduna Central Senatorial District. General Hospital Kafanchan is located within Jama'a Local Government Council in Kaduna South Senatorial District (Kaltungo *et al*., 2013).

Kaduna State is located in the North-West geo political zone of Nigeria. The capital city of the State is Kaduna. Kaduna city along with Zaria and Kafanchan are the main urban areas of the State. Kaduna State lies at latitude 10°20′ north and longitude 7°45′ east and covers an area of 45,711.2 km^2. According to the National Population Coommission, the 2006 census puts the population of Kaduna State at 6,113,503 (Demographics, 2021). It accounts for 4.3% of Nigeria's total population. Kaduna lies in the savanna ecological belt. It experiences a rainy (wet) season between April and October and a harmattan (dry and dusty) season between November and March. The area experiences an average annual rainfall of 1099 mm and average daily temperature of 28°C. Malaria occurs all year round, with peaks during the middle to late rainy season (Aliyu *et al.,* 2017). The map of Kaduna State showing the hospital locations in the three senatorial districts is shown in Figure 3.1.

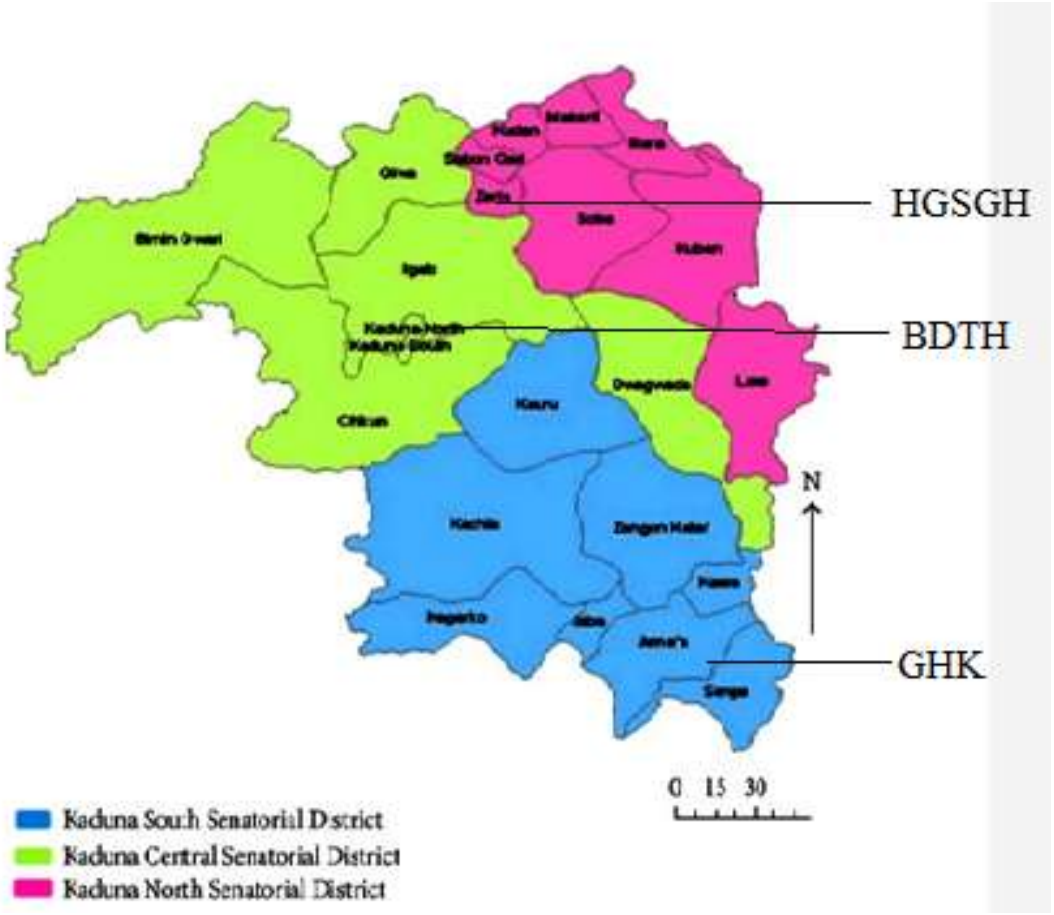


Figure 3.1: Hospital locations in the three senatorial districts of Kaduna State

Source: Kaltungo *et al*. (2013)

Key:GHK= General Hospital Kafanchan, BDTH=Barau Diko Teaching Hospital, HGSGH=Hajiya Gambo Sawaba General Hospital

3.2 Study Design

The study was a cross-sectional hospital based study that lasted for six months (May to October 2018)

3.3 Ethical Clearance

Ethical clearance was obtained from the Ethical Committee of Kaduna State Ministry of Health as well as Barau Diko Teaching Hospital Board of Ethics, Kaduna.

3.4 Inclusion Criteria and Exclusion Criteria

All febrile patients presenting symptoms of malaria (fever, chills, headache, body and joint pains, sweats, malaise) that were directed to the laboratory for malaria parasite (MP) test and gave consent were included. All patients directed to the laboratory for laboratory tests other than malaria test and those who did not give consent were excluded.

3.5 Sample Size

The sample size was determined using a prevalence of 22.4% (Aliyu *et al.*, 2017) and the following formula as described by Naing *et al.* (2006):

$$n=\frac{Z^2 p(1-p)}{d^2}$$

n= number of samples

p=prevalence of previous study =22.4%=0.224

z=standard normal distribution at 95% confidence limit=1.96

d=absolute desired precision of 5%=0.05

z=1.96

$$n=\frac{1.96^2 * 0.224(1-0.224)}{0.05^2}$$

$$n = \frac{3.8416 * 0.224 * 0.776}{0.0025}$$

n= 267 samples

For proper distribution, a total of 300 blood samples (100 blood samples from each hospital within the senatorial districts) were collected for this study.

3.6 Administration of Consent Forms and Structured Questionnaire

Consent forms and structured questionnaire (Appendix IV) were administered to consenting individuals who met the inclusion criteria. These were used to obtain bio-data, demographic data and other information relevant to this research.

3.7 Sample Collection

Venipuncture technique was employed for blood sample collection by a trained laboratory technician. Soft tubing tourniquet was fastened to the upper arm of the patient to enable the index finger feel a suitable vein. The puncture site was then cleansed with alcohol swab and venipuncture made with the aid of a 21 guage (21G) needle attached to a syringe. When 2 milliliters of blood were collected, the tourniquet was released and the needle removed immediately while the blood samples were transferred into an EDTA bottle (Cheesbrough, 2009)

3.7.1 Preparation of blood films

Thick and thin blood films were prepared immediately after the blood samples were collected according to the technique outlined by Cheesbrough (2009). A drop of each blood sample was placed in the center of a grease-free clean glass slide, and spread immediately using a smooth edged slide spreader to make a thin film. The thin films were allowed to air dry before being fixed with methanol. The thick films were made by transferring a drop of blood to another clean slide

and spread in such a way that it was possible to see (but not read through) newsprint, it was then allowed to dry properly. The blood films were lysed with water and stained using 10% Giemsa working solution for 30 minutes. After staining the blood films, they were allowed to air-dry (Cheesbrough, 2009).

3.7.2 Examination of stained blood film slides

The stained blood films were examined under the microscope using immersion oil and 100X objective lens after focusing. Presence of ring forms, trophozoites or gametocytes of *Plasmodium falciparum* or other *Plasmodium* sp was recorded. A blood smear was considered negative if no parasite was seen after 10 minutes of search or examination under 100X high power fields of microscope.

The prevalence (%) of malaria was determined by the number of positive cases over the number of specimens collected.

$$\text{Prevalence} = \frac{\text{Number of positives}}{\text{Total number of samples}} \times 100$$

3.8 Rapid Diagnostic Test (RDT)

The Rapid Diagnostic Test was done using CareStart™ Malaria HRP II (Access Bio,Inc, Somerset, NJ), which contains a membrane strip pre-coated with monoclonal antibody across a test strip. The monoclonal antibody (test line) is specific to the Histidine-rich protein 2 of the *Plasmodium falciparum*. CareStart™ Malaria HRP II has 98% sensitivity and 97.5% specificity.

A sample pipette was used to take 5µl of whole blood, which was transferred into the sample well by squeezing the sample pipette. Two drops of assay buffer was added into the assay buffer well and the result was read after 20 minutes. A

positive reaction was indicated by the presence of two lines at the regions labeled T and C on the test cassette, while a negative test was indicated by the presence of only one line at the C region of the test cassette. A test was declared invalid when no line was seen in the C region of the test cassette.

3.9 Determination of Genotype by Haemoglobin Electrophoresis

Cellulose acetate method of haemoglobin electrophoresis was carried out on all malaria positive samples as follows: A drop of blood from all malaria positive blood samples was placed on a clean white tile and mixed with three drops of water to lyse the red blood cells. With the aid of an applicator, the haemolysate was placed on a cellulose acetate paper. This was followed by electrophoresis in Tris-EDTA-borate buffer solution at pH 8.6 for 15 minutes at electromotive force of 250v. Haemolysates from blood samples of Haemoglobin AS and Haemoglobin AC were run as controls (Egesie *et al.*, 2008).

3.10 Deoxyribonucleic Acid (DNA) Extraction

Total DNA was extracted from 25 of the 71 malaria positive blood samples using Zymo Research Quick-DNA ^{TM} Miniprep Plus Kit (Irvine, California).

Proteinase K was reconstituted by adding 1,060 µl of storage buffer to the 20 ml tube of proteinase K and stored at -20^{o}C prior to DNA extraction. The DNA extraction was carried out as follows: For each of the blood samples, 200 µl was transferred into a microcentrifuge tube; this was followed by the addition of 200 µl of biofluid and cell buffer and 20 µl of proteinase K. The mixture was thoroughly mixed and incubated at 55^{o} C for 10 minutes, after which the tubes were removed and one volume (420 µl) of genomic binding buffer was added to digest the sample and mixed thoroughly by pipetting up and down. The mixture was transferred in a zymo-spin column in a collection tube. This was centrifuged at 12, 000 x g for 1 minute after which the collection tube was discarded along

with the flow through. Four hundred microlitre (400 µl) DNA pre-wash buffer was added to the spin column in a new collection tube and centrifuged at 12, 000 x g for 1 minute. The collection tube was emptied and 700 µl of gDNA wash buffer was added to the spin column and centrifuged at 12, 000 x g for 1 minute. The collection tube was emptied and 200 µl of gDNA wash buffer was added to the spin column and centrifuged again for 1 minute, after which the collection tube was discarded along with the flow through. To elute the DNA, the spin column was transferred to a clean microcentrifuge tube and 50 µl of DNA elution buffer was added, incubated for 5 minutes at room temperature, and centrifuged at 12, 000 x g for 1 minute. The eluted DNA was kept at -20^{o} C prior to PCR and sequencing.

