ANTIMALARIAL SUSCEPTIBILITY AND DRUG RESISTANCE CHARACTERIZATION STUDIES OF *PLASMODIUM* SPECIES ISOLATES FROM PATIENTS IN SABON-WUSE, NIGER STATE, NIGERIA

BY

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DECLARATION

I declare that the work in the dissertation entitled "Antimalarial Susceptibility and Drug Resistance Characterization Studies of *Plasmodium* species Isolates from Patients in Sabon-Wuse, Niger State, Nigeria" has been performed by me in the Department of Pharmaceutics and Pharmaceutical Microbiology under the supervision of Professor J. O. Ehinmidu and Professor (Mrs.) A. R. Oyi. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

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_____	_____	_____
Name of Student	Signature	Date

CERTIFICATION

This dissertation entitled "Antimalarial Susceptibility and Drug Resistance Characterization Studies of *Plasmodium* species Isolates from Patients in Sabon-Wuse, Niger State, Nigeria" meets the regulations governing the award of Master of Science (Pharmaceutical Microbiology) of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary contribution.

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DEDICATION

This dissertation is dedicated to Almighty God, the Most High and everlasting one.

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ABSTRACT

About half of Nigerian population experience at least one episode of malaria per year, resulting in high morbidity and mortality and loss of economic value. Retrospective study of the profile of malarial patients and the antimalarial drugs prescribed at Umaru Musa Yaradua memorial Hospital, Sabon-Wuse, Niger State, Nigeria were undertaken. The *Plasmodium* species among the malarial patients were also studied. The susceptibility of the predominant species to the prescribed antimalarial drugs was investigated. PCR analysis of the DNA of the resistant *Plasmodiuum* species isolates against the test antimalarials were studied using Qiagen DNeasy blood and tissue kit method. The retrospective study showed that Artemisinin Combination Therapy (ACT) was the predominantly prescribed drugs while the month of May and October had the highest incidence of malarial infection in the years studied. *Plasmodium falciparum* (85.7%) was the predominant malaria parasite isolated from malaria patients in Sabon-Wuse, Niger State, Nigeria. *Plasmodium malariae* accounts for only 14.5% of the malaria parasite observed in the area. Only 5% of the *P. falciparum* parasites were sensitive *in vitro* to Chloroquine. PCR analysis of the DNA indicated the presence of *Pfcrt-Ra* Gene in the study area, which further confirms resistance to Chloroquine. *Plasmodium falciparum* isolates were observed to be sensitive *in vitro* to Artesunate-Amodiaquine drug in the study area. The investigation also shows that all the samples analysed had multi antimalarial drug resistant gene *Pfcrt*/FB (76T). A high percentage (87.5%) of the samples analysed displayed the multidrug resistant gene *Pfcrt*/FB (76K) at 76 bp. The result of the PCR analysis of the DNA corroborates the observed *in vitro* susceptibility studies. Thus, there is the need for periodic antimalarial surveillance in order to curb emergence of multi antimalarial drug resistance as observed in Sabon-Wuse.

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ABBREVIATIONS

ACT	Artemesinin Combination Therapy
CDC	Centre for Disease Control and Prevention
IC	Inhibitory concentration
MDGs	Millennium Development Goals
NEEDS	National Economic Empowerment and Development Strategy
PPL	Peak plasma level
P. falciparum	*Plasmodium falciparum*
*Pf*ATPase	*Plasmodium falciparum* Adenosine Triphosphatase
Pfcrt	*Plasmodium falciparum* Chloroquine resistant Gene
Pfmdr1	*Plasmodium falciparum* multidrug resistant Gene
P. knowlesi	*Plasmodium knowlesi*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. reichenowi	*Plasmodium reichemnwi*
P. vivax	*Plasmodium vivax*
PCR	Poymerase Chain Reaction
RDT	Rapid diagnostic test
RFLP	Refraction Fragment Length Polymorphism
SERCA	Sacro-endoplasmic reticulum calcium dependent ATPase
UMYMH	Umaru Musa Yaradua Memorial Hospital
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Malaria is a disease caused by the parasite called *Plasmodium* species that is transmitted from human-to-human by the female *Anopheles* mosquito. Malaria is a preventable and treatable disease. If malaria is diagnosed and treated early, the duration of the infection can be considerably shortened, which in turn reduces the risk of complications and death. The word “malaria” comes from 18th century Italian *mal* meaning "bad" and *aria* meaning "air". Most likely, the term was first used by Dr. Francisco Torti in Italy, when people thought the disease was caused by foul air in marshy areas (Opeskin, 2009).

Malaria remains a huge burden for human populations living in tropical areas. More than two million malaria cases globally were recorded in 2010, with the heaviest mortality rates in children living in sub-Saharan Africa (WHO, 2011). During the past decade, there was an increase in malaria control interventions and this has resulted in considerable reductions in morbidity and mortality associated with malaria in parts of Africa (O’Meara *et al*., 2010, Steketee and Campbell, 2010). The WHO recommended malaria control measures include long-lasting insecticidal nets (LLIN), indoor residual spraying programmes (IRS) and access to artemisinin combination therapy (WHO, 2010). Despite these efforts, malaria continues to pose a major public health threat in many African countries (WHO, 2011).

Currently, malaria can be caused by five *Plasmodium* species which include *Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax* and, more recently, *Plasmodium knowlesi* (Singh *et al*., 2004). *Plasmodium falciparum* is the most prevalent in `Africa and the most pathogenic of these, but in most malaria endemic regions multiple

sympatric species are found and co-infection within individual human hosts or the mosquito vector population is common. In Africa, *P. malariae* is the species most frequently found in sympatric with *P. falciparum* (Collins and Jeffery, 2007). *Plasmodium knowlesi* is found in southeast Asia (Naing *et al.*, 2011).

Early symptoms of malaria includes; chills, sweats, fever, muscle pain, nausea, and vomiting which may appear 7 to 30 days after initial contraction of the parasite. The incubation period varies depending on the strain of the parasite. Since these symptoms resemble the flu, malaria can be difficult to diagnose in places where it is not endemic, such as the United States (Mali, *et al*., 2012). With severe malaria, caused by *P. falciparum*, victims begin to experience more intense symptoms including neurological focal signs, anaemia, confusion, dypsnea, and can result in coma and death if untreated especially in children under the age of five years and pregnant women (Phillips, 2001). Severe organ damage may also occur and infected organs include the brain, liver, spleen and kidney. Chronic malaria, where disease relapse occur months or years after exposure, is occasionally associated with *P. vivax or P. ovale* infection due to latent parasites remaining in the liver. Again, these symptoms are shared with many other diseases (Mali *et al*., 2012). Consequently, the only way to diagnose malaria is to conduct laboratory tests. For definitive diagnosis, a drop of the patient's blood is stained (usually with Giemsa stain) and examined under a microscope for the presence of parasites. Diagnostic kits using a dye-labeled antibody that binds to the parasite are also available and are especially useful in the field and for rapid diagnoses (CDC, 2014). These tests are becoming more important in the diagnosis of malaria. They do not require a microscope or a skilled technician. In Africa and other countries without adequate medical infrastructure, these tests are an essential tool in detecting the parasite.

Health and socio-economic problems caused by malaria are enormous. The development and spread of malaria parasite resistance to drugs have led to global recognition and commitment to public health problems of malaria. Areas with concentrated populations of people with low socioeconomic status typically do not have resources for effective prevention and subsequent treatment. For example, a study conducted by Gwatkin and Guillot showed "58% of malaria deaths occurred in the poorest 20% of the world's population, a higher percentage than any other disease of major public health importance" (Worrall *et al*., 2005). Public health interventions intended to reach areas with people of low socioeconomic status may not be reaching their target population. Barat *et al.* (2004) noted that in developing countries, the poor often live in the most remote areas and are socially or culturally marginalized. Consequently, while programs in support of malaria prevention and treatment are sometimes available, they are not reaching the people who need them the most.

Resistance to antimalarial drugs is often manifested in subtle ways which are only apparent when the treatment is accompanied by detailed follow-up. Accurate and effective surveillance systems for monitoring antimalarial drug efficacy have been recognized as an essential basis for decisions on the use of drugs (WHO, 2003). Surveillance is carried out at different levels of sophistication: from basic drug efficacy tests through to more detailed *in vitro* characterization of drug resistant strains of parasite and clinical information. The World Health Organisation (WHO) has recognized that malaria treatment failure rate of 5% to 14% signals an alert phase in malaria monitoring while failure rate of 15% to 24% signals the need for action (WHO, 2002). In the past fifty years, extensive development of these anti-malarial drugs has provided a tremendous selection pressure on human malaria parasite which has enabled the parasite to evolve mechanisms of resistance. The emergence of resistance, particularly in *P. falciparum* has been a major contributor to the global resurgence of malaria

in the last three decades (Fevre *et al*., 1999; Baird, 2000). 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.

Measurement of drug resistance with malaria causative organisms (i.e. *Plasmodium* species) is complex. Studies of clinical and parasitological outcomes are the main sources of information on which national malaria control programme and treatment policy should be based. However, other studies such as the molecular studies and *in vitro* analysis are needed to confirm drug resistance. The aim of *in vitro* studies is to measure the intrinsic sensitivity of parasite to antimalarial drugs while molecular markers are used to identify genetic mutations related to antimalarial drug resistance in the parasite genome (WHO, 2010a). The pharmacokinetic study often characterizes antimalarial drug absorption, distribution, metabolism and elimination in the body. While each method makes a contribution to a more complete understanding of antimalarial drug resistance, therapeutic study remains the gold standard for guiding drug policy (WHO, 2010b).

1.2. Statement of Research problem

Drug resistance in malaria is an important public health concern. According to the latest estimates, there were about 198 million cases of malaria with an estimated 584,000 death globally (WHO, 2014). Most malaria caused deaths were reported to occur among children living in Africa where a child dies every minute from malaria. The Democratic Republic of the Congo and Nigeria account for over 40% of the estimated total malaria caused deaths globally (WHO, 2014). Amid such a disease burden, the development of resistance has significant influence on the control of malaria in affected countries. Hence there is need for

the study of changing patterns of drug resistance which will improve our current understanding of its development. The resistance pattern 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 this study area. Studies such as this will give current information on antimalarial susceptibility pattern of plasmodium species isolated in this locality.

1.3 Justification

Nigeria has more reported cases of malaria and deaths due to malaria than any other country in the world (CDC, 2012). Malaria drugs are working well in many parts of the world, however, there is serious concern that malaria parasites are once again developing widespread resistance to antimalarial drugs. Key drivers of antimalarial drug resistance

- Unusual genetic structure of malaria parasites in regions known for antimalarial drug resistance
- Counterfeit or substandard drugs
- Unregulated or poorly administered antimalarial drug use
- Artemisinin drug use without a complementary combination treatment, such as Artesunate.

Thus, 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. This study is focused on monitoring of drug resistance in Niger state and understanding the mechanism of drug-resistance.

The *in vitro* micro-test provides information on the quantitative drug response of *Plasmodium* parasites irrespective of the patient's immune status. It is an epidemiological tool for

assessing baseline sensitivity and for monitoring the drug response of plasmodium parasites over time and place and therefore can provide background information for the development and evaluation of drug policies. Changes in parasite drug sensitivity *in vitro* can be an indicator of future therapeutic failure. Unlike the *in vivo* tests, the results of an *in vitro* test are not disturbed by on-going malaria transmission.

1.4. Aim and Objectives

1.4.1 Aim of the Study

The aim of this study is to determine the susceptibility of *Plasmodium* species isolated from patients attending Umaru Musa Yaradua Memorial Hospital (UMYMH), Sabon-Wuse to the commonly prescribed antimalarial drugs.

1.4.2 Specific objectives

The specific objectives of the study were:-

a) To determine the prevalence of malaria in Umaru Musa Yaradua Memorial Hospital (UMYMH), Sabon-Wuse, Niger State, Nigeria.
b) To determine the antimalarial drugs commonly prescribed in the hospital.
c) To determine the type of *Plasmodium* species isolates among patients attending the hospital
d) To determine the susceptibility of *Plasmodium* species isolates to some antimalarial drugs commonly prescribed in Umaru Musa Yaradua Memorial Hospital (UMYMH), Sabon-Wuse, Niger State, Nigeria.
e) To characterize the multi-drug resistance factor present among the resistant *Plasmodium falciparum* isolates.

1.4.3 Research Hypothesis

Null Hypothesis

The *Plasmodium* species isolates obtained from patients attending Umaru Musa Yaradua Memorial Hospital (UMYMH), Sabon-Wuse, Niger State, Nigeria are not susceptible to the commonly prescribed antimalarial drugs in the hospital.

Alternate Hypothesis

The *Plasmodium* species isolates obtained from patients attending Umaru Musa Yaradua Memorial Hospital (UMYMH), Sabon-Wuse, Niger State, Nigeria are susceptible to the commonly prescribed antimalarial drugs in the hospital.

1.5 Limitation of Research

The study was carried out in the only Government Hospital in the town. Patients attending private hospital for malaria cases were not considered. The number of blood samples considered for *in vitro* micro-test was limited by the fire hazard that affected the Haematological Laboratory of the General Hospital, Minna where analysis of samples was conducted.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Outline

The history of malaria predates humanity, as this ancient disease evolved before humans did. Malaria, a widespread and potentially lethal infectious disease, has afflicted people for much of human history, and has affected settlement patterns (Carter *et al.*, 2002). The prevention and treatment of the disease have been investigated in science and medicine for hundreds of years, and, since the discovery of the parasite which causes it, attention has focused on its biology. These studies have continued up to the present day, since no effective Malaria vaccine has yet been developed and many of the older antimalarial drugs are losing effectiveness as the parasite evolves high levels of drug resistance. As malaria remains a major public health problem, causing 250 million cases of fever and approximately one million deaths annually, understanding its history is important.

Malaria is an ancient disease and references to what was almost certainly malaria occur in a Chinese document from about 2700 BC, clay tablets from Mesopotamia from 2000 BC, Egyptian papyri from 1570 BC and Hindu texts as far back as the sixth century BC. The early Greeks, including homer in about 850 BC, Empedocles of agrigentum in about 550 BC and Hippocrates in about 400 BC, were well aware of the characteristic poor health and enlarged spleens seen in people suffering from malaria disease living in marshy places. For over 2500 years the idea that malaria fevers were caused by miasmas rising from the swamps persisted. the word malaria comes from the Italian mal'aria meaning spoiled air, although this has been disputed. Scientific studies incriminated mosquitoes as malaria vectors, first for avian malaria by Ronald Ross in 1987 and then for human malaria by the Italian scientists Giovanni and co-workers between 1898 and 1900 (Francis, 2010).

Over a century later it seems appropriate to attribute the various discoveries concerning the malaria parasites and their transmission as follows. Laveran was the first person to find *Plasmodium* parasites in the blood of patients suffering from malaria in 1880. MacCullum was the first to observe and report the sexual stages of a malaria-like parasite. In 1898 Grassi, Bignami and Bastienelli were the first to demonstrate that human malaria parasites were actually transmitted by mosquitoes. Ross reported that a blood-sucking insect could not take up infective organisms from an infected individual but could also transmit them later when it fed on an uninfected host (Schalagenhauf, 2004).

2.2 The Malaria Parasites

2.2.1Taxonomy

The causative agents of malaria disease are protozoa of the genus Plasmodiidae, suborder haemosporidiidae, order Coccidia. There are over 100 species of *Plasmodium* and these are found in the blood of mammals, reptiles and birds. They are recognized taxonomically by the presence of the two types of asexual division: schizogony, in the vertebrate host; and sporogony, in the insect vector. The great majority of malarial parasites are transmitted by mosquitoes, and the parasites of humans are exclusively transmitted by anophelines. Importantly, the parasites of humans are of two subgenera, *Laverania* and *Plasmodium*. The subgenus *Plasmodium* includes *P. falciparum*, the most pathogenic causative agent of malaria, and the closely related *P. reichenowi*, a parasite of the higher primates such as chimpanzee (Robert and Herbert, 2003). The subgenus *Laverania* includes the remaining parasites of humans, namely *P. vivax, P. malariae* and *P. ovale*. Parasites of the other mammals also fall into subgenera: *Plasmodium* and *Vinckeia*; the latter includes the parasites of lemurs and the lower mammals (Robert and Herbert, 2003).

2.2.2 Life Cycle of Human Malaria Parasite

The life cycle of the malaria parasite (*Plasmodium*) is complicated and involves two hosts, humans and female *Anopheles* mosquitoes. The disease is transmitted to humans when an infected *Anopheles* mosquito bites a person and injects the sporozoites (the injective stage) into the blood. This is shown in Fig. 2.1, where the illustration shows a mosquito taking a blood meal.

The sporozoites infect liver cells **(2)** and mature into schizonts **(3)**, which rupture and release merozites **(4)** (of note, in *P. vivax* and *P. ovale* a dormant stage (hypnozotes) can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later). After this initial replication in the liver (exo-erythrocytic schizogony A), merozites infect red blood cells **(5)**. The ring stage trophozoites mature schizonts which rupture releasing merozites **(6)**. Some parasites differentiate into sexual erythrocytic stages (gametocytes) **(7)**. Blood stage parasites are responsible for clinical manifestations of the disease such as intermittent fever due to the synchronous lysis of the infected erythrocytes.

The gametocytes, male (microgametocytes) and female (macro gametocytes), are ingested by an *Anopheles* mosquito during a blood meal **(8).** The parasites' multiplication in the mosquito is known as the sporogonic cycle **(C)**. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes **(9)**. The zygotes in turn become motile and elongated (ookinetes) **(10)** which invade the midgut wall of the mosquito, which they develop into occysts **(11)**. The occysts grow, rupture, and release sporozoites **(12)**, which make their way to the mosquito salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle **(1).**

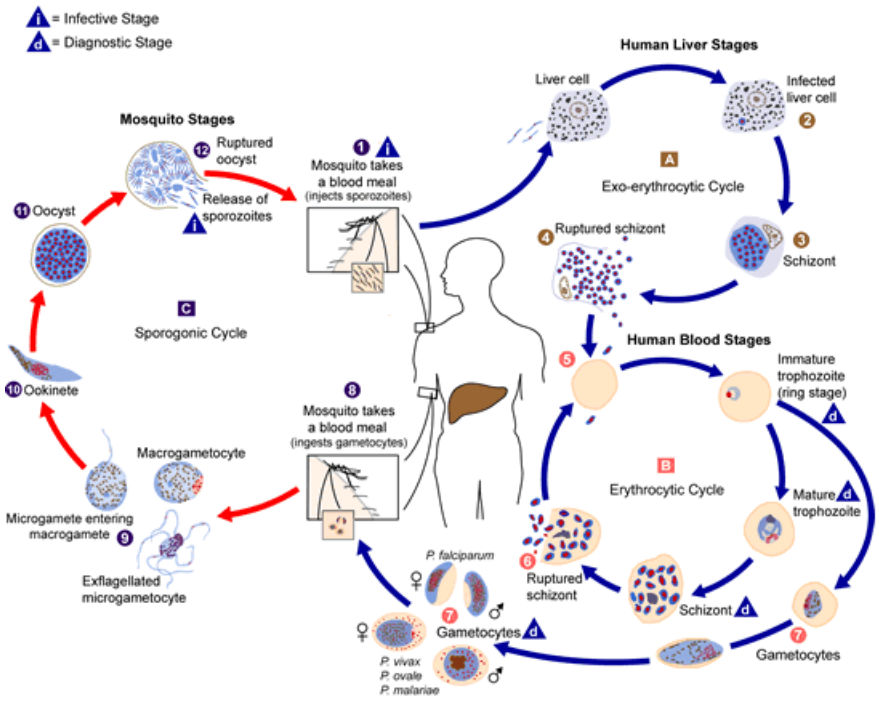


Fig. 2.1: Life cycles of malaria parasite, *Plasmodium* species (Source: CDC, 2012)

2.3 Malaria

2.3.1 Epidemiology

Malaria is one of the most important challenges to public health with about 300 to 500 million global cases reported annually. More than 1 million people have been reported to die from the malaria disease. Most of these are children under the age 5 years. Over 90.0% of the malaria cases and 75% of the deaths occur in sub-Saharan Africa (SSA). These childhood deaths, were reported to be due to cerebral malaria and anaemia which constitute 20% to 25% of child mortality in Africa (WHO, 2000; Teklehaimanot and Mejia, 2008).

Malaria generally occurs in areas where environmental conditions allow both the vectors and parasites multiply. Malaria today is commonly found in tropical and subtropical area s and altitudes below 1500 metres, although in the past, malaria was endemic in North America, Europe, northern Asia and Korean peninsula. However, these malaria distributions were observed to be affected by climatic changes and population movements. P. falciparum has been reported to be the predominant species in the world (Fig. 2. 2). *P. vivax* and *P. ovale* are traditionally thought to occupy complementary niches. *P. ovale* has been observed to be predominating in Sub-Saharan Africa. *P. vivax* and P. ovale are not always clearly distinguished on the basis of morphologic characteristics alone, but the use of molecular tools has been reported useful to help clarify their diagnosis and exact distribution. *Plasmodium malariae* has wide global distribution found in places such as South America, Asia and Africa, but it is less frequent than *P. falciparum* in terms of association with cases of malaria disease. *P. knowlesi* is mainly found in Southeast Asia.

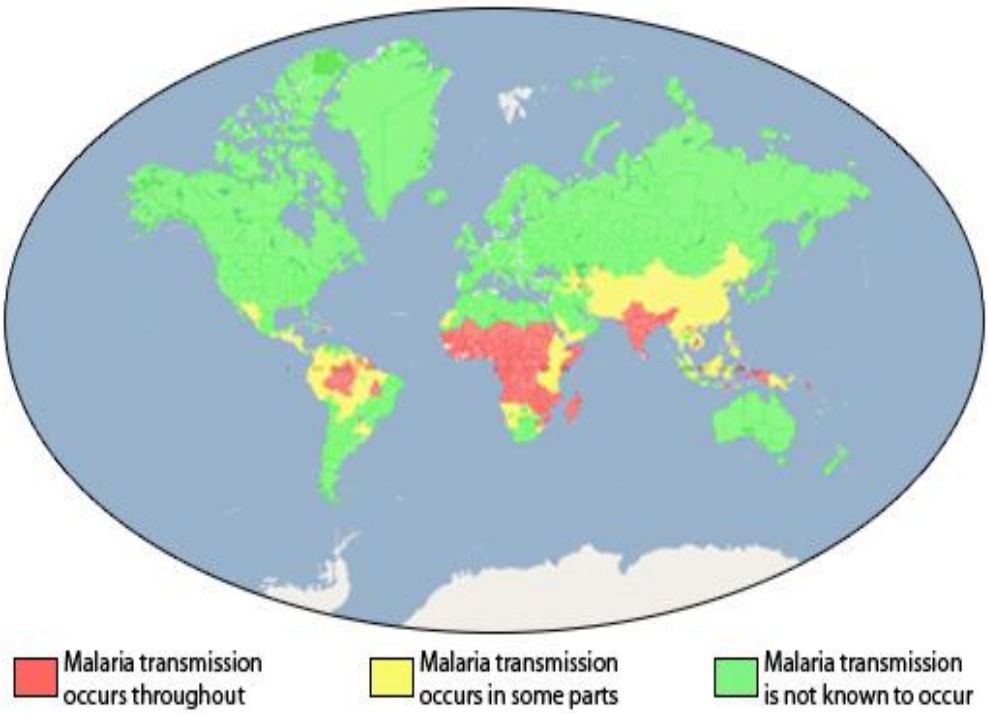


Fig. 2.2: Geographical distribution of malaria disease (source: CDC, 2014).