3.11 Primers Used For Polymerase Chain Reaction

The genes and primer sequences used for the polymerase chain reaction are shown below in Table 3.1. The target genes were; *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) gene which is a single copy, 13-exon gene, localized on chromosome 7 and codes for a digestive vacuole trans-membrane protein, which plays a key role in chloroquine resistance. *Plasmodium falciparum* multidrug resistance transporter 1 (*pfmdr1*) gene which codes for a large 12 transmembrane domain ABC-transporter (PfMRP1), located in the parasite plasma membrane (Klokouzas *et al*., 2003), *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) gene (codes for *P. falciparum* dihydrofolate reductase enzyme), *Plasmodium falciparum* dihydropteroate synthase (*pfdhps*) gene which codes for dihydropteroate synthase enzyme of *P. falciparum*. *Plasmodium falciparum* atpase*6* (*pfatpase6*).

Table 3.1: Priimer Sequences Used For Polymerase Chain Reaction

S/N	Gene	Primer sequence	Size (bp)	SNPs (codons)
1	*Pfcrt*	F: GGAGGTTCTTGTCTTGGTAAAT R: ATATTGGTAGGTGGAATAGATTCT	315	391T/A,392G/C, 399G/T 400A/G,402T/A,404A/C (codons C72S, M74I,N75E, K76T)
2	*Pfmdr1*	F: TGTTGAAAGATGGGTAAAGAGCAGA R: TCGTACCAATTCCTGAACTCACTT	514	256A/T,257A/T (Codon N86Y/F)
3	*Pfdhps*	F: GATTCTTTTTCAGATGGAGG R: TTCCTCATGTAATTCATCTGA	770	1482T/G,1483C/T/G,1486C/G,1794A/G,1918C/G,2013G/T/A, (codon S436A/F/C, A437G, K540E, A581G, A613S/T)
4	*Pfdhfr*	F: TGATGGAACAAGTCTGCGACGTT R: CTGGAAAAAATACATCACATTCATATG	594	148T/C,152A/T,153T/C, 175T/C, 323G/A/C,490A/T (codons C50R,N511, C59R, S108, I164L)
5	*Pfatpase6*	F: AAAATAAATACCACATCAACACAT R: TCAATAATACCTAATCCACCTAAA	437	2306G/A (codon769N)

(Zhang *et al*., 2008) Key: *pfcrt=Plasmodium falciparum* chloroquine resistance transporter, *pfmdr1=Plasmodium falciparum* multidrug resistance 1, *pfdhfr=Plasmodium falciparum* dihydrofolate reductase, *pfdhps= Plasmodium falciparum* dihydropteroate synthase. *Pfatpase6= Plasmodium falciparum* atpase*6,* SNPs=Single Nucleotide Polymorphisms

3.12 Amplification of Genetic Markers of *Plasmodium falciparum*

The *pfcrt, pfmdr1, pfdhfr, pfdhps* and *pfatpaes6* genes were amplified by multiplex PCR as follows: Multiplex PCR master mix cocktail was prepared by adding 3.0µl of the extracted DNA, 2.5 µl of 10X PCR buffer, 1.5 µl of 50mM $MgCl_2$, 1.0 µl of 2.5 Mm DNTPs, 1.0 µl of Taq polymerase (5 µ/ µl) , 5 µl of nuclease free water, 1.0 µl each of 5pMol of each of the forward and reverse primers in table 3.1 to give a total volume of 25 µl. This was run using the following programme: Initial detauration at 94^{o}C for 5 minutes followed by 9 cycles of denaturation at 94 ^{o}C for 15 seconds, annealing at 65 ^{o}C for 20 seconds, extension at 72 ^{o}C for 30 seconds and another 35 cycles of denaturation at 94 ^{o}C for 15 seconds, annealing at 55 ^{o}C for 20 seconds, extension at 72 ^{o}C for 30 seconds, final extension at 72 ^{o}C for 7 minutes.

The PCR amplicons were separated by electrophoresis on a 1.5% (w/v) agarose gel stained with ethidium bromide. The gel was visualised on UV transilluminator gel imaging system (gel documentation unit) and system photographed, band positions were determined and compared to molecular weight markers.

3.13 Amplification of *pfatpase6* Gene

The *pfatpase6* gene was amplified using the *pfatpase6* primers in Table 3.1 above as follows: The master mix cocktail was prepared by adding 2.0 µl of 100ng/ul DNA, 2.5 µl of 10x PCR buffer, 1.5 µl of 50mM $MgCl_2$, 1.0 µl of DMSO, 2.0 µl of 2.5Mm DNTPs, 0.15 µl of Taq Polymerase (5 µ/µl), 1.0 µl forward primer, 1.0 µl reverse primer and 13.85 µl nuclease free water to make a total of 25 µl. This was run using the following programme: Nine cycles of initial denaturation at 94˚C for 15 minutes, denaturation at 94˚C for 40 seconds, annealing at 60˚C for 120 seconds, extension at 72˚C for 40 seconds.

This was followed by another 35 cycles of denaturation at 94˚C for 40 seconds, annealing at 50˚C for 120 seconds, extension at 72˚C for 40 seconds, final extension at 72˚C for 10 minutes.*The PCR amplicons were separated by electrophoresis and visualised on UV transilluminator gel imaging system (gel documentation unit), after which gel pictures were taken from which band positions were determined and compared to molecular weight markers.*

3.14 Sequencing of *Pfatpase* Gene

Amplicon purification was done using QiaQuick DNA gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amplicon of interest was cut off, sliced into pieces and 500 µl of phosphate buffer (PB) was added and mixed thoroughly. The sample was applied into the spin column and centrifuged for 20-30sec at 13000 rpm to bind. The flow through was discarded and the Qiaquick column was placed back into the same tube. For the washing step, 0.75ml of Buffer PE was added to the Qiaquick column and centrifuged for 30sec at 13000 rpm. The flow through was discarded and the column was placed in the same tube and centrifuged for 1 minute to remove excess ethanol from buffer PE. The Qiaquick spin column was placed in a new 1.5ml microcentrifuge tube and 50 µl of Buffer EB (10 mM Tris Cl, pH 8.5) was added to the center of the Qiaquick membrane and centrifuged for 1 minute to elute the nucleic acid. Five to 10 µl of purified PCR product was used to prepare the sequencing mix using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1. (Applied Biosystem, Foster City, CA, USA). Forty cycles of thermocycle programme (96°C for 10s, 50°C for 5s and 60°C for 4min) were run using a thermocycler. The cycle sequencing products were cleaned up using the Sephadex based filtration (Edge Biosystems) method.

Capillary electrophoresis of products on the Applied Biosystems 3130 automated DNA sequencer was carried out. GenBank sequence database was used to evaluate the result of the sequenced DNA. This software is available at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/).

3.15 Phylogenetic Analysis

The multiple sequence alignment programme Clustal W was used to obtain an optimal nucleotide sequence alignment file after comparing with sequences deposited in GenBank. Phylograms were obtained by MEGA X based on aligned nucleotide sequences.

3.16 Statistical Analysis

Data obtained were analysed using Statistical Package for Social Sciences (SPSS) version 21. Chi-square and Odds ratio statistical tools were used to determine associations. P value ≤ 0.05 was considered as significant and results are presented in tables, figures and plates.

CHAPTER FOUR

4.0 RESULTS

4.1 Prevalence of Malaria Among the Study Participants

The prevalence of malaria among the study participants by microscopy and Malaria Rapid Diagnostic Test (MRDT) is presented in table 4.1. Out of the 300 blood samples examined using microscopy and Malaria Rapid Diagnostic Test; 71 (23.7%) tested positive by microscopy while 65 (21.7%) tested positive by MRDT. The higher prevalence (23.7%) was recorded with microscopy and only *Plasmodium falciparum* was detected.

4.2 Sensitivity and Specificity of Malaria Rapid Diagnostic Test (MRDT)

The sensitivity and specificity of malaria rapid diagnostic test (MRDT) using microscopy test as standard is shown in Table 4.2. Sensitivity of 92 % and specificity of 100 % were calculated from the table.

4.3 Percentage Occurrence of Malaria Among Study Participants According to the Hospitals in the Three Senatorial Districts of Kaduna State

The percentage occurrence of malaria among study participants according to the hospitals in the three senatorial districts of Kaduna State is presented in figure 4.1. From all the 300 samples screened, prevalence of 25%, 30%, and 16% were obtained in the hospitals within Kaduna North, Kaduna South and Kaduna Central respectively. The difference in prevalence between hospitals in the three senatorial districts was not statistically significant ($p=0.062$).

Table 4.1. The Prevalence of Malaria Among the Study Participants by Microscopy and Malaria Rapid Diagnostic Test

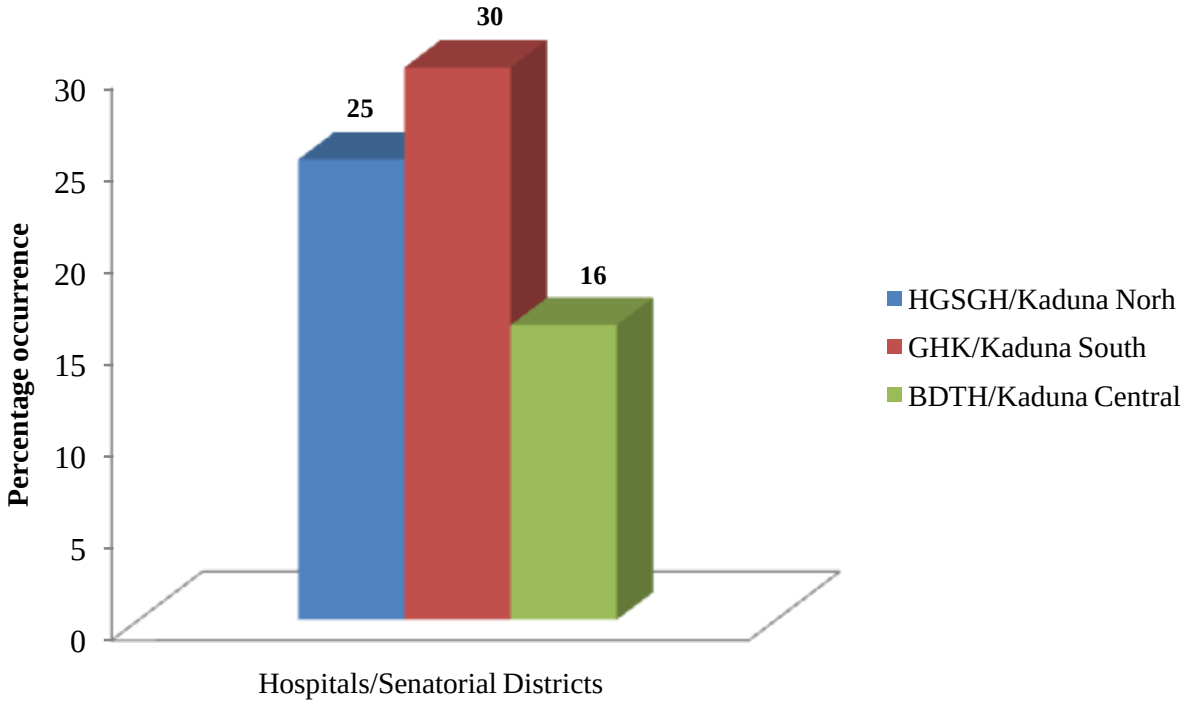
Test Method	No. examined	No. positive	% prevalence
Microscopy	300	71	23.7
RDT	300	65	21.7