Evidence has shown that Nigeria has the largest population at risk of malaria in Africa. The disease is a major health problem in the country, with stable transmission throughout the country. It accounts for about 50% of out-patient consultation, 15 percent of hospital admission, and also prime among the top three causes of death in the country (National Malaria Control plan of action 1996 to 2001). Approximately 50% of the Nigerian population experience at least one episode per year with an official estimate of an average of four malaria attack per person per year (WHO, 1995 and WHO, 2002).

The magnitude of incidence and death due to malaria is a multiple of all other tropical diseases put together. It is responsible for over 90% of reported cases of tropical disease in Nigeria. Evidence from WHO (2005) shows that malaria incidence throughout Nigeria had been on the increase over the years ranging between 1.12 million at the beginning of 1990 and 2.225 million by the turn of the millennium 2000 and2.61 million in 2003 (Alaba and Alaba, 2002).

According to the latest estimates in Africa (WHO, 2014) there were about 198 million cases of malaria with an estimated 584,000 deaths. Malaria mortality rates have fallen by 47% globally since year 2000 and by 54% in WHO African Region. These reduction in malaria cases are associated with the scaling-up of WHO recommended malaria control measures, such as long-lasting insecticidal nets (LLIN), indoor residual spraying programmes (IRS) and access to artemisinin combination therapy (WHO, 2014). Most malaria caused deaths are reported to occur among children living in Africa where a child dies every minute from malaria. Country- malaria level burden estimates available for 2010 show that an estimated 80% of malaria deaths occur in just 14 countries. The Democratic Republic of the Congo and Nigeria account for over 40% of the estimated total malaria caused deaths globally.

2.3.2 Diagnosis of malaria

Malaria must be recognized promptly in order to treat the patient in time and to prevent further spread of infection in the community via local mosquitoes. Malaria should be considered a potential medical emergency and should be treated accordingly. Delay in diagnosis and treatment is a leading cause of death in malaria patients in the United States.

Malaria can be suspected based on the patient's travel history, symptoms, and the physical findings at examination. However, for a definitive diagnosis to be made, laboratory tests must demonstrate the malaria parasites or their components.

Diagnosis of malaria can be difficult where malaria is not endemic any more (such as in the United States), health-care providers may not be familiar with the disease. Clinicians seeing a malaria patient may forget to consider malaria among the potential diagnoses and not order the needed diagnostic tests. Laboratory scientists may lack experience with malaria and fail to detect parasites when examining blood smears under the microscope.

In some malaria-endemic areas, malaria transmission is so intense that a large proportion of the population is infected but not made ill by the parasites. Such carriers have developed just enough immunity to protect them from malarial illness but not from malarial infection. In that situation, finding malaria parasites in an ill person does not necessarily mean that the illness is caused by the parasite**s.**

Clinical diagnosis is based on the patient's signs and symptoms and on physical findings at examination. The first symptoms of malaria which are most often fever, chills, sweats, headaches, muscle pains, nausea and vomiting are often nonspecific because they overlap with common life-threatening diseases such as bacterial and common viral infections

(Mwangi, 2005). Likewise, the physical findings are often nonspecific which includes elevated temperature, perspiration and tiredness. In severe malaria (caused by *Plasmodium falciparum*), clinical findings (confusion, coma, neurologic focal signs, severe anemia, respiratory difficulties) are more striking and may increase the index of suspicion for malaria. If possible, clinical findings should always be confirmed by a laboratory test for malaria. Microscopy remains the gold standard for laboratory confirmation of malaria. When performed in optimal conditions, the technique has a remarkable sensitivity. It is specific and enables the identification of the parasite at the species level as well as quantitatively. It is also reasonably easy to perform and cheap (Marcel, 2003). However, microscopy depends on the quality of the reagents, the microscope, and on the experience of the microscopy scientist.

Various test kits are available to detect antigens derived from malaria parasites. Such immunologic ("immunochromatographic") tests most often use a dipstick or cassette format, and provide results in 2-15 minutes. These "Rapid Diagnostic Tests" (RDTs) offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available. Malaria RDTs are currently used in some clinical settings and programs. However, before malaria RDTs can be widely adopted, several issues remain to be addressed, including improving their accuracy; lowering their cost; and ensuring their adequate performance under adverse field conditions. It is recommended that all RDTs are followed-up with microscopy to confirm the results and if positive, to quantify the proportion of red blood cells that are infected. The use of this RDT may decrease the amount of time that it takes to determine that a patient is infected with malaria.

Parasite nucleic acids are detected using polymerase chain reaction (PCR). Although this technique may be slightly more sensitive than smear microscopy, it is of limited utility for the

diagnosis of acutely ill patients in the standard healthcare setting. PCR results are often not available quickly enough to be of value in establishing the diagnosis of malaria infection.
PCR is most useful for confirming the species of malaria parasite after the diagnosis has been established by either smear microscopy or RDT.

Serology detects antibodies against malaria parasites, using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA). Serology does not detect current infection but rather measures past exposure. In situations in which laboratory facilities are not available, a presumptive diagnosis may need to be made on clinical features alone, sometimes with the help of a suitable algorithm develop to suit local conditions. This will enable the physician to start treatment early and confirm the diagnosis later (Marcel, 2003).

WHO recommends that all persons of all ages in all epidemiological settings with suspected malaria should receive a parasitological confirmation of diagnosis by either rapid diagnostic test (RDT) or microscopy (WHO, 2010b).

2.3.3 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 (Gallup and Sachs, 2001 and 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 welfare. For instance, malaria has been reported to be responsible for about a 1.3 percent 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 (Westling *et al.*, 1997 and Alaba, 2007).

Given the above scenarios, the drive of any government in the third world countries e.g. Nigeria to achieve the targets National Economic Empowerment and Development Strategy (NEEDS) and the Millennium Development Goals (MDGs) is currently threatened by the seemingly intractable trend of the ancient scourge which has compounded national and household poverty through intensive loss of productive time to attack and death in extreme cases. This is compounded by the development of resistance to the traditional first-line drug (Chloroquine), while the newly introduced therapy (ACT) would be largely beyond the reach of many due to poverty, if it was not heavily subsidized by international organisations. Malaria has been reported endemic throughout Nigeria and that over 50% of the country's population are living below poverty line, and hence malaria incidence may increase

significantly, because many may not be able to afford the newly introduced drugs due to poverty.

There are several reports on the relationship between malaria and the economic growth in the literatures (WHO, 2000; Teklehaimanot and Mejia, 2008; Alaba and Alaba, 2009). 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.

2.4 Treatment of Malaria

The treatment of malaria is depended on three main factors such as: 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* resistant peculiar to that location. The effective antimalarial drugs for different malaria must be prescribed by a doctor who is familiar with malaria treatment protocols.

Resistance to antimalarial drugs has been documented for *P. falciparum*, *P. malariae* and *P. vivax*. In *P. falciparum*, resistance has been observed in all currently used antimalarial drugs (Amodiaquine, Chloroquine, Mefloquine, Quinine, and Sulfadoxine-pyrimethamine) and, more recently, in artemisinin derivatives (WHO, 2010b). Chloroquine phosphate is the anti-malarial drug of choice for all malarial parasites except for chloroquine-resistant *Plasmodium* strains. Although, some strains of *P. malariae* have been reported susceptible to chloroquine, however, *P. falciparum*, *P. vivax*, and *P. ovale* strains have been documented as resistant to chloroquine which is usually indicated by drug-treatment failure in the individual patient. The specialized laboratories to test for patient's parasites for anti-malarial susceptibility are not used frequently. Consequently, treatment is often based on *Plasmodium species* diagnosed which usually is blind treatment with attendant consequence of drug-resistance for country or world region where the patient became infested. The WHO's anti-malarial treatment policy (WHO, 2006) has 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 have shown that *P. falciparum*-infected individuals have parasites ACT drugs-treatment failure.

2.4.1 Classification of antimalarial drugs

Antimalarial medications are designed to inhibit or kill malaria parasites. The drugs may also be used for prevention of *Plasmodium* species infections of individuals visiting a malaria-endemic region who have no immunity (Malaria prophylaxis), or routine intermittent treatment of certain groups in endemic regions (Intermittent prevention therapy) (WHO, 2010b).

Antimalarial drugs can be classified by the chemical group to which they belong to and this in turn determines the stage of the life cycle they affect. The principal antimalarial agents, classified according to their drug group and activity are:

1. The 4-methanolquinoline derivatives, such as the cinchona alkaloids and mefloquine. The main cinchona alkaloid quinine and its optical isomer quinidine are rapid-acting blood schizontocides with some gametocytocidal activity. Mefloquine also acts as a blood schizontocide.
2. The 4-aminoquinolines, such as chloroquine, hydroxychloroquine, and amodaquine, are rapid-acting blood schizontocides with some gametocytocidal activity.
3. The 8-aminoquinolines, such as primaquine and tafenoquine, are tissue schizontocides; primaquine also has gametocytocidal activity and some activity at other stages of the parasite's life cycle.
4. The biguanides, such as proguanil and chlorproguanil, have dihydrofolatereductase inhibitory activity and thus inhibit folate synthesis in the parasite. They are tissue

schizontocides and slow-acting blood schizontocides with some sporontocidal activity.

5. The diaminopyrimidines, such as pyrimethamine. Pyrimethamine is a dihydrofolatereductase inhibitor and has actions similar to those of the biguanides. It is usually administered with other antimalarials that inhibit different stages of folate synthesis, such as a sulfonamide or sulfone, to form synergistic combinations.
6. The dichlorobenzylidinelumefantrine, a blood schizontocide given in combination with the artemisinin derivative artemether.
7. The hydroxynaphthoquinones, such as atovaquone. Atovaquone has blood schizontocidal activity and is usually given in combination with proguanil.
8. The 9-phenanthrenemethanols, such as the blood schizontocidehalofantrine.
9. The sesquiterpene lactones, such as artemisinin and its deratives, act mainly as blood schizontocides.
10. The sulfonamides, such as sulfadoxine and sulfametopyrazine, which are dihydropteroate synthase inhibitors and thus inhibit folate synthesis. They act mainly as blood schizontocides and are usually administered with pyrimethamine.
11. The tetracyclines, such as doxycycline and tetracycline, are blood schizontocides and also have some activity against tissue forms.
12. The lincosamide, clindamycin, which is also sometimes used, has a similar action to the tetracyclines.
13. The sulfone, dapsone, which has similar antimalarial actions and uses to the sulfonamides (WHO, 2006).

2.4.2 Pharmacology of Antimalarial Agents

2.4.2.1*Chloroquine*. Drugs such as Chloroquine, Primaquine, Proguanil, Amodiaquine and Sulfadoxine/Pyrimethamine were developed during World War II to protect the troops from malaria(Greenwood, 1995). Chloroquine, a synthetic 4-aminoquinoline, proved to be the most efficient and important antimalarial and was widely used throughout the world. However, after ten to twelve years of use, Chloroquine resistance appeared in *P. falciparum*. By the late 1970s Chloroquine resistance had reached Africa and has since spread across sub-Saharan Africa (Kuhn *et al*., 2003).