Key: No. = Number

Table 4.2. Sensitivity and Specificity of RDT Using Microscopy as Gold Standard

		Microscopy		
		Positive	**Negative**	**Total**
RDT	**Positive**	65.0	0.0	65.0
	Negative	6.0	229.0	235.0
	Total	**71.0**	**229.0**	**300**

(Appendix VI: Formula for the calculation of sensitivity and specificity)



P=0.062 Chi square=5.572 df=2

Figure 4.1. Percentage Occurrence of Malaria Among Study Participants According to the Hospitals in the Three Senatorial Districts of Kaduna State

Key: HGSGH= Hajiya Gambo Sawaba General Hospital, GHK= General Hospital Kafanchan, BDTH= Barau Diko Teaching Hospital

4.4 Relationship between Malaria and Some Demographic Factors

The relationship between malaria and some demographic factors is shown in Tables 4.3a and 4.3b. Out of the 300 participants in this study, there were 115 males and 185 females. A total of 71 persons had malaria; out of which 28 were males and 43 were females. Males had a higher prevalence (24.3%) than females (23.2%), the p value was p=0.827. The association between malaria and sex was not statistically significant.The odds ratio showed association which was also not statistically significant because the confidence interval contained 1 which is the null value (Odds ratio =1.063, 95% Confidence interval= 0.616 - 1.834).

The highest malaria prevalence (33.3%) was recorded in the age group ≤10, followed by the age groups 31-40 (31.3%), 11-20 (24.7%), and 21- 30(17.2%). The age group ≥41 had the least prevalence (9.1%). The association between malaria and age was statistically significant (p=0.029).

Table 4.3a also shows the prevalence of malaria in relation to the number of children in the family. Participants in families with ≤5 children had a prevalence of 22.5% followed by those with 6-10 children (25.0%), 11-15 children (42.9%),16-20 children (50.0%). The group with more than 21 children had only one respondent who had zero prevalence. There was no statistically significant difference in the association between malaria and number of children in the family (p value 0.476).

Prevalence of malaria based on the type of housing is presented in Table 4.3b. Those living in compound houses had a prevalence of 25.0% which was higher than the 19.4% found among those living in self-contained apartments (p=0.334, Odds ratio=0.724, Confidence interval=0.376-1.396, df =1). Those with monogamous families had a lower prevalence

(21.5%) than those in polygamous families with 27.6% (p=0.237, Odds ratio=0.719, Confidence interval at 95%=0.416 -1.244, df =1). In both cases, the association with malaria was not statistically significant.

The table also shows that married people had a prevalence of 31.8% which was higher than the 16.0% and 12.0% prevalence in single and divorced people respectively. There was a significant (p=0.005) association between malaria and the marital status of participants.

The highest prevalence (30.6%) according to educational status was found among those with no education (Nil), this was followed by the prevalence of 28.6% among those who only passed through adult literacy, primary school education (27.0%), secondary school education (26.6%) and tertiary education (17.2%). The lowest prevalence was found among those who had non-formal education (9.1%). There was no statistically significant difference in the asociation between malaria and educational status (p value 0.378).

Based on occupation, the finding showed that civil servants had the least prevalence (17.1%).Traders had a prevalence of 24.5% followed by those who were unemployed (24.3%), farmers (20.0%) and artisans with 18.2%. The highest prevalence (36.4%) was recorded among those involved in other forms of occupations. The association between malaria and occupation was not statistically significant (P value 0.836).

Table 4.3a. Relationship between Malaria and Some Demographic Factors Among the Study Participants.

		Microscopy					
Demographic factors	**No. examined**	**No. positive**	**% Prevalence**	χ^2	**P value**	**OR**	**95% CI**
Sex							
Male	115	28	24.3	0.048	0.827	1.063	0.616-1.834
Female	185	43	23.2				
Age (years)							
≤10	75	25	33.3	10.807	**0.029***		
11-20	73	18	24.7				
21-30	87	15	17.2				
31-40	32	10	31.3				
≥41	33	03	9.1				
Number of children per family							
≤5	236	53	22.5	3.515	0.476		
6-10	52	13	25.0				
11-15	7	3	42.9				
16-20	4	2	50.0				
≥21	1	0	0.0				

Key**:** *=significant at p≤0.05, χ^2=Chi square, %=percentage, OR=Odds Ratio, CI=Confidence interval, No.= number

Table 4.3b. Relationship between Malaria and some Demographic Factors Among the Study Participants.

Demographic factors	No. screened	Microscopy		χ^2	P value	OR	95% CI
		No. positive	% Prevalence				
Type of housing							
Self-contained	72	14	19.4	0.935	0.334	0.724	0.376-1.396
Compound house**	228	57	25.0				
Type of family							
Monogamous	195	42	21.5	1.397	0.237	0.719	0.416-1.244
Polygamous	105	29	27.6				
Marital status							
Single	144	23	16.0	10.633	**0.005***		
Married	148	47	31.8				
Divorced	8	1	12.5				
Educational status							
Primary	74	20	27.0	5.317	0.378		
Secondary	79	21	26.6				
Tertiary	93	16	17.2				
Adult Literacy	7	2	28.6				
Nil	36	11	30.6				
Non-formal	11	1	9.1				
Occupation							
Civil servant	35	6	17.1	2.092	0.836		
Trader	49	12	24.5				
Artisan	11	2	18.2				
Farmer	5	1	20.0				
Unemployed	189	46	24.3				
Others	11	4	36.4				

Key**:** *=significant at $p \leq 0.05$, χ^2=Chi square, %=percentage, OR=Odds Ratio, CI=Confidence interval, No.=number **= houses sharing facilities like wells, toilets.

4.5 Prevalence of Malaria in Relation to Pregnancy Among the Participants

Prevalence of malaria in relation to pregnancy among the participants is presented in Table 4.4. Out of the 185 female participants, only 35 were pregnant, the remaining 150 were not. Seven (7) out of the 35 pregnant women had malaria, while 36 of the non-pregnant women were malaria positive. The pregnant women had a prevalence of 20.0% which was lower than the 24.0% found among the non-pregnant women. The difference between malaria and pregnancy was not statistically significant (χ^2 = 0.255, p value=0.614, Odds=0.792, 95% Confidence interval=0.319-1.965).

4.6 Prevalence of Malaria in Relation to the Presence of Bushes/ Ditches Around Homes of Study Participants

The prevalence of malaria in relation to the presence of bushes/ ditches around homes of study participants is presented in Table 4.5. Out of the 300 participants, 236 had bushes/ditches around their homes; the remaining participants (64) did not have bushes/ditches around their homes. Those who had bushes/ditches around their homes had a prevalence of 23.3% while those without bushes/ditches around their homes had 25.0%. The difference between malaria and the presence of bushes/ditches around the homes of study participants was not statistically significant (χ^2 =0.080, P value= 0.777, Odds ratio=0.912, Confidence interval = 0.480-1.731, df =1).

4.7 Prevalence of Malaria in Relation to the Use of Insecticide Treated Bed Nets

The prevalence of malaria in relation to the use of insecticide treated bed nets is presented in Table 4.6. Those who were sleeping under insecticide treated bed nets had a lower prevalence of (21.7%) than those who were not sleeping under insecticide teated bed nets

with 29.7% prevalence. The difference observed between malaria and use of insecticide treated bed nets was not statistically significant (χ^2 =1.999, P=0.157, Odds ratio=0.654, 95% CI=0.363-1.181).

4.8 Prevalence of Malaria in Relation to Use of Insecticide Spray at Home

Prevalence of malaria in relation to use of insecticide spray at home is presented in Table 4.7. The highest prevalence (30.0%) was found among those that do not spray insecticides at home. Those who were using insecticide spray at home had a prevalence of 20.0%. The difference in prevalence of malaria with use of insecticide spray was statistically significant (χ^2=3.856, P=0.050, OR=0.583, CI=0.340-1.002).

4.9 Haemoglobin Electrophoresis Pattern of Malaria Positive Participants

The haemoglobin electrophoresis pattern of malaria positive participants is presented in Figure 4.2. Participants with haemoglobin genotype AA had the highest prevalence of malaria (73%), followed by those with genotypes AS (23%), AC (3%) and SS (1%). The actual values of the result are presented in Appendix III.

4.10 Agarose Gel Electrograph Showing the Presence of *pfcrt, pfmdr1, pfdhfr* and *pfdhps* detected by Multiplex PCR

Agarose gel electrograph showing the presence of *pfcrt, pfmdr1, pfdhfr* and *pfdhps* detected by multiplex PCR is presented in Plate I. The gel electrophoresis of the resultant multiplex PCR products indicated that they were of the expected sizes.

4.11 Analysis of the Number and Percentages of the Genes Amplified by Multiplex PCR

Analysis of the number and percentages of the genes amplified by multiplex PCR is presented in Table 4.8. Out of the 25 DNA samples screened 20 (80%) *pfcrt*, 9 (36%) *pfmdr1*, 15 (60%) *pfdhfr* and 2 (8%) *pfdhps* genes were amplified by multiplex PCR.

4.12 Frequency of *Plasmodium falciparum* Parasites with Multiple Drug Resistance Genes

The frequency of *Plasmodium falciparum* parasites with multiple drug resistance genes is presented in Table 4.9. Twenty four (24%) percent (6/25) of the parasites had the *Pfcrt*/ *Pfmdr1* genes, 8% had *Pfmdr1/pfdhps*, 8% had *pfdhps/pfmdr1*, 16% (4/25) had *Pfmdr1*/*pfcrt*/*pfdhfr*, 4% (1/25) had *Pfcrt/pfmdr1/pfdhps* resistance genes.

4.13 Agarose Gel Electrograph Indicating the Amplification of *pfatpase6* Gene

Agarose gel electrograph indicating the amplification of *pfatpase6* gene is presented in Plate II. Eleven out of the 15 DNA samples analysed (73.3%) showed amplification of the *pftpase6* gene.