Structure of Chloroquine: The D, L and DL forms of Chloroquine have equal potency in duck malaria, but the D-isomer is somewhat less toxic than the L-isomer in mammals. A chlorine atom attached to position 7 of the quinoline ring (Fig. 2.3) confers the greatest antimalarial activity in both avian and human malarias. Research on the structure-activity relationships of Chloroquine and related alkaloid compounds continues in an effort to find new effective antimalarial drugs with improved safety profiles that can be used successfully against Chloroquine and multidrug-resistant strains of *P. falciparum* (examples include the bisoquinolines and short-chain chloroquines) (Kaur *et al*., 2010). The oral formulations are the sulphate and phosphate salt. The parental formulation is in the hydrochloride salt. Both the sulphate and phosphate salts are readily solube in water (in ratio 1:3) (Basco, 2007).

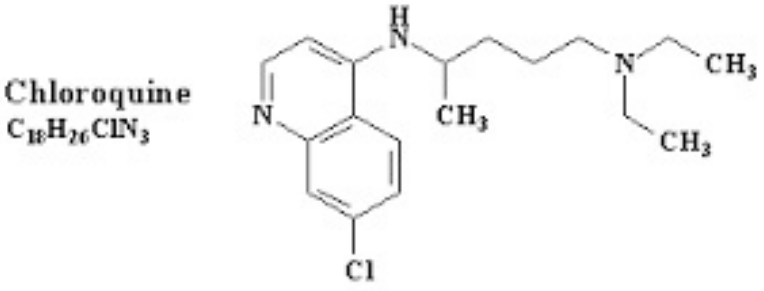


Fig. 2.3: Chemical structure of Chloroquine

Mode of action: The mechanism of plasmodical action of chloroquine (CQ) is not completely understood. A clue to the mechanism of action of CQ came from the observation that it is active only against the erythrocytic stages of malaria parasites. It is not active against preerytrocytic or hypnozite-stage parasites in the liver (Peters, 1970), nor against mature gametocytes (Sinden, 1982). Studies using ultra structural device has suggested that the parasites food vacuole in the target of chloroquine's activity. There is also evidence to show that chloroquine interfes with the parasites mechanism for detoxifying ferriprotoporphyrin IX and superoxide anions resulting from its digestion of haemoglobin so that the malaria parasite is killed by accumulation of these toxic products (Rosenthal, 2001). Thus, the selectivity of action of quinoline drugs appears to derive from the fact that they target a parasite specific process, namely some aspect of haemoglobin digestion.

Therapeutic uses of chloroquine: Chloroquine is used for prophylaxis and treatment of malaria due to susceptible stains of *Plasmodium ovale, P. vivax and P. malariae*. It has also been used for susceptible strains of *P. falciparum* but it is important to note that in the majority of the world *P. falciparum* is now resistant to chloroquine, which should therefore not be given as treatment. Chloroquine is a rapid-acting blood schizontocide with some gametocytocidal activity against *P. ovale, P. vivax, P. malariae,* and immature gametocytes of *P. falciparum* (WHO, 2006).

2.4.2.2 *Amodiaquine*. Amodiaquine is a mannich-base type 4-aminoquinoline derivative with a mode of action similar to that of Chloroquine (Basco, 2007). This antimalarial was briefly recommended for prophylaxis in travellers during the 1980s, but was subsequently abandoned after reports that it can induce immune-mediated granulocytopenia in some patents (Hatton, et al., 1986). Amodiaquine is more palatable than chloroquine and may be

more effecyive. It is not recommended as a first line of treatment (WHO, 1993). To prevent emergence and spread of drug resistant malaria parasites WHO recommends that Amodiaquine be given with artesimin derivative, such as Artesunate, for the treatment of falciparum malaria (WHO, 2006).

Structure of Amodiaquine: Amodiaquine is closely related to chloroquine, differing only by having a p-hydroxxyanillino aromatic ring in its side chain (Fig. 2.4). Amodiaquine is a more active inhibitor of the growth of *P. falciparum in vitro* than chloroquine (Ekweozor *et al*., 1987), which has a significantly reduced activity.

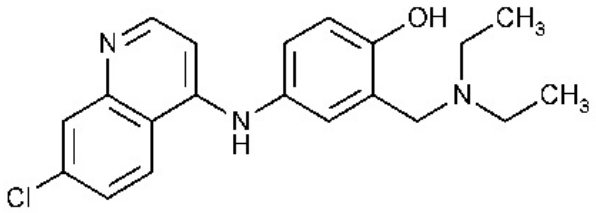


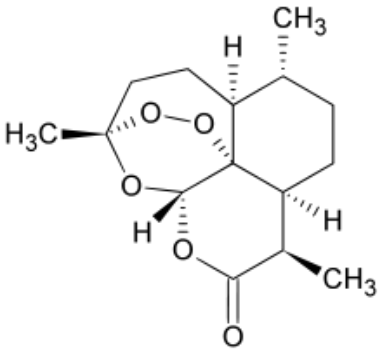
Fig. 2.4: Chemical structure of Amodiaquine

Mode of action: The mechanism of Amodiaquine action is similar to that of Chloroquine (WHO, 2006). Amodiaquine competitively inhibits CQ accumulation and vice versa, suggesting that these compounds share similar mechanism of accumulation (Fitch, 1973).

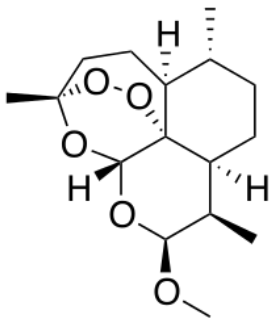
Therapeutic uses of Amodiaquine: Amodiaquine is effective against some chloroquine resistant strains of P. falciparum, although there have been reports of cross resistance among malaria parasites (WHO, 2010b). To prevent the emergence and the spread of drug resistant parasites WHO recommends that amodiaquine be given with artemisin derivative, such as artesunate, for the treatment of falciparum malaria, amodiaquine is not recommended for the prophylaxis of malaria because of resistance and the risk of major toxicity. Amodiaquine is given by mouth as the hydrochloride, butt doses are expressed in terms of amodiaquine base. For the treatment of *P. falciparum* malaria and uncomplicated chloroquine-resistant *P. vivax* malaria a total dose of 30mg/kg is given over 3 days (10 mg/kg daily for three days) (Olliaro and Mussano, 2003).

2.4.2.3 ***Artemisinin and its derivatives*****.** Artemisinin, a sesquiterpene lactone endoperoxide derived from the weed *qinghao* (Artemisia annua), also called sweet wormwood or annual wormwood, was used by Chinese herbal medicine practicioners for more than 2000 years, initially to treat haemorrhoids (Klayman, 1985). As early as 340 A.D, Ge Hong prescribed tea made from *qinghao* as a remedy for fevers and in 1956, Li Shizhen, a famous herbalists recommended it to relieve the symptoms of malaria and specified that the extract be prepared in cold water (Klayman, 1985). By 1972, Chinese scientists had extracted and crystallized the major antimalarial ingredient now known as artemisinin,

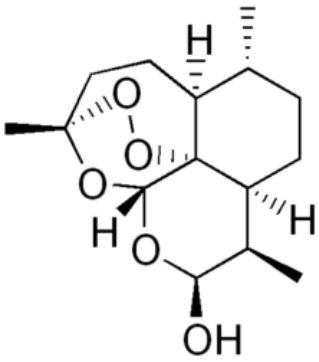
Structures of Artemisinin and its derivatives: Three semisynthetic derivatives with improved potency and bioavailability have since largely replaced the use of artemisinin (Fig. 2.5). These include dihydroartemisinin, a reduced product; artemether, an oil-solube methyl ether; and artesunate, the water solube hemisuccinate ester of dihydroartemisinin (WHO, 2006).



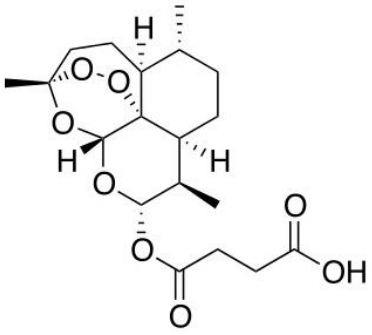
Artemisinin



Artemether



Dihydroartemisinin



Artesunate

Fig. 2.5: Chemical Structure of artemisinin and its derivatives (Agtmael *et al*., 1999)

Mode of action: Artemisinin is a sesquiterpene lactone that contains a labile peroxide bridge (David, 2001). The mechanism of artemisini action involves two steps (Meshnick, 2001). First, heme iron within the parasite catalyzes cleavage of the endoperoxide bridge. This is followed by rearrangement to produce a carbon-centered radical that alkylates and damages macromolecules in the parasite, likely including the ortholog of sarco/endoplasmic reticulum Ca^{2+}-ATPase (Eekstein-Ludwig *et al*., 2003). Artemisinin and its derivatives exhibit antiparasitic activity *in vitro* against other protozoa, including *Leishmania* major and *Toxoplasma gondii*, and have been used alone or in combination in patients with schistosomiasis (Utzinger *et al*., 2003). Artemisinin has been largely replaced by the more potent dihydroartemisinin and its derivatives i.e. artemether, artemotil and artesunate (WHO, 2010b).

Therapeutic uses of artemisinin and its derivatives: The artemisinins generally are not used alone because of their low efficacy and to prevent the selection of resistant parasites. Nevertheless, artesunate has proven exceedingly useful when combined with other antimalarials for the first-line of treatment of malaria, in a series of trials in Africa, South America and Asia (Adjuik *et al*., 2004).

2.5 Types of Antimalarial Drug Resistance Mechanisms

Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria treatment (Lawrence, 2010). Resistance started with chloroquine, followed by resistance to sulfadoxine-pyrimethamine and mefloquine. Recently, the first evidence of artemisinin resistance has been found in Cambodia (Lawrence, 2010). A better understanding of the mechanism of antimalarial resistance will provide the tools to prevent and control the spread of resistance.

2.5.1 Mechanism of Chloroquine resistance

Although the mechanism of resistance of Chloroquine has been extensively studied, it is not yet fully elucidated (Lawrence, 2010). It has been demonstrated (Kikkilaya, 2011) that Chloroquine acts by getting accumulated in the food vacuole where it inhibits heme polymerase (Fig. 2.6). The accumulation is due to the fact that Chloroquine is a diprotic weak base and, at physiological pH (~7.4), it can be found in its un-protonated (CQ), mono-protonated (CQ^{+}) and di-protonated (CQ^{++}) forms. The uncharged chloroquine is the only membrane permeable form of the molecule and it freely diffuses into the erythrocyte up to the digestive vacuole. In this compartment, chloroquine molecules become protonated and, since membranes are not permeable to charged species, the drug accumulates into the acidic digestive vacuole (Yayon *et al.*, 1984) where it is believed to bind haematin, a toxic by-product of the haemoglobin proteolysis (Bray *et al.*,1998), preventing its incorporation into the haemozoin crystal (Slater *et al.*, 1993). The free haematin seems to interfere with the parasite detoxification processes and thereby damage the *Plasmodium* membranes (Sagioka *et al.*, 1987).