Table 4.4. Prevalence of Malaria in Relation to Pregnancy Among Female Participants

		Microscopy					
Pregnancy	**No. examined**	**No. positive**	**% Prevalence**	χ^2	**P value**	**OR**	**95 % CI**
Yes	35	7	20.0	0.255	0.614	0.792	0.319-1.965
No	150	36	24.0				
Total	185	43	23.2				

Key**:** χ^2=Chi square, %=percentage, No.=number, OR=Odds Ratio, CI=Confidence interval

Table 4.5. Prevalence of Malaria in Relation to the Presence of Bushes/ Ditches Around Homes of Study Participants

Presence of Bushes/ Ditches	No. examined	Microscopy		χ^2	P value	OR	95% CI
		No. positive	% Prevalence				
Yes	236	55	23.3	0.080	0.777	0.912	0.480-1.731
No	64	16	25.0				
Total	300	71	23.7				

Key: χ^2=Chi square, %=percentage, No.=number, OR=Odds Ratio, CI=Confidence interval

Table 4.6. Prevalence of Malaria in Relation to the Use of Insecticide Treated bed Nets (ITN)

ITN	Microscopy						
	No. examined	No. positive	% Prevalence	χ^2	P value	OR	95% CI
Yes	226	49	21.7	1.999	0.157	0.654	0.363-1.181
No	74	22	29.7				
Total	300	71	23.7				

Key: χ^2=Chi square, %=percentage, No.= number, ITN= Insecticide Treated Net, OR=Odds Ratio, CI=Confidence interval

Table 4.7. Prevalence of Malaria in Relation to use of Insecticide Spray at Home

Insecticide spray	No. examined	Microscopy		χ^2	P value	OR	95% CI
		No. positive	% Prevalence				
Yes	190	38	20.0	3.856	**0.050***	0.583	0.340-1.002
No	110	33	30.0				
Total	300	71	23.7				

Key**:** *=significant at p≤0.05, χ^2=Chi square, %=percentage, No. = number, OR=Odds Ratio, CI=Confidence interval

.

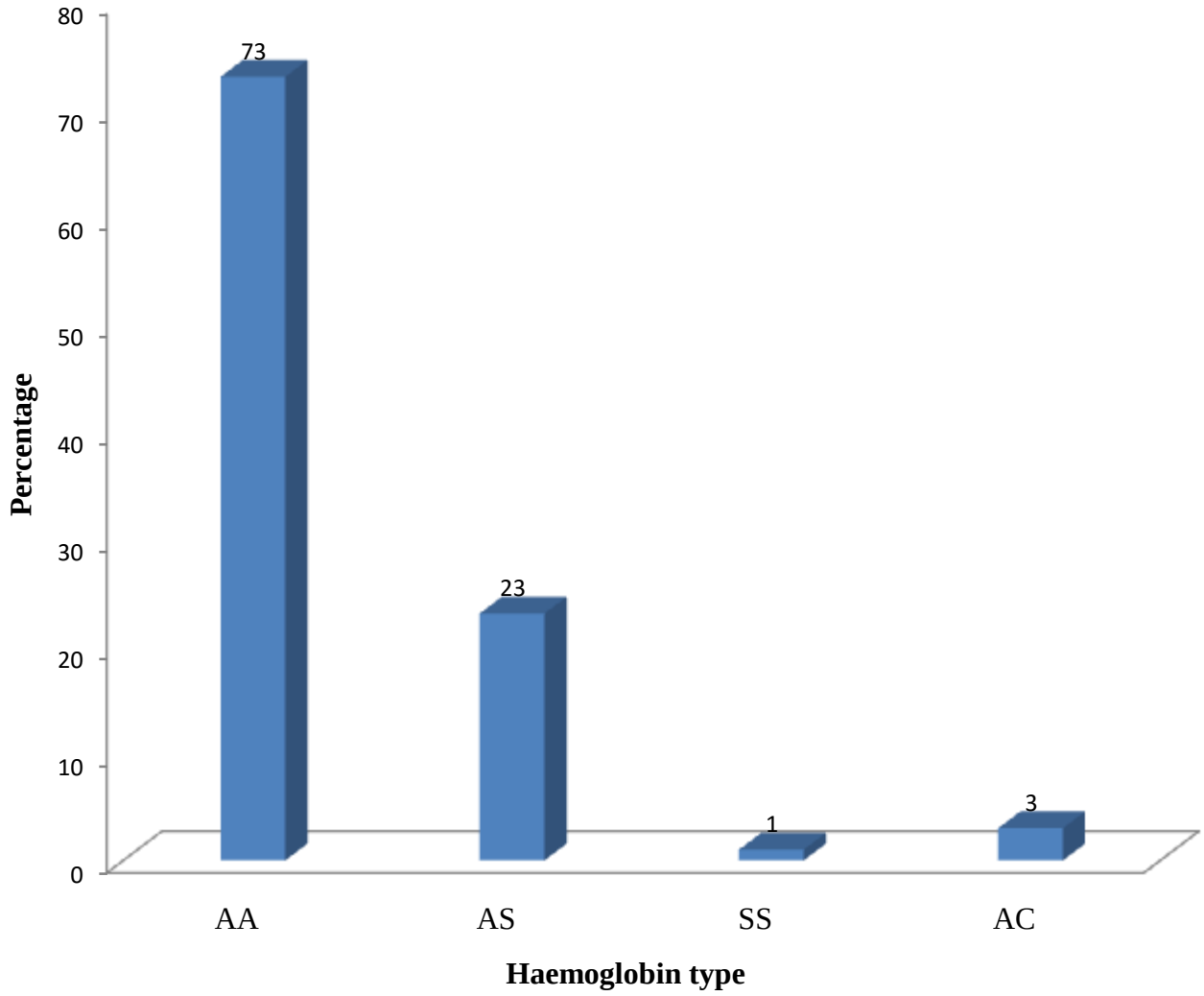


Figure 4.2. Haemoglobin Electrophoresis Patterns of Malaria Positive Participants in the Study Area

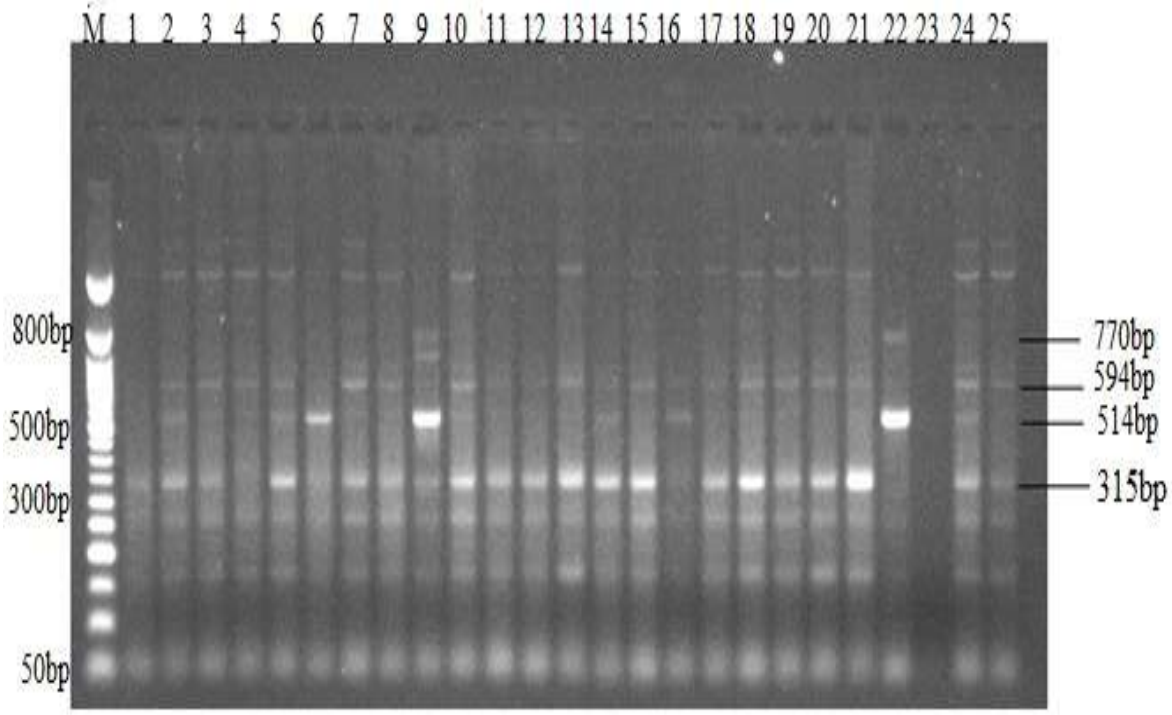


Plate I. Agarose Gel Electrograph for the Detection of *pfcrt* (315bp), *pfmdr1* (514bp), *pfdhfr* (594bp), and *pfdhps* (770bp) genes

Key: Lane M= Molecular weight marker (50bp), Lanes 1-25= *Plasmodiun falciparum* positive samples, bp= base pairs

Table 4.8 Frequency and Prevalence of the *P. falciparum* Genes Detected

Genes	N=25	Frequency	% Prevalence
Pfcrt		20	80
Pfmdr1		9	36
Pfdhfr		15	60
Pfdhps		2	8

Key: N= number examined, *pfcrt=Plasmodium falciparum* chloroquine resistance transporter, *pfmdr1=Plasmodium falciparum* multidrug resistance I, *pfdhfr= Plasmodium falciparum* dihydrofolate reductase, *pfdhps= Plasmodium falciparum* dihydropteroate synthase

Table 4.9 Frequency of *Plasmodium falciparum* Parasites with More than One Drug Resistance Gene

Genes	N=25	Frequency	% Prevalence
Pfcrt/ Pfmdr1		6	24
Pfmdr1/pfdhps		2	8
pfdhps/pfmdr1		2	8
Pfmdr1/pfcrt/pfdhfr		4	16
Pfcrt/pfmdr1/pfdhps		1	4

Key: N=Number examined, *pfcrt=Plasmodium falciparum* chloroquine resistance transporter, *pfmdr1=Plasmodium falciparum* multidrug resistance I, *pfdhfr= Plasmodium falciparum* dihydrofolate reductase, *pfdhps= Plasmodium falciparum* dihydropteroate synthase.

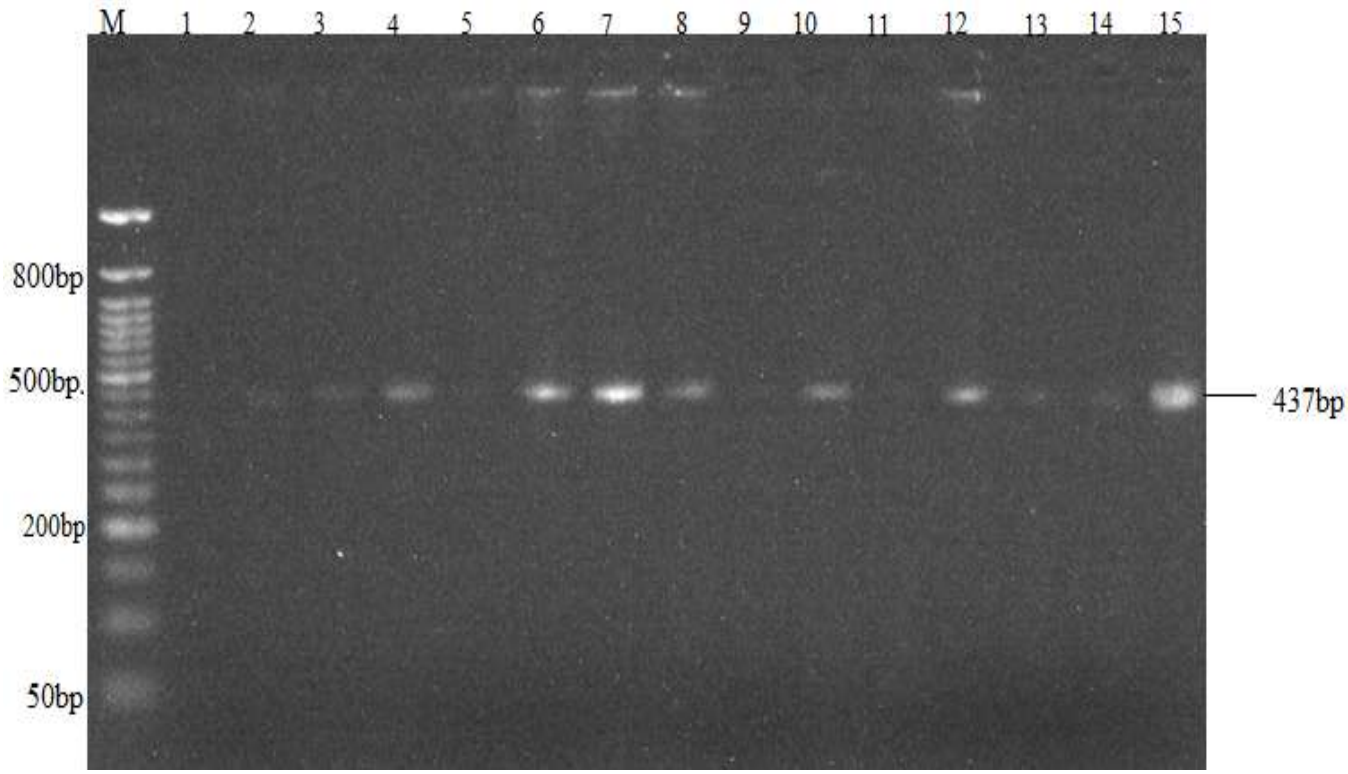


Plate II: Agarose Gel Electrograph of Amplified *pfatpase6* Gene

Key: Lane M: Molecular weight marker (50bp), Lanes 1-15: *Plasmpdium falciparum* positive samples, bp= base pairs

4.14 Confirmation of *pfatpase6* Gene Sequences

The result for confirmation of *pfatpase6* gene sequences on National Centre for Bioinformatics Information (NCBI) is shown in Table 4.10 The table shows the description, query cover, E value, percentage identity and accession number of the sequences that were generated after carrying out a nucleotide blast in the National Center for Bioinformatics Information database. One out of the seven sequences had 99.72 % and three had 99.19 % identity with the sequences in the gene bank. Two sequences had over 98.78 and 98.68% percent identity, while one had 96.78% identity with our sequences.

4.15 Phylogenetic Tree

A phylogenetic tree showing the relatedness between the *pfatpase6* sequences in this study and sequences obtained from the NCBI GenBank is presented in figure 4.3. *Plasmodium falciparum* ATPase6_1 to 7 are sequences from this study. The other *Plasmodium falciparum* ATPase6 sequences were obtained from NCBI Genbank. *Plasmodium_vivax*_E1-E2_ATPase/hydrolase sequence was used as outgroup for the phylogenetic analysis. The phylogenetic tree that was created after carrying out multiple sequence alignment (Appendix VI), showed that, all the *pfatpase6* sequences were closely related as compared to the outgroup (*Plasmodium_vivax*_E1-E2_ATPase/hydrolase).

Table 4.10 Confirmation of *pfatpase6* Gene Sequences on NCBI Database

Sequence code	Description	Query cover (%)	E value	% Identity	Accession number
*Pfatpase*1	*Plasmodium falciparum* serca gene for P-type calcium transporting ATPase.	92	0.0	99.72	AB501644.1
*Pfatpase*2	*Plasmodium falciparum* strain 3D7 chromosome: 1	91	0.0	99.19	AL844501.2
*Pfatpase*3	*Plasmodium falciparum* genome assembly, chromosome: 1	91	0.0	99.19	LR131466.1
*Pfatpase*4	*Plasmodium falciparum* gene for P-type calcium transporting ATPase, complete cds, isolate: Iran-01	91	0.0	99.19	AB576270.1
*Pfatpase*5	*Plasmodium falciparum* isolate VIET9 sercoplasmic/endoplasmic reticulum Ca2+ ATPase gene	82	3e-158	96.78	KC577001.1
*Pfatpse*6	*Plasmodium falciparum* gene for P-type calcium transporting ATPase, complete cds, isolate: Bgl-22	95	0.0	98.97	AB576339.1
*Pfatpase*7	*Plasmodium falciparum* strain 7G8 genome assembly chromosome: 1	94	0.0	98.68	LR536674.1

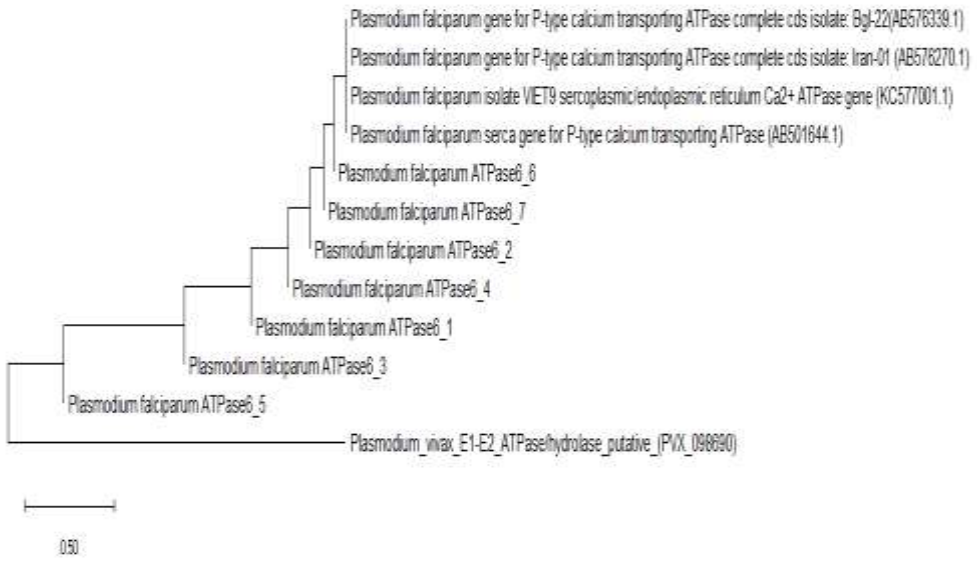


Figure 4.4. Phylogenetic Tree (neighbor joining tree) of *pfapase6* Sequences

Key: *Plasmodium falciparum* ATpase6_1 to 6 = sequences from this study, the other *pfatpase6* sequences were obtained from NCBI, and *Plasmodium_vivax*_E1-E2_ATPase/hydrolase = Outgroup.

CHAPTER FIVE

5.0 DISCUSSION

Out of the 300 blood samples examined, the only *Plasmodium* species detected was *Plasmodium falciparum,* with a prevalence of 23.7% was obtained by microscopy, while 21.7% was obtained by Malaria Rapid Diagnostic Test (MRDT). This is in consonance with reports from similar studies which identified *Plasmodium falciparum* as the prevailing malaria parasite in the study area (Pondei *et al*., 2012; Abah and Temple, 2015; Nyirakanani *et al*., 2018; Wogu and Nduka, 2018). This is probably because *Plasmodium falciparum* is the most prevalent malaria parasite in the African region (Nigeria inclusive), accounting for 99.7% of the estimated malaria cases in 2017(WHO, 2018b).

The prevalence obtained with microscopy was higher than what was obtained with MRDT. This shows that more *Plasmodium falciparum* infections were detected by microscopy than MRDT. This is in agreement with Idoko *et al.* (2015) and Wogu and Nduka (2018) who reported a higher prevalence with microscopy than MRDT.

The sensitivity and specificity test carried out for MRDT using microscopy as standard showed that the MRDT had sensitivity and specificity of 92 % and 100 % respectively.

The prevalence of malaria in this study is lower than the 46.5%, 28.3%, and 25.3% reported by Idoko *et al*. (2015), Benjamin *et al*. (2016), and Okogwu *et al*. (2018) respectively in Kaduna State. The low prevalence in this study could be as a result of increased awareness on malaria, and the use of preventive measures against the vector.

The false negative results and lower prevalence recorded in this study by RDT as compared to microscopy may be as a result of mutation of the Histidine-Rich Protein-II gene (Oyeyemi *et al*., 2015; Beshir *et al*., 2017).

According to Beshir *et al.* (2017) deletions or mutations of the *Plasmodium falciparum* Histidine-Rich Protein-II gene can affect the performance of HRP-II based malaria rapid diagnostic test and lead to false negative results; they also stated that performance of HRP-II based RDTs can be affected by factors such as antigenic variability of the target protein, antigen persistence in the bloodstream following elimination of parasites, and parasite density below the RDT threshold of detection. Also, the possible presence of anti-HRP-II antibodies in the patients, may suggest why some tests were negative despite being positive by microscopy; high levels of anti-HRP-II antibodies attach to soluble *Plasmodium falciparum* HRP II in the bloodstream and block its detection by RDT (Biswas *et al*., 2015; Ho *et al*., 2014; Markwalter *et al*., 2018).

Factors that may decrease performance of the RDT kit include the following: the storage temperature the RDT kit was subjected to by marketers could also be among the reasons for the low sensitivity. Exposure to high temperatures is likely to be a major contributor to poor performance of malaria RDTs.