Resistant strains are able to efflux the drug by an active pump mechanism and release the drug at least 40 to 50 times faster than sensitive strains, thereby rendering the drug ineffective. There is an increase in the surface area of the resistant parasites, permitting more efficient pinocytosis. Binding of chloroquine with haemoglobin breakdown product to form toxic complexes is also prevented. Chloroquine resistance has also been clearly associated with multiple mutations in genes encoding for membrane transporters located in the digestive vacuole, namely the chloroquine resistance transporters PfCRT and the multi-drug resistance transporter 1 PfMDRI (Lawrence, 2010).

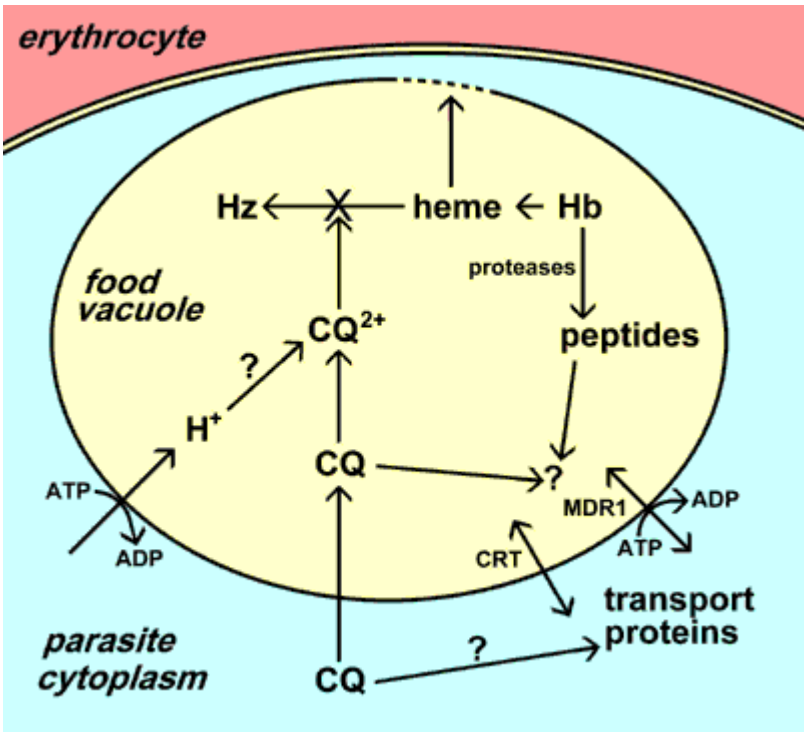


Fig. 2.6: Mechanism of Chloroquine drug action (Wiser, 2003)

2.5.2 Mechanism of antifolate resistance

Folate metabolism is the target of several antimalarials as well as drugs used against other pathogens. Reduced folates serve as co-factors in a many one-carbon transfer reactions involved in the biosynthesis of amino acids and nucleotides. Due to its high rate of replication the malaria parasite has a high demand for nucleotides as precursors for DNA synthesis, and thus is particularly sensitive to antifolates (Fig. 2.7). The two primary targets of antifolate metabolism are the de novo biosynthesis of folates and dihydrofolatereductase (DHFR) as denoted with the boxed arrows in the diagram. Folate is synthesized from 3 basic building blocks, GTP, p-aminobenzoic acid (pABA), and glutamate, in a pathway involving 5 enzymes. One of these enzymes, dihydropteroate synthase (DHPS), is inhibited by sulpha-based drugs. Sulfadoxine and dapsone are two common antimalarials that inhibit DHPS.DHFR is an enzyme that reduces dihydrofolate to tetrahydrofolate. Inhibiting DHFR will lead to an arrest in DNA synthesis and subsequent parasite death. Pyrimethamine and proguanil are the two most common DHFR inhibitors used as antimalarials. Most often inhibitors of DHPS and DHFR are used in combination. Fancidar, which is a combination of sulfadoxine and pyrimethamine, is widely used for the treatment of uncomplicated *falciparum* malaria. Specific point mutations in these enzymes lead to a lower affinity for the drugs, which appear to be responsible for antifolate drug resistance (Lawrence, 2010).

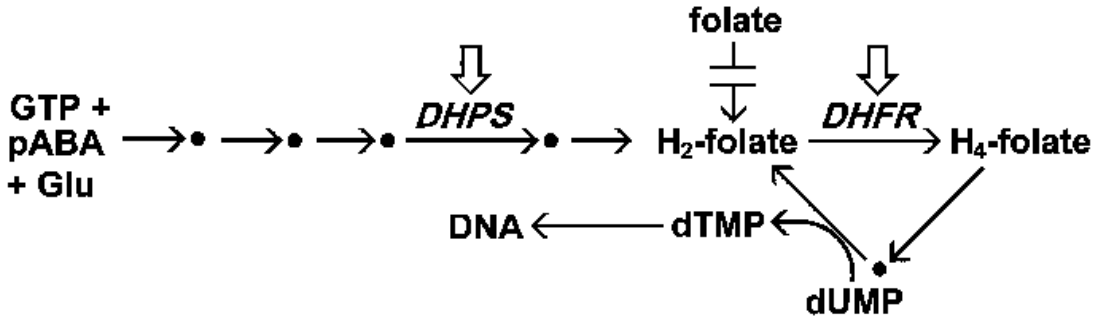


Fig. 2.7: Simplified scheme of folate metabolism (Wiser, 2003)

2.5.3 Mechanism of artemisinin compounds resistance

These are peroxide antimalarial which release carbon centred free radicals when they come in contacts with heme. Examples are artesunate and artemether, which are derived from Chinese herb qingho (Aremisiaannua). They were introduced recently as malaria treatment (www. Malariasite.com). Several studies indicate PfaTP6, an active transporter enzyme found on membranous structures within the parasite cytoplasm, play a key role in artemisinins effectiveness. Stable resistance to artemisinin and atesunate, a derivative of artemisinin, has been induced in laboratory lines of a rodent parasite, suggesting that resistance to artemisinin derivatives as potential therapeutic failure. Indeed, some resistance has recently been reported in Cambodia and Thailand (Lawrence, 2010).

2.6 *In Vitro* Tests

The *in vitro* assay is a research tool that is commonly used for monitoring drug resistance (Basco, 2007). The problem related with the assessment of antimalarial drug resistance *in vivo* led to the introduction of a number of *in vitro* tests (WHO, 2010a) for the measurement of antimalarial drug susceptibility. *In vitro* tests avoid many of the factors that influence the *in vivo* test by removing parasites from the host and placing them in suitable controlled experimental growth conditions. This test accurately reflects the intrinsic antimalarial drug resistance (Boland, 2001).

A number of studies have been done using the in vitro tests (Ramadhan *et al*., 2000; Oyedeji *et al*., 2005; Folarin *et al*., 2008; Olasehinde *et al.*, 2014) to monitor antimalarial drug resistance in endemic regions of the world, Nigeria inclusive. Most of the researches done in Nigeria are in the southwest Nigeria, and a few in the Northern Nigeria.

2.7 Molecular Characterization of Antimalarial Resistance Genes

Plasmodium falciparum malaria parasite has become resistant to most avoidable antimalarial drugs, such as chloroquine, amodiaquine and Suifadoxine-pyrimethamine (WHO, 2005) . To circumvent the problem of drug resistance, national health authorities of many concerned countries, with the support of World Health Organisation, have resorted to the use of arthemisinin-based combination therapies (ACTs) for the first-line treatment of uncomplicated malaria. However, a decrease in the sensitivity to arthemisinin has been recently documented in Cambodia, necessitating an increased vigilance in monitoring drug – resistant malaria (Dondorp *et al*., 2010). In Nigeria, malaria is still a major public health problem.

Detection of parasite genetic material through polymerase-chain reaction (PCR) techniques is becoming a more frequently used tool in the diagnosis of malaria, as well as the diagnosis and surveillance of drug resistance in malaria (Olasehinde *et al*., 2014). Specific primers have been developed for each of the four species of human malaria. One important use of this new technology is in detecting mixed infections or differentiating between infecting species when microscopic examination is inconclusive (Beck, 1999). In addition, improved PCR techniques could prove useful for conducting molecular epidemiology investigations of malaria clusters or epidemics (Purfield *et al*., 2004).

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* method (Basco, 2007). 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 (Beck, 1999). Primary disadvantages of these methods are overall high cost, high degree of training required, need for special equipment, absolute requirement for electricity, and potential for cross-contamination between samples (Olasehinde *et al*., 2014; Berzins and Anders, 1999). Sidhu *et al*., (2002) also reported that confirmation of the association between given mutations and actual drug resistance is difficult, especially when resistance involves more than one gene locus and multiple mutations.

Chloroquine resistance is manifested by impaired CQ uptake by the parasite vacuole, which is correlated with mutations in several genes. A single mutation K76T in the P. falciparum chloroquine resistance transporter gene (*pfcrt*) has been demonstrated to be a major determinant of CQ resistance in *P. falciparum* (Yang et al., 2007; Fidock *et al*., 2000; Sidhu *et al*., 2002). All P. falciparum clinical samples that are resistant to CQ contain K76T mutation (Djimde *et al*., 2001).

Other epistatic markers such as P. falciparum multidrug resistant 1 (pfmdr1) gene also affect the final outcome of CQ resistance (Reed et al., 2000; Babiker *et al*., 2001). Five single nucleotide polymorphisms (SNPs) of pfmdr1 N86Y, Y183F, S1034C, N1042D and D1246Y were identified in field isolates from different regions of the world (Yang *et al*., 2007).
Sidhu *et al*. (2005) reported that that N1042D of *pfmdr*1, a prevalent mutation in South America, contributes to quinine instead of chloroquine resistance, suggesting that *pfmdr*1 mutations can affect parasite susceptibility to a wide range of antimalarials depending on the parasite's genetic background.

The mechanism of action of artemisinin is not well understood. One of the hypotheses is based on the specific inhibition of *P. falciparum* ATPase 6 (PfATP6), an orthologue gene product of the mammal sacro-endoplasmic reticulum calcium dependent ATPase (SERCA). Initial laboratory studies have suggested that L263E substitution in PfATP6 affects that active site and induces conformational changes, reducing the affinity between the enzyme and artemisinin. Subsequent studies carried out on field isolates have shown an association between increased 50% inhibitory concentration (IC_{50}) for artemether and either a single amino acid substitution S769B (in South American strains) or double amino acid substitutions E431K and A623E (in Africa strains) (Jambou *et al*., 2005).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

The following equipment , reagent and chemicals were used for the study.

3.1.1 Equipment

Tissue culture plates 12 x 8 wells (Becton Dickinsin Labware, New Jersy, model – Falcon 3072), Micropipette- 0-50 µL, 0-100 µL and 100-1000 µL (Micropet), Microscope (Fischer Scientific Company), Microscope slides, Autoclave (GULFEX Medical and Scientific, England, model – LS-B75L), Anaerobic jar (Gaspak system, model – BQBBL), Incubator (Biochemical, model SHP-250), Weighing balance (OHAUS, model – SPJ303), Sterile pipette tips and Heparinise falcum tube (Skytec Medical).

3.1.2 Drugs

Choroquine diphosphate powder (IPCA Laboratories Ltd), Amodiaquine dihydrochloride (Pfizer Afrique de l'Ouest) and Artesunate (Greenfield Pharm. Co. Ltd).

3.1.3 **Chemical**

RPMI 1640 liquid medium (Sigma Aldrich), Giemsa stain, Methanol (Gainland Chemical Co. United Kingdom), Buffer tablets (Hopkin and Williams, Chadwel Heath, Essex, England).

3.1.4 Others

Aluminium foil, Rack, plastic covered wire, Alcohol swabs, Glass writing pencil, Forceps

3.2 Methods

3.2.1 The Study Centre and Ethical Clearance

a. Study Centre

The study was carried out at Umaru Musa Yaradua Memorial Hospital (UMYMH), Sabon-Wuse, Niger State, Nigeria. Sabon-Wuse is a town located on Abuja-Kaduna highway with a population of 84,000 (NPC, 2007). Sabon-Wuse is the Headquarter of Tafa Local Government Area.

b. Ethical Clearance

The study protocol was approved by the Ethics Committee of Umaru Musa Yaradua Memorial Hospital Management Board (Appendix I).

3.2.2 Retrospective Study

The medical records of reported malaria cases for 2011 and 2012 were collated from the medical records office of the hospital. Data extracted included:-

1. No of reported malaria cases
2. Age group (age 0-5, 6-18 and adult (above 18 years))
3. Sex (male/female)
4. Occupation (student, civil servant, business, housewife and unknown)
5. Antimalarial drugs prescribed

The format of the data sheet is shown in Appendix II. The records were analysed using frequency analysis.

3.2.3 Profile of *Plasmodium* species isolates

A total of 160 patients (aged between 1-70 years) who reported to the hospital with history of fever (axiliary temperature > 37.5°C) were enrolled in this part of the study. Each age group

(0 – 5 years, 6 – 18 years, 19 – 30 years and above 30 years) had 40 patients (20 male and 20 females). The blood samples were collected and tested for the presence and type of *Plasmodium* species. This study was carried out between March and July 2014.

3.2.4 Patient and Study Protocol

Blood samples were collected, with the assistance of Medical laboratory scientists, from patients attending the hospital. About 350 male and female patients (aged between 1-70 years) who reported to the hospital with history of fever (axiliary temperature > 37.5°C) were enrolled after determining the level parasitemia. The patients reported not to have taken any antimalarial medication during the preceding four weeks. Informed consent was obtained from the patients or, in case of children, from their parents. All patients that had mono infection with *P. falciparum* and parasitaemia level being >1000 - < 80,000 asexual parasites per µl of blood were then used for the *in vitro* study (WHO, 2001). This part of the research lasted from September 2014 to August 2015. All patients that have taken antimalarial drugs within 2 weeks preceding the study, or with any other symptoms or signs of non-malaria etiology were excluded from the study.

3.2.5 Performance of the *in vitro* micro-test

3.2.5.1 ***Antimalarial drug dilutions and preparation of plates*****.** Stock solutions of antimalarial drugs, viz: Chloroquine, Amodiaquine and Artesunate, and a combination of Artesunate-Amodiaquine were prepared in appropriate solvents (Appendix III). The solutions were then filtered with 0.22 µm membrane filter (Millipore) aseptically. Further dilution of each drug was made to obtain a working solution of desired concentrations. From the working solutions, seven two fold dilutions of each drug were made and used to dose 96-well flat bottom micro culture plates and left to dry in an incubator at 37°C (Russel *et al*., 2013).

The 96-micro culture plates (Appendix IV) consists of rows A to H, row A is drug free well while rows B-H represent the drug concentrations.

3.2.5.2 *In vitro* cultivation of *Plasmodium falciparum* isolates and drug susceptibility test.

For the *in vitro* tests, the standard culture techniques by Haynes *et al.* (1976) and Trager and Jensen (1976) were applied, while the drug susceptibility test followed the standard procedure for schizont inhibition (WHO, 2001). The whole *in vitro* procedure from the beginning was carried out under strict aseptic conditions. Approximately 50 µl of the blood media mixture (1:10 dilution of malaria positive blood) from each patient was transferred to wells on the micro culture plates pre-dosed with varying concentrations of antimalarial drugs using the Eppendorf pipette with a sterile tip. This was done in descending order, starting from well A (control) and ending at well H with the highest concentration of the test drug. One row of 8 wells (A-H) of each test drug was used for individual blood samples. A sterile pipette tip was used each time a new row of wells were to be filled with the Blood-Medium mixture (BMM) in order to avoid contamination of the control well A. Each patient's serial number was inscribed on the lids of the plates directly over the appropriate column. The plates were closed with the sterile lids and gently agitated to reconstitute the drug deposits. The plates were then placed carefully in an anaerobic jar (with hydrogen gas generator and pellet catalyst).The anaerobic jar was then closed. The jar was then placed in an incubator set at 37°C, and incubated for 30 hours (WHO, 2001). At the end of the incubation the tests were "harvested" from each of the wells by removing the medium-plasma supernatant, and the sediment placed, with the aid of an Eppendorf pipette, as thick film on a microscope slide. After thorough drying, the thick films on the slides were stained with Giemsa solution at a dilution of 1% $w/_v$ in buffered water (pH 7.2) for 15min (WHO, 1991).

3.2.6 Examination of the Post-culture Blood Slide

The reading commenced with the control (well A), enumerating schizonts and other asexual forms separately, to a total of 200. The count that showed a total of ≥ 20 schizonts or $\geq 10\%$ of all asexual parasites was taken to be valid and the reading proceeded with three or more nuclei out of a total of 200 asexual parasites (i.e. Schizonts and Trophozoites). If however, the schizont maturation was less than 10%, the plates were placed back into the candle jar and further incubated for a longer period of time. All the samples were harvested at the end of 30 hours. The counts read in each wells were expressed as percentage of the control as shown in Table 3.1.

The individual percentage Schizont maturation inhibitions were converted to probit, while the various drug doses used were converted to log doses. The results were fed directly into Microsoft Excel. Using linear regression lines, their Inhibitory Concentrations (IC) at $IC_{50,}$ $IC_{90,}$ IC_{95} and IC_{99} were determined. Drug resistant *P. falciparum* parasites were identified as isolates with Chlororoquine: IC_{50} >114 nM; Amodiaquine: IC_{50} > 80 nM. For artesunate, as there has been no clear cut-off level for artemisinin resistance (WHO, 2012 and Na-Bangchang *et al*., 2013), Phompradit *et al.* (2011) considered IC_{50} > 2.8 nM as declined sensitivity to artesunate.

Table 3.1: Schizont maturation inhibition table

Control	Samples with antimalarial under test	
Number of schizont per 200 parasites after incubation	Number of schizonts per 200 parasite after incubation	% of schizonts relative to control sample (control samples 100%)
M	N	A=N/M x 100/1

The inhibition of parasite growth for each of the isolates used was calculated as follows

$$\text{Percentage inhibition} = 100 - \frac{Number\ of\ Schizont\ in\ test\ wells}{Number\ of\ Schizont\ in\ control\ wells} \times 100$$

3.2.7 Molecular Analysis of Resistant Strains

Blood samples were collected from the randomly infected individuals where resistance has been noticed against the test antimalarials investigated. Genomic DNA from the blood samples were extracted using the Qiagen DNeasy Blood and Tissue Kit (cat. 69506). The procedure for the Genomic DNA isolation is as follows:

Approximately 100 µl of the blood sample was aliquoted and then transferred into a well labelled 1.5 ml microcentrifuge tubes, each tube contained 20 µl of proteinase K. The volume was adjusted with 1x PBS to 220 µl then 200 µl Buffer AL (Appendix V) was added to each of the tubes and mixed by vortexing. The tubes were then incubated at 56°C for 10 min. A 200 µl of ethanol (96–100%) was added and mixed thoroughly by vortexing. The mixture was pipetted into a DNeasy Mini spin column in a 2 ml collection tube and centrifuged at 6000 x g (8000 rpm) for 1 min. The flow-through and collection tube were discarded. The spin columns were placed in new 2 ml collection tubes. A 500 µl of Buffer AW1 (Appendix V) were added to the spin column and centrifuged for 1 min at 6000 x g. The flow-through and collection tube were discarded and the spin columns were placed in new 2 ml collection tubes, 500 µl of Buffer AW2 (Appendix V) were added to the tubes and centrifuged for 3 min at 20,000 x g (14,000 rpm). The flow-through and collection tube were discarded and the spin columns were carefully removed so that it does not come into contact with the flow-through. The spin columns were then transferred into new 1.5 ml or 2 ml microcentrifuge tubes, then 200 µl of Buffer AE (Appendix V) was added to the centre of the spin column for elution of the genomic DNA and then incubated for 1 min at room temperature (25^{o}C) and centrifuged for 1 min at 6000 x g. DNA quality and concentration were checked by running 2 µl of the diluted DNA sample on 1% agarose gel.

Accurate DNA quantification was carried out using a NANODROP®2000 spectrophotometer (Thermo Scientific Inc.). PCR amplification was then performed according to Olasehinde *et al*. (2014) and described as follows:

3.2.7.1 PCR for detection of *Pfcrt* gene. The primary PCR components, in a final volume of 20µL, was 2.5mM $MgCl_2$, 640µM deoxynucleotide triphosphate (dNTPs), buffer 10x, 10pM of each primer, 1U of Taq polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, CA, USA) and 2µL of DNA samples). The primers and thermal cycling conditions used for the PCR analysis are contained in Tables 3.2 and 3.3 respectively.

3.2.7.2 Nested PCR and RFLP for *Pfcrt* mutation-specific detection. The PCR products from the amplification reactions were evaluated by electrophoresis on 2% agarose gels containing ethidium bromide. 10 µl of the nested PCR product reaction mixture were treated directly with 3U of the restriction enzyme *Apo* I for 6 to 16 hours at 50°C as recommended by the manufacturer (New England Biolabs, Beverly, MA). The enzyme *Apo* I recognize and cut the 76K codon and leaves the 76T codon found in chloroquine-resistant *P. falciparum*. The conditions used for thermal cycling conditions are contained in Table 3.4.

3.2.7.3 PCR and RPLP assay for (SERCA) *PfATPase*6. *Plasmodium falciparum* positive samples were amplified by PCR using *PfATPase6*-specific primary and nested primer pairs. DNA extract for each sample was subjected to nested PCR amplification with primers flanking nucleotide codon 2307 of the *PfATPase6* gene. Both the primary and secondary reactions comprised 2 µl template, 0.25 µM primer, 1.5 mM MgCl2, 200 µM dNTP's, 1x PCR buffer and 1U Taq DNA polymerase, in 25 µl reactions. One site, at *PfATPase6* nucleotide codon 1916, serves as the internal control for the restriction digestion assay, which

is always cut by the enzyme. Csp6 I digestion assay comprised 4 µl of secondary PCR amplicon (432 bp), 1x buffer and 1.5U of Csp6 I restriction enzyme, in 30 µl reactions. Digestion assays were incubated for 12 hours at 37^{o}C. PCR amplicon and restriction digests were analyzed by electrophoresis on a 2% ethidium bromide-stained agarose gel and visualized under UV transillumination. Restriction digests were loaded in 15 µl volumes per lane. Band sizes were measured using Syngene gel imaging analysis software. The conditions used for thermal Cycling Conditions Used for *PfATPase*6 PCR and RPLP assay (SERCA) are contained in Table 3.5.