Transport from the manufacturer, and road transport within a country, are particularly vulnerable times. Prolonged exposure to high humidity could also rapidly degrade RDTs, and may occur after removal from the envelope or if the envelope is damaged. Most manufacturers recommend RDT storage between 2^{o}C and 30^{o}C. Expiry dates are generally set according to these conditions. If kits are stored at temperatures exceeding the recommended limits, it is likely that the shelf life of the RDTs will be reduced and

sensitivity affected prior to the expiry date (Malaria RDT, 2005). This is in agreement with Chiodini *et al*. (2007) who reported that exposure of RDT kit to high temperatures could result in its poor performance in the tropics. Denaturation of antibodies in the test membrane can impair binding to antigen at high temperature. Heat can also cause damage to the nitrocellulose membrane, thus changing its flow characteristics or causing the antibody to detach from the membrane (Chiodini *et al*., 2007).

This study found higher prevalence of malaria among study participants in General Hospital Kafanchan. This is in agreement with findings of Bajoga *et al*., (2019) which reported higher malaria prevalence in Local Government Areas found in Kaduna South. This may be because majority of the Local Government Areas in Kaduna south senatorial district usually had high rainfall than the other senatorial districts as reported by Yunusa *et al*. (2017).

High rainfall has been associated with increase in the number of breeding sites of *Anopheles* mosquito, which is the major culprit in malaria transmission. In many places, malaria transmission is seasonal, with the peak during and just after the rainy season (WHO, 2019). According to Confalonieri *et al*. (2007) periods of unusually high rainfall, altered humidity or warmer temperatures can result in modified distribution and duration of malaria, as well as increased transmission; even in areas where control is strong. Small *et al.* (2003) reported that precipitation and temperature are key drivers of malaria case variations across Africa, while acknowledging the complexities of some climatic factors.

The prevalence of malaria in the present study was found to be higher in males than females; the odds ratio value showed that the association was significant at 95 % confidence interval. This agrees with the studies of Nyarko and Cobblah (2014) and

Nmadu *et al*. (2015) who reported higher malaria prevalence in males than females. This finding however, is in contrast to that of Otajevwo (2013) and Jenkins *et al*. (2015) who reported a higher infection rate in females than males.

Females had lower prevalence of malaria in this study than males. The high prevalence in males may be due to the fact that men are less likely to sleep under the insecticide treated bed nets than females (WHO, 2010a). According to Olapeju *et al*. (2018) ITN use tends to be higher among females than males especially in households without sufficient ITNs. In some societies, men have a greater occupational risk of contracting malaria than women if they work in mines, fields or forests at peak biting times. Leisure activities and sleeping arrangements may also be contributing factors, as men are more likely to sleep outdoors or be found outdoors during the active biting hours of *Anopheles* mosquito (WHO, 2007; Monroe *et al*., 2019). This can increase the human-vector contact, and consequently lead to *Plasmodium* infection (WHO, 2007).

Other studies in Africa have shown no association between gender and malaria risk (Ghebreyesus *et al*., 2000; Brooker *et al*., 2004) and risk fluctuating between sexes across a number of seasons (Giha *et al*., 2000).

In Nigeria, malaria accounts for 60% of outpatient visits and 30% of hospitalizations are among children under five years of age. Malaria has the greatest prevalence, close to 50%, in children aged 6-59 months in the South West, North Central, and North West regions (Nigeria Malaria Fact Sheet, 2011).

This study showed higher malaria prevalence in children less than 10 years old. This is in agreement with the findings of Geleta and Ketema (2016) who reported that the incidence of *Plasmodium falciparum* positive cases was higher among the children age groups

(mostly <10 years).This also agrees with the findings of Valerian *et al.* (2013) which reported a high prevalence of malaria parasitaemia and anaemia among inpatient children less than five years. A similar study in Western Kenya reported a malaria prevalence of 82.5% in hospitalized children (Obonyo, *et al*., 2007). According to a study by Williams *et al*. (2005c), immune protection from malaria increased with age from only 20% in the first 2 years of life to a maximum of 56% by the age of 10 years, returning thereafter to 30% in participants greater than 10 years old.

The high prevalence in the age group ≤10 years is probably because the age-range 0-5 years falls in this group, and many studies including the World Health Organization have reported an increased malaria prevalence in the age-group 0-5 years (Askling *et al.*, 2005; Metanat, 2015; UNICEF, 2018; WHO, 2018a).It may also be because majority of the children in the group have not yet developed partial immunity to malaria and are thus susceptible (CDC, 2019).

This study revealed increasing prevalence of malaria with increase in the number of children per family. Even though the association was not statistically significant, it was observed that families with high number of children had higher prevalence of malaria. The least prevalence was found among families with ≤5 children. This increase in malaria prevalence with increase in the number of children may be as a result of malnutrition, congestion, inadequate housing, and inability to provide insecticide treated bed nets and adequate health care to all members of the family, due to the financial burden associated with having too many children in a family. This is in consonance with the study of Nyarko and Cobblah (2014) in Ghana, who reported high malaria prevalence among children whose parents were poor.

Housing is an important determinant of health and quality of life (Morakinyo *et al*., 2018). The design of a house could posibly contribute to the incidence of *Plasmodium falciparum* infection (Konradsen *et al*., 2003; Lindsay *et al*., 2003). Researchers have reported that living in an improved housing played a significant role in the eradication of malaria in the United States and Europe (Morakinyo *et al*., 2018). It played a vital role in controlling human exposure to mosquitoes. Pinder *et al*. (2016) also reported lower odds of malaria among occupants of improved house.

The higher malaria prevalence in compound houses (with shared facilities) than self-contained houses found in this study may be because shared facilities such as tap water, wells, toilets and bathrooms may not always be cared for, or used properly, due to the large number of people using them, it is therefore possible to find stagnant water around those areas which could serve as breeding sites for mosquitoes and consequently lead to more exposure of humans to mosquitoes and *Plasmodium* infection. Nyirakanani *et al*. (2018) reported high malaria prevalence in occupants of houses that have mosquito breeding sites near them. According to Morakinyo *et al*. (2018) persons living in improved housing have limited mosquito breeding sites. They reported that persons living in partially improved and non-improved housing consistently were more likely to have *Plasmodium* infection compared with those living in improved housing.

Stephenson *et al.* (2006) reported reduced healthcare seeking behaviours among those in polygamous homes. According to Arthi and Fenske (2018) resources per capita are likely to be lower in polygamous households due to their large family size compared to monogamous households. The large size of polygamous households may impose financial constrains that could lead to inability to afford adequate malaria preventive measures such

as ITNs and insecticide sprays to all members of the family. All these may explain why this study found higher malaria prevalence among those in polygamous households than those in monogamous households. The association was not statistically significant.

There was a significant association ($p<0.05$) between the marital status of the participants in this study and malaria prevalence. The highest prevalence was recorded among those who were married, followed by singles and divorced participants. This is similar to the findings of Nyirakanani *et al*. (2018) which reported higher malaria prevalence in those who were married, than in those who were divorced. This finding is however in contrast to that of Nyarko and Cobblah (2014) which revealed high prevalence of malaria in divorced participants, followed by married participants and those who were never married (singles). The high prevalence among the married reported in this study may be because there is high tendency for married persons to give their ITNs to their children or younger siblings.

The study of Dike *et al*. (2006) in Southeastern Nigeria reported that a higher level of education was associated with improved knowledge and practices in relation to appropriate prevention and treatment strategies for prevention and treatment of malaria. In this study, there was lower malaria prevalence among those who had primary, secondary, tertiary, adult literacy and non-formal forms of education compared to those who had no education at all. This is in agreement with the report of Shayo *et al*. (2015) who reported higher malaria prevalence among non-educated respondents.

Those who had other types of occupations in the current study had the highest prevalence (36.4%) followed by traders. This means that they were involved in jobs that increased their exposure to mosquito bites than the occupations listed in this study. Unemployed

participants also recorded high prevalence of malaria compared to civil servants. This may be due to their inability to afford health care, insecticide treated bed nets, insecticide sprays and other forms of preventive measures for malaria. This agrees with the study of Yusuf *et al*. (2010) which reported that poor households experience more malaria due to inadequate treatment and lack of access to preventive measures.

Pregnant women are particularly vulnerable to malaria as pregnancy reduces a woman's immunity to malaria, increasing their risk of illness, severe anaemia, and death, while the risk of spontaneous abortion, stillbirth, premature delivery, and low birth weight increases for the foetus (Steketee *et al.,* 2001; WHO, 2007). The use of microscopy in diagnosis of malaria infection in adult population that comprises pregnant women can pose some challenges due to placental sequestration of parasites and thus reduce the sensitivity of microscopy (Oyeyemi *et al*., 2015).

Preventing malaria in pregnant women through intermittent preventive treatment (IPTp) with sulfadoxine-pyrimethamine, which is administered during antenatal care visits, is an effective way of reducing maternal anaemia and low birth weight. Nearly every country in sub-Saharan Africa with a high malaria burden has adopted intermittent preventive treatment for pregnant women as part of its national malaria control strategy (UNICEF, 2018). The World Health Organisation recommends Intermittent Preventive Treatment against malaria for pregnant women. The current recommendation is to give at least two doses of a safe and effective antimalarial (currently, sulphadoxine-pyrimethamine) to all pregnant women living in malaria endemic areas (WHO, 2010b). The regular use of ITNs by pregnant women as well as intermittent preventive treatment during pregnancy are vital interventions in the prevention of malaria among pregnant women (UNICEF, 2018).

In this study, pregnant women had a lower prevalence (20.0%) than non-pregnant women (24.0 %). The low prevalence observed among pregnant women in this study may be because, majority of the pregnant women enrolled in the study were undergoing ante-natal care, and thus, they were taking antimalarial drugs as prophylaxis.

The presence of bushes/ditches at home was not significantly associated with malaria in this study, even though those who had bushes/ditches around their houses had a lower prevalence (23.3%) than those without bushes/ditches around their houses (25.0%). This is in contrast to the findings of Nkuo-Akenji *et al*. (2006) who reported that malaria prevalence and parasite density was higher in children living in houses surrounded by bushes/garbage and swamps/stagnant pools of water when compared with those inhabiting cleaner environments. The association in this study was not statistically significant.

The current study revealed decreased malaria prevalence among participants using ITN at home, compared to those who were not. This suggests that the use of insecticidal bed nets helped to decrease the prevalence of malaria. The protective effect of insecticide-treated net use as demonstrated in this study adds to the vast body of evidence supporting the effectiveness of insecticide-treated bed nets for protection against malaria and other vector-borne diseases in this setting (Lengeler, 2004; Winskill *et al*., 2011). A study by Yusuf *et al*. (2010) in Nigeria found that those households which reported having and using bed nets had less malaria cases among children than those without mosquito bed nets. Long Lasting Insecticidal Nets (LLINs) have been successful in reducing malaria incidences by either reducing or not allowing human exposure to the vector mosquitoes (Sunil and Vijay, 2014). According to the report of Syed *et al.* (2011), there has been a substantial improvement in possession and usage of insecticidal bed nets especially for the two most

vulnerable groups (under-five children and pregnant women). Indeed sleeping under insecticide-treated nets (ITNs) on a regular basis is one of the most effective ways to prevent malaria transmission and reduce malaria related deaths (UNICEF, 2018).