Table 3.2: Primers Used For Polymerase Chain Reaction

S/N	Primer	Oligonucleotides sequence	Base pair	Resistance	Reference
1	*Pfcrt*- RA	CCGTTAATAATAAATACAGGC	1.6kb	Chloroquine Transporter	Dorsey *et al*., 2001
	Pfcrt- F	CTTTTAAAAATGGAAGGGTGT			
2	*Pfcrt*- FB	GGCTCACGTTTAGGTGGA	76kb	Chloroquine	Olasehinde *et al*., 2014
	Pfcrt- RB1	TGAATTTCCCTTTTTATTTCCAA A	76T		
	Pfcrt- RB2	GTTCTTTTAGC AAAAATCT	76K	MDR	Olasehinde *et al*., 2014
3	*Pfmdr*1-MDR 1F	ATGGGTAAA GAGCAGAAAGA			
	*Pfmdr*1- MDR 2R	AACGCAAGTAATACATAAAGTCA			
	*Pfmdr*1-MDR 3F	TGGTAACCTCAGTATCAAAGAA			
	*Pfmdr*1-MDR 4R	ATAAACCTAAAAAGGAACTGG			
4	*Pfdhfs*-F	ATGATTCTTTT TCAGATG	747bp	Sulphadoxine	Olasehinde *et al*., 2014
	Pfdhfs-R	CCAATTGTGTGATTTGTCCAC			
	*P*fdhps-F1	GTTGAACCTAAACGTGCTG			
	Pfdhps-R1	ATTACAACATTTTGATCATTC			
5	*Pf*ATPase6-F	TGA GCA TGG CAC AAG TIT	432bp	ATPase	Olasehinde *et al*., 2014
	*Pf*ATPase6- R	TCA ATA ATACCTAATCCACCTAAATA			

Table 3.3: Thermal Cycling Conditions Used For PCR

Step	Temperature ^{0}c	Time	Number of Cycles
Initial denaturation	95	5 minutes	1
Denaturation	94	30sec	40 cycles
Annealing	52	40 sec	1
Extension	72	30sec	1
Final extension	72	5mins	1
Final hold	4	Total time is 11mins, 40 sec.	

Table 3.4: Thermal Cycling Conditions Used For Nested PCR

Step	Temperature ^{0}c	Time	Number of Cycles
Initial denaturation	94	5 minutes	1
Denaturation	94	30sec	40 cycles
Annealing	52	40 sec	1
Extension	72	45sec	1
Final extension	72	5mins	1
Final hold	4	Total time is 11mins, 55 sec.	

Table 3.5: Thermal Cycling Conditions Used for *PfATPase*6 PCR and RPLP assay (SERCA)

Step	Temperature ^{0}c	Time	Number of Cycles
Initial denaturation	94	2 minutes	1
Denaturation	94	45sec	25 cycles
Annealing	46	45 sec	1
Extension	65	1 minute	1
Final extension	65	2mins	1
Final hold	4	Total time is 6mins, 25 sec.	

CHAPTER FOUR

4.0 RESULTS

4.1 Survey of Malaria Prevalence in Sabon-Wuse

4.1.1 Prevalence of malaria

The total number of reported malaria cases in UMYMH, Sabon-Wuse, Nigeria was 3,350 in 2011 with an average of 275 per month. In 2012, the total number of reported cases of malaria in UMYMH, Sabon-Wuse was 3,837 with an average of 319 cases per month. Sabon-Wuse has one secondary hospital. The monthly distribution of the reported cases is presented in Fig. 4.1. The reported malaria cases in UMYMH, Sabon-Wuse increased from 3,350 cases in 2011 to 3,837 cases in 2012. That is, the rate of increase of reported malaria cases is 14.5%. The rate of increase of reported malaria cases in UMYMH, Sabon-Wuse exceeded the population growth rate reported to be between 1.93% and 2.67% in Nigeria (CIA World Factbook, 2015).

The number of reported cases of malaria per month in 2011 significantly differs (95% C.I.) from the monthly average (275 cases per month) in February, March, May, June, August and October. While the reported cases in February, March, August and October are lower than the monthly average, the reported cases in May and June are above the monthly average. In 2012, the number of reported cases of malaria per month significantly differ (95% C.I.) from the monthly average (319 cases per month) in February, March, May, July, September, October and December. There was a significant increase in the number of reported cases in July and october, while there was a decrease in reported cases in February, March, May, September and December.

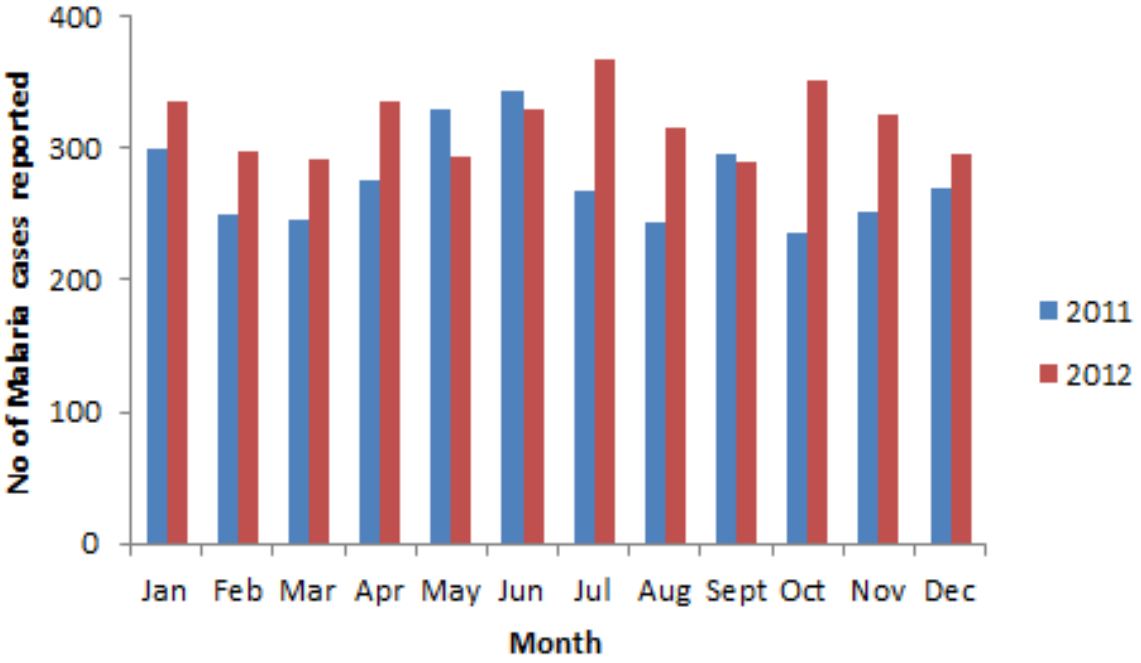
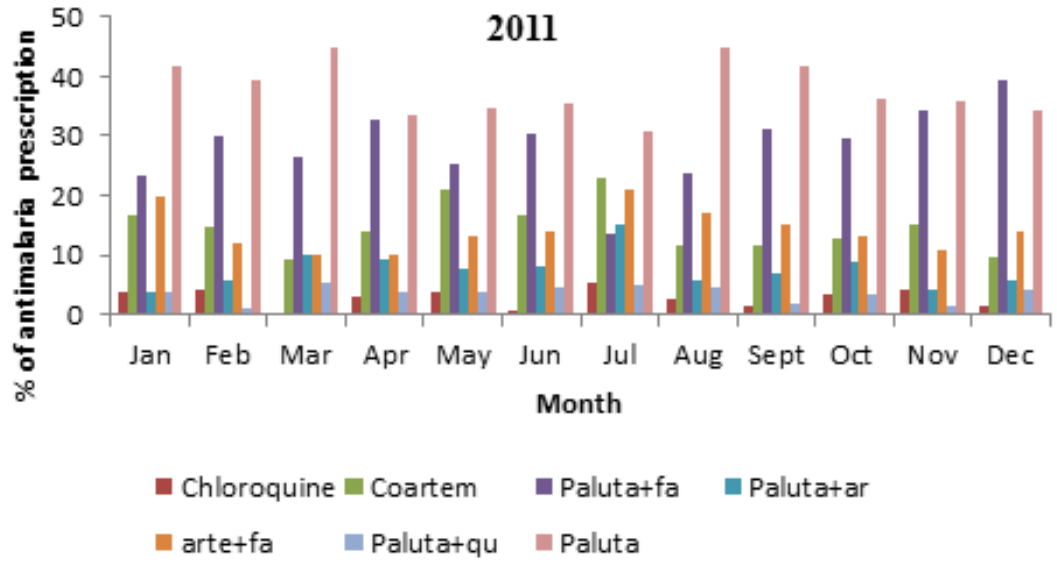


Fig. 4.1: Prevalence of reported malaria cases in UMYMH, Sabon-Wuse in 2011 and 2012

4.1.2 Drugs prescribed

Figure 4.2 shows the different drugs prescribed for reported malaria cases in UMYMH, Sabon-Wuse, Niger State, Nigeria. The antimalarial drugs observed prescribed in this study were Chloroquine, Coartem (Artemether + lumefantrine), Paluther (Artemether injection), Fansidar (Sulphadozine/pyrimethemine), Artesunate and Quinine. The summary of drugs observed prescriibed is presented in Table 4.1. In 2011, a total of 1,243 (37.3%) of reported malaria patients were treated with Paluther, while in 2012 the number of reported malaria patients treated with Paluther was 1,444 (37.8%). The number of reported malaria cases treated with Coartem in 2012 was 564 (14.8%). The most observed prescribed antimalarial drug was Paluther, while the least observed prescribed antimalarial drug was Chloroquine. These results show that malaria incidence is increasing in this region and ACT antimalarial drugs were observed to be commonly prescribed.



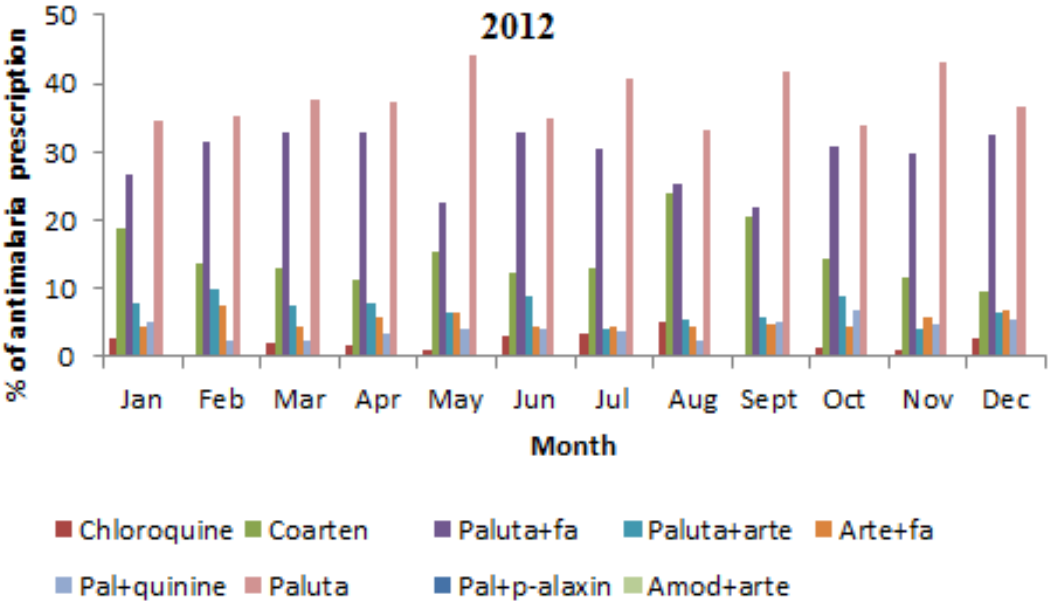


Fig.4.2: Antimalarial Drugs prescribed in UMYMH, Sabon-Wuse

Table 4.1: Summary of Patients Profile with Antimalarial Drugs Prescription in UMYMH, Sabon-Wuse, Niger State, Nigeria

Year	A	B	C	D	E	F	G
2011	92	491	936	254	170	119	1243
2012	80	564	1119	263	201	160	1444

Key:

A= Chloroquine B= Coartem C= Paluther + Fansidar D= Paluther + Artesunate

E= Artesunate +Fansidar F= Paluther + Quinine G= Paluther

4.2 Profile of *Plasmodium* species

The blood samples from 160 male and female patients who reported to the hospital with history of fever (auxiliary temperature >37.5°C) were screened for *Plasmodium* species. It was found that 84 (52.4%) had *Plasmodium* species, of which 38 (45.2%) were male and 46 (54.8%) were female. It was also observed that out of 84 patients with *Plasmodium* species, 72 (85.7%) had *Plasmodium falciparum* and 12 (14.3%) had *Plasmodium malariae*, with male, 45.8% and female 54.2% (Fig. 4.4).