This study revealed high malaria prevalence in participants who were not using insecticide spray (Indoor Residual Spraying) at home to kill mosquitoes compared to those who were using insecticide spray. That means there was a reduction in malaria prevalence among those who were using insecticides to control mosquitoes at home. The World Health Organisation (WHO, 2010a) reported that the number of people protected as a result of insecticide usage increased from 13 million in 2005 to 75 million in 2009. A number of field studies have also reported the effectiveness of Indoor Residual Spraying (IRS) in reducing malaria prevalence, but it is difficult to generalize from any single study how effective IRS is at reducing malaria prevalence because various studies have shown conflicting results (Ranson *et al*., 2009; Kim *et al.*, 2012). Aspects of geography, entomology, human behavior, and community acceptance of the IRS program could contribute to why IRS is more successful in one community than in another. In 2009, IRS conferred protection from malaria to 75 million persons, or 10% of the population of Africa (WHO, 2010a), and contributed to the decrease in this disease. Several other studies have also reported similar finding (WHO, 20010b; Kim *et al.*, 2012; Hamusse *et al.*, 2012; Tukei *et al*., 2017).

Haemoglobin genotype is known to influence the prevalence of malaria in endemic areas (Akanbi, 2015). The results of this study revealed that 73% of the malaria positive participants had the haemoglobin genotype AA (HbAA), which was higher than the percentages in the other genotypes (HbAS [23%]) and HbAC [3%]). This is similar to the

reports of Albiti and Nsiah (2014) and Akanbi (2015). The prevalence of participants with HbAS and HbAC were quite low compared to HbAA. This suggests that individuals with HbAS and HbAC are probably able to resist malaria better than HbAA. This is in agreement with the findings of Aidoo *et al*. (2002), Ayi *et al.* (2004), Williams *et al*. (2005c), Albiti and Nsiah (2014) and Archer *et al*. (2018). In another study by Williams *et al*. (2005d), HbAS was 50% protective against mild clinical malaria, 75% protective against admission to the hospital for malaria, and almost 90% protective against severe or complicated malaria. The level of susceptibility to malaria has been reported to be higher in individuals with HbAA when compared with those with HbAS and HbAC, thus, the high frequency of HbAC and HbAS in malaria endemic areas has been attributed to a decrease in malaria morbidity and mortality (Uneke *et al*., 2007; Archer *et al*., 2018). The protective role displayed by HbAC and HbAS in malaria infection is as a result of reduced cytoadhesion of infected red blood cell to microvasculature and impaired rosette formation as a result of the presence of abnormal PfEMP1 antigen on HbAC and HbCC (Fairhurst *et al*., 2012).

Earlier studies (Luzzatto *et al*., 1970; Friedman *et al* 1979) had associated low oxygen tension in HbAS RBCs with impairment in the invasion and growth of *Plasmodium falciparum* parasites in the HbAS RBCs which causes infected RBCs to sickle under low oxygen tension and lead to their premature destruction in the spleen, thus, reducing parasitaemia and providing protection. Archer *et al*. (2018) reported that resistance to *Plasmodium falciparum* in sickle cell erythrocytes is driven by oxygen dependent-growth inhibition. Their experiments showed that low oxygen (1% oxygen concentration) indeed

stalled the growth of *Plasmodium falciparum* and no DNA replication was evident at that oxygen concentration.

Rosette formation (binding of *Plasmodium falciparum* infected RBCs to each other) which is thought to lead to microcirculatory obstruction in cerebral malaria was found to be impaired in *P. falciparum* infected HbAS RBCs under deoxygenated conditions; this may be due to increased sickling of the RBCs in deoxygenated condition or reduced expression of erythrocyte surface adherence protein (Cholera *et al*., 2008). Decreased rosette formation and the resulting decreased circulatory obstruction might contribute to protection against severe malaria observed in HbAS individuals.

The protective effect of HbC may result from a reduced ability of *Plasmodium falciparum* to grow and multiply in RBCs containing HbC (Fairhaust *et al*., 2005). HbC exerts its protection through a specific effect on cytoadherence, mediated by the altered display of surface expressed parasite proteins (Brittain *et al.*, 2007; Cyrklaff *et al*., 2011).

Subjects with HbSS appear to be less susceptible than normal Hb (HbAA) to developing malaria but are highly susceptible to the catastrophic consequences of malaria particularly severe anaemia if they do become infected with the parasite (McAuley *et al*., 2010; Williams and Obara, 2011).

The primers used in this study were designed by Zhang *et al*. (2008) to amplify regions containing single nucleotide polymorphisms (SNPs) covering genetic markers reported to be associated with the resistance of *Plasmodium falciparum* to some of the most commonly used antimalarial drugs such as chloroquine, mefloquine, amodiaquine, sulfadoxine-pyrimetamine and artemether. *Pfcrt, pfmdr1, pfdhfr, pfdhps* genes were

detected at expected amplicon sizes from the malaria positive samples in this study. *Pfcrt* (80%) had the highest prevalence, followed by *pfdhfr* (60%), *pfmdr1* (36%) and *pfdhps* (8%). The presence of these genes, if expressed, will pose a significant danger to the management and control of malaria (WHO, 2018b). This is because *Plasmodium* parasites carrying drug resistance genes have been shown to cause treatment failure which by extention leads to increased malaria prevalence.

The wide spread of chloroquine-resistant parasites prompted the WHO to recommend Artemisinin-based Combination Therapy for the treatment of malaria in endemic regions. According to Mayengue *et al*. (2005) and Ndounga *et al*. (2005) the first report describing the prevalence of polymorphisms in *pfcrt* conferring chloroquine resistance showed that all the *Plasmodium falciparum* isolates were carrying the *pfcrt* alleles.

The prevalence of *pfmdr1* (36%) gene was also slightly high in this study. The current study successfully amplified segments of *pfcrt* as well as *pfmdr1* bearing known mutations associated with chloroquine resistance. Twenty four percent of the parasites had *Pfcrt*/ *Pfmdr1* mutant genes together.The high prevalence of the *pfcrt* shows that chloroquine resistance is still very likely in the studied population despite its withdrawal as first line drug for the treatment of uncomplicated malaria in Nigeria in 2005 (Ogundipe, 2012; Olawande, 2017). In their report, Sidhu *et al.* (2002) provided conclusive evidence that *pfcrt* mutations increased susceptibility to artemisinin and quinine, and minimally affected amodiaquine activity.

In this study, *pfdhfr* (60%) had a higher prevalence than *pfdhps* (8%) which suggests that there are more *Plasmodium falciparum* parasites bearing the *pfdhfr* gene in the study population than those bearing the *pfdhps* genes. Mutations in the *pfdhfr* and *pfdhps* genes

have been associated with resistance to pyrimethamine and sulfadoxine respectively (Lo *et al*., 2013). In Nigeria, sulfadoxine is given in combination with pyrimethamine as sulfadoxine-pyrimethamine (SP) to pregnant women during intermittent preventive treatment (IPT). The high prevalence of *pfdhfr* genes may indicate possible resistance to pyrimethamine and consequently reduce the efficacy of SP as a combination therapy.

The possession of more than one drug resistance gene in some of the *Plasmodium falciparum* parasites in this study may increase their resistance to different antimalarials, and probably lead to high rate of treatment failure with these drugs.

In this study, only the portion of *pfatpase6* gene carrying the 2306G/A SNP at codon S769N was detected. Maslachah *et al.* (2017) reported in their study that all the resistant *Plasmodium falciparum* isolates contained *pfatpase6* S769N mutation which is associated with artemisinin resistance. A similar finding was also reported by Jambou *et al.* (2005) and Jung *et al*. (2005).

The *pfatpase6* gene sequences in this study were confirmed by carrying out nucleotide blast search on National Centre for Bioinformatics Information (NCBI) database. The query cover, expected value (E value) and percent identity confirmed our sequences to be those of *pfatpase6.* All the *pfatpase6* nucleotide sequences had query cover of over 90 % and E values of 0.0 except one with 82% query cover and 3e-158 E value. Majority of the sequences had high percentage identity with sequences in the Genbank, thus confirming our sequences to be for *pfatpase6*. The sequence alignment and phylogenetic tree revealed the similarities and relatedness between *pfatpase6* sequences in this study and the reference sequences in Ghana, Iran, Greater Mekong Subregion (Vietnam) and Bangladesh. The phylogenetic tree showed that all the *pfatpase6* gene sequences (both the ones from this

study and those from NCBI) had the same origin and were closely related. However, the sequences from NCBI were from one clade; arising from a common ancestor (monophyletic) thus they were more closely related than to the *pfatpase6* sequences from this study.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The prevalence of malaria reported in this study, as obtained by microscopy and RDT were 23.7% and 21.7% respectively.

The age and marital status of participants were the only demographic factors significantly associated with malaria in this study. The use of insecticide spray at home helps to reduce malaria prevalence.

The high frequency of HbAA genotype in malaria positive participants suggests that people having the aforementioned haemoglobin type are less protected from malaria than those having HbAS, HbAC and HbSS.

The study demonstrated the presence of *pfcrt, pfmdr1, pfdhfr, pfdhps and pfatpase6* genes, which are associated with resistance.

The *pfatpase* sequences in this study were related to published *pfatpase6* sequences in the GenBank.

6.2 Recommendations

Based on the results of this study, it is recommended that:

1. Malaria RDT should not be used exclusively for the laboratory diagnosis of malaria. Microscopy alongside malaria RDT should be used for malaria diagnosis. Malaria RDT alone should only be used where microscopy is not possible.
2. Special attention shoud be given to children ≤ 10 years to protect them against malaria.

3. The use of ITN and insecticide residual spray should be emphasized for malaria control.
4. Due to the presence of resistance genes to chloroquine, SP and artemisinin, the use of antialarial drugs should be controlled to prevent the spread of resistance genes.
5. Further research should be carried out to determine the direct effect of expressing *P. falciparum* genes carrying SNPs on antimalarial drug resistance.

6.3 Contributions to knowledge

1. An overall malaria prevalence of 23.7% was obtained with microscopy and 21.7% prevalence was obtained with RDT.
2. The age and marital status of participants were the only demographic factors significantly associated with *Plasmodium falciparum* malaria in this study.
3. The use of insecticide spray at home was significantly associated with reduced malaria prevalence.
4. The highest percentage of malaria (73%) was found among participants with haemoglobin genotype AA (HbAA).
5. The drug resistance genetic markers; *pfcrt* (80%), *pfmdr1* (36%), *pfdhfr* (60), *pfdhps* (8%) and *pfatpase6* (73.3%) were detected among the *Plasmodium falciparum* parasites in the study area. Chloroquine and SP are very likely to be under threat of resistance.

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APPENDICES

APPENDIX I Ethical Approval from Kaduna State Ministry of Health

MINISTRY OF HEALTH AND HUMAN SERVICES
KADUNA STATE, NIGERIA

MOH/ADM/744/VOL.1/484 23RD APRIL, 2018

NOTICE OF EXPEDITED REVIEW AND APPROVAL

MOLECULAR DETECTION OF DRUG RESISTANT PLASMODIUM FALCIPARUM IN SOME PARTS OF KADUNA STATE, NIGERIA

Name of Principal Investigator :	BENJAMIN GIDEON YAKUSAK
Address of Ethical Approval :	DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA
Date of receipt of Application :	26TH JANUARY, 2018
Date of Ethical Approval :	20TH APRIL, 2018

This is to inform you that the Research described in the submitted Protocol, the Consent Forms, advertisements and other participant information materials have been reviewed and given full approval by the the Health Research Ethics committee (HREC).

If there is delay in starting the research or any change, inform the HREC so that the dates of approval can be adjusted accordingly.

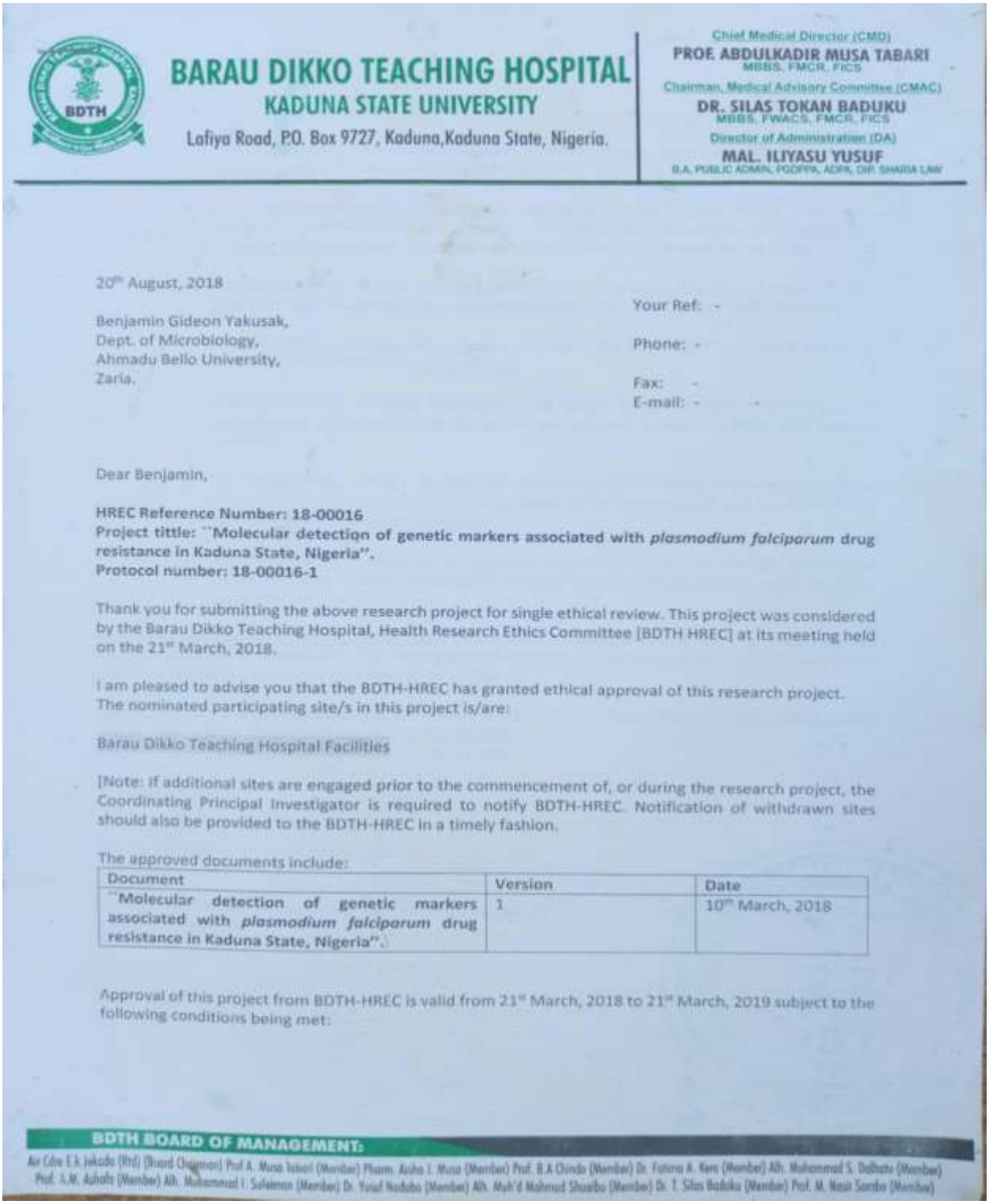
However, Researcher is kindly requested to submit a copy of his/her findings to the State Ministry of Health, please.

Dr. BUTAWA NN
Chairman

SMOH
24/04/18

Independence Way, P.M.B 2014, Kaduna, Kaduna State-Nigeria
Tel: +234 (0)8184078093 Website: www.khtg.gov.ng Email: health@khtg.gov.ng

APPENDIX II Ethical Approval from Barau Dikko Teaching Hospital Kaduna



BARAU DIKKO TEACHING HOSPITAL
KADUNA STATE UNIVERSITY
Lafiya Road, P.O. Box 9727, Kaduna, Kaduna State, Nigeria.

Chief Medical Director (CMD)
PROF. ABDULKADIR MUSA TABARI
MBBS, FMCR, FICS
Chairman, Medical Advisory Committee (CMAC)
DR. SILAS TOKAN BADUKU
MBBS, FWACS, FMCR, FICS
Director of Administration (DA)
MAL. ILIYASU YUSUF
B.A. PUBLIC ADMIN, PGDPPA, ADPA, DIP. SHARIA LAW

20th August, 2018

Benjamin Gideon Yakusak,
Dept. of Microbiology,
Ahmadu Bello University,
Zaria.

Your Ref: -
Phone: -
Fax: -
E-mail: -

Dear Benjamin,

HREC Reference Number: 18-00016
Project tittle: ``Molecular detection of genetic markers associated with *plasmodium falciparum* drug resistance in Kaduna State, Nigeria''.
Protocol number: 18-00016-1

Thank you for submitting the above research project for single ethical review. This project was considered by the Barau Dikko Teaching Hospital, Health Research Ethics Committee [BDTH HREC] at its meeting held on the 21st March, 2018.

I am pleased to advise you that the BDTH-HREC has granted ethical approval of this research project. The nominated participating site/s in this project is/are:

Barau Dikko Teaching Hospital Facilities

[Note: If additional sites are engaged prior to the commencement of, or during the research project, the Coordinating Principal Investigator is required to notify BDTH-HREC. Notification of withdrawn sites should also be provided to the BDTH-HREC in a timely fashion.

The approved documents include:

Document	Version	Date
``Molecular detection of genetic markers associated with *plasmodium falciparum* drug resistance in Kaduna State, Nigeria''.	1	10th March, 2018

Approval of this project from BDTH-HREC is valid from 21st March, 2018 to 21st March, 2019 subject to the following conditions being met:

BDTH BOARD OF MANAGEMENT:
Air Cdre E.K. Jakada (Rtd) (Board Chairman) Prof A. Musa Yabori (Member) Pharm. Aisha I. Musa (Member) Prof. B.A Chinda (Member) Dr. Fatima A. Kere (Member) Alh. Muhammad S. Dalhatu (Member)
Prof. A.M. Ashafa (Member) Alh. Muhammad I. Suleiman (Member) Dr. Yusuf Nadabo (Member) Alh. Muh'd Mahmud Shuaibu (Member) Dr. T. Silas Baduku (Member) Prof. M. Nasir Sambo (Member)

APPENDIX III Result of Haemoglobin Electrophoresis Shown as Percentages in Figure 4.2.

Haemoglobin Genotype	Number Positive	Percentage
HbAA	52	73
HbAS	16	23
HbAC	2	3
HbSS	1	1
Total	71	100

APPENDIX IV Structured Questionnaire

DEPARTMENT OF MICROBIOLOGY, FACULTY OF SCIENCE

AHMADU BELLO UNIVERSITY, ZARIA

STRUCTURED QUESTIONNAIRE

RESEARCH TOPIC: MOLECULAR DETECTION OF GENETIC MARKERS ASSOCIATED WITH *PLASMODIUM FALCIPARUM* DRUG RESISTANCE AMONG PATIENTS ATTENDING SOME HOSPITALS IN PARTS OF KADUNA STATE, NIGERIA

Please answer the questions sincerely; information provided here is confidential. Thank you.

Hospital Name……. Reference No: ……..

BIODATA AND DEMOGRAPHIC DATA

1) Sex: Male ( ) Female ( )

2) Age: ( )

3) Marital Status: Married ( ) Single ( ) Divorced ( )

4) Type of family: Monogamous ( ) Polygamous ( )

5) Number of children per family: ( )

6) Place of residence: Self-contained ( ) Compound house ( )

7) Level of Education: Primary ( ) Secondary ( ) Tertiary ( ) Adult literacy ( )

Non ( ) Non-formal ( )

8) Occupation: Civil servant ( ) Trader ( ) Artisan ( ) Farmer ( ) Unemployed ( ) Others ( )

9) Are you pregnant? Yes ( ) No ( )

10) Do you sleep under insecticide treated bed net? Yes ( ) No ( )

11) Do you use insecticide spray at home? Yes ( ) No ( )

12) Are there bushes/gutters around your house? Yes ( ) No ( )

APPENDIX V Formula for the Calculation of Sensitivity and Specificity

$$\text{SENSITIVITY} = \frac{\text{True positive}}{\text{Total with disease}} = \frac{\text{True positive}}{\text{true positive} + \text{false negative}} \text{ X } 100$$

$$\text{SENSITIVITY} = \frac{65}{65+6} \text{ X } 100 = 92\%$$

$$\text{SPECIFICITY} = \frac{\text{True negative}}{\text{Total without disease}} = \frac{\text{True negative}}{\text{true negative} + \text{false positive}} \text{ X } 100$$

$$\text{SPECIFICITY} = \frac{229}{229+0} \text{ X } 100 = 100\%$$

Appendix VI Extract of Multiple Sequence Alignment of *pfatpase6* Sequences