Out of 40 patients screened in each age group, 16 (40%) had *Plasmodium species* in age group 1 -5 yr. For age groups 6 – 18yr, 19 – 30 yr and 31- 70 yr, the number of patients with *Plasmodium* species *we*re 19 (47.5%), 21 (52.5%) and 28 (70%) respectively (Fig. 4.5).

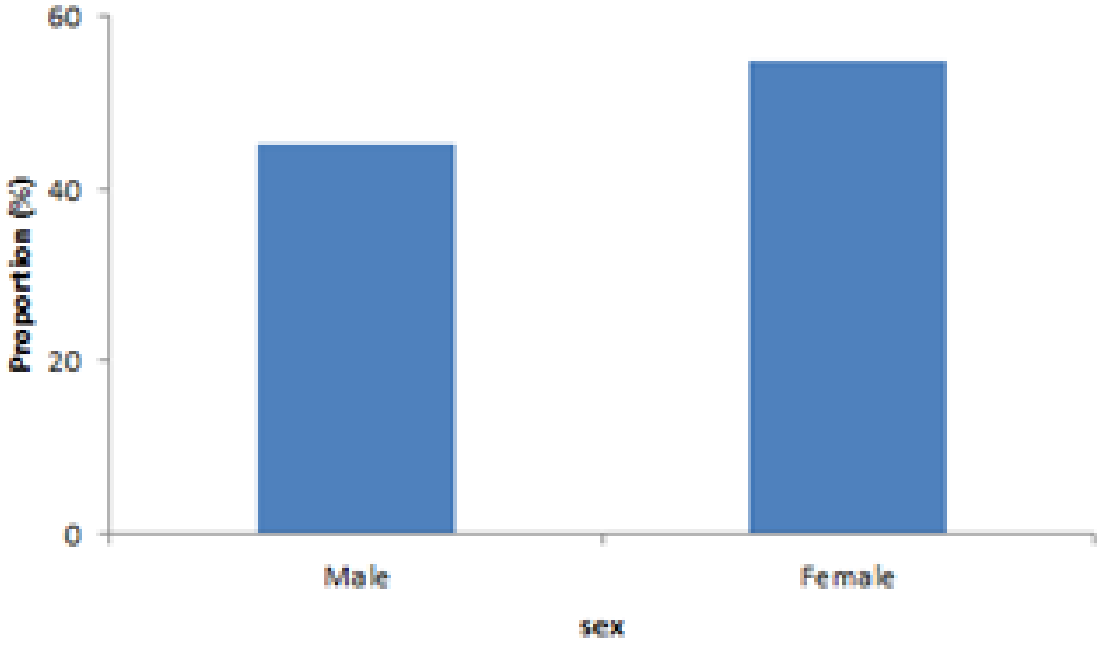
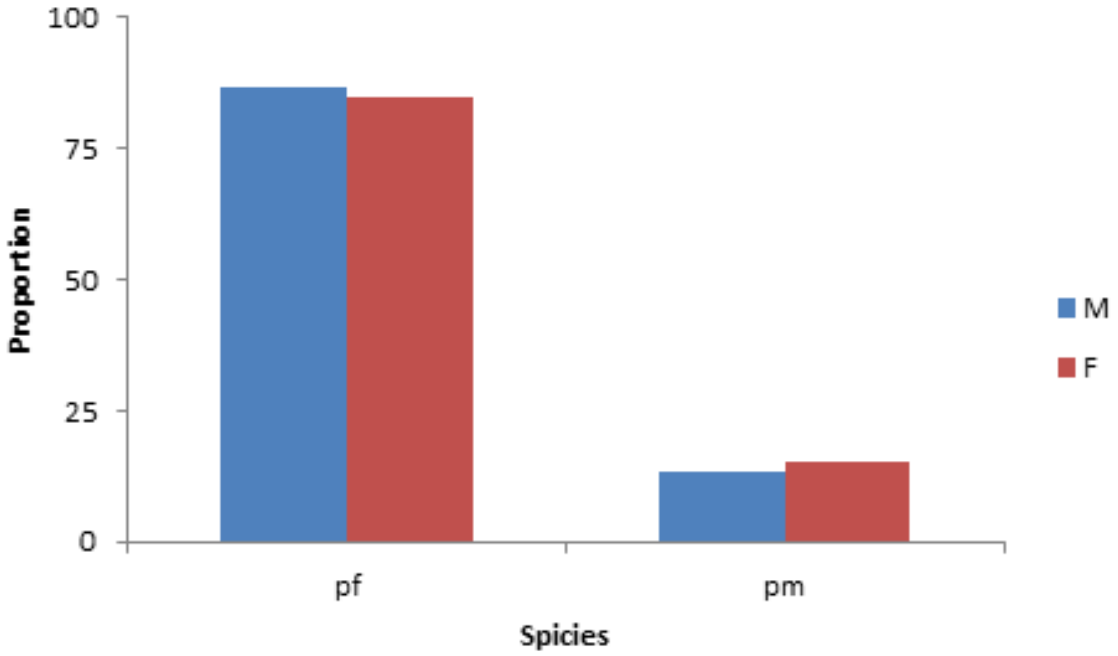


Fig. 4.3: Proportion of patient with *Plasmodium* species



Key: M=male, F=Female, pf = *Plasmodium falciparum*, pm=*Plasmodium malariae*

Fig. 4.4: Variation of *Plasmodium* species with sex

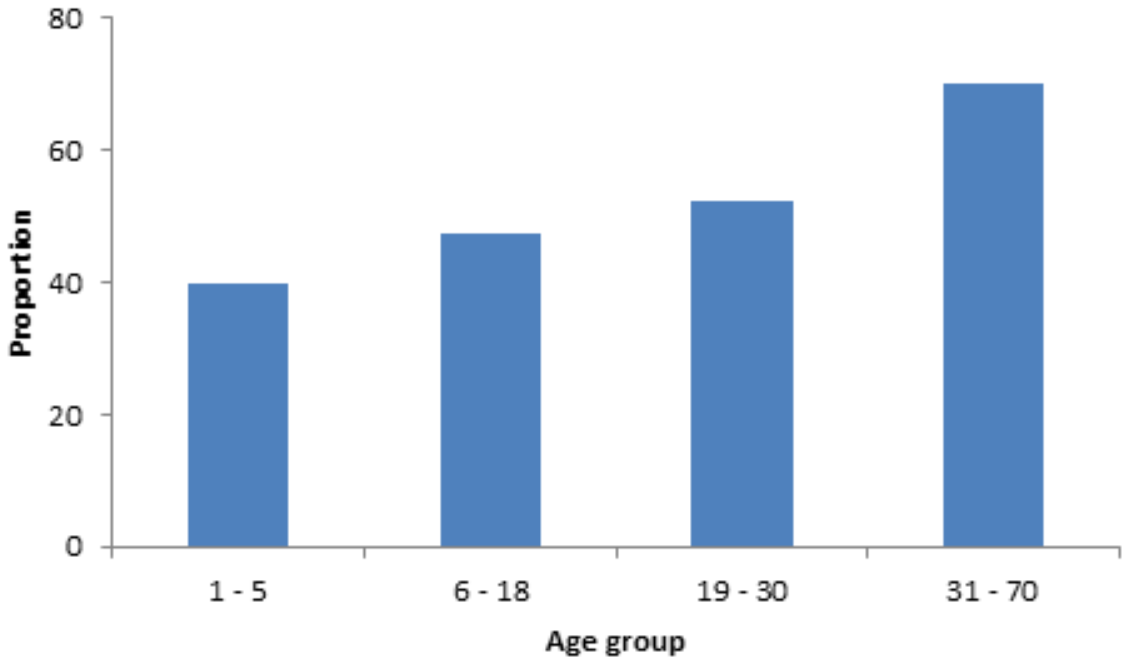


Fig. 4.5: Proportion of patient with *Plasmodium species* with age

4.3 *In vitro* Assay

4.3.1 Screening of patient

A total of 304volunteered patients that attended the hospital were screened. Out of these, 258 patients (84.9%) admitted not to have taken antimalarial drugs 14 days prior to attending the hospital, while 46 patients (15.1%) admitted to have taken antimalarial drugs 14 days prior to attending the hospital. 141 patients (54.7%) tested positive for *Plasmodium falciparum*. Out of the 141 patients, 93 (66%) had high parasitemia count (Table 4.2).

Table 4.2: Incidence of parasitemia in the blood samples of patients screened

Patient category	High count (> 1000 parasite /µl blood)	Low count (< 1000 parasite /µl blood)	No malarial parasite	Total
Patient with no antimalarial history within 14 days	93	48	117	258
Patient with antimalarial history within 14 days	8	24	14	46
Total	101	72	131	304

4.3.2 Evaluation of *In vitro* susceptibility *P. falciparum* isolates

A total of 93 *Plasmodium falciparum* isolates were processed, but 20 isolates were successfully evaluated for the antimalarial drugs, viz; Chloroquine, Amodiaquine, Artesunate and combinations of Artesunate-Amodiaqu4ine. Some samples were discarded due to failure of schizonts to mature satisfactorily, while others were lost due to fire accident in the laboratory during the study. The schizont maturation counts in Chloroquine, Amodiaquine, Artesunate and a combination of Artesunate-Amodiaquine were obtained (Appendix VI) and schizonts percentage inhibition for the test antimalarial drugs are presented in Tables 4.3, 4.4, 4.5 and 4.6.

Table 4.3: Degree of Chloroquine inhibition of schizont maturation (%) at different antimalarial concentrations (μM)

Parasitemia blood samples	A Control	B 0.2	C 0.4	D 0.8	E 1.6	F 3.2	G 6.4	H 12.8
1	0.0	27.3	49.1	69.1	81.8	94.5	100.0	100.0
2	0.0	15.5	52.1	69.0	78.9	94.4	100.0	100.0
3	0.0	46.2	67.7	84.6	93.8	100.0	100.0	100.0
4	0.0	37.6	54.1	80.0	89.4	96.5	100.0	100.0
5	0.0	24.3	51.4	71.6	85.1	93.2	100.0	100.0
6	0.0	51.6	75.0	82.8	89.1	96.9	100.0	100.0
7	0.0	31.4	60.8	64.7	72.5	80.4	92.2	100.0
8	0.0	37.3	55.2	70.1	86.6	91.0	95.5	100.0
9	0.0	29.6	57.4	66.7	81.5	92.6	100.0	100.0
10	0.0	25.5	42.9	60.2	79.6	89.8	95.9	100.0
11	0.0	27.5	47.8	69.6	73.9	89.9	95.7	100.0
12	0.0	26.9	55.2	68.7	76.1	86.6	100.0	100.0
13	0.0	59.5	78.4	91.9	100.0	100.0	100.0	100.0
14	0.0	39.5	60.5	86.0	97.7	100.0	100.0	100.0
15	0.0	65.9	81.8	89.8	96.6	100.0	100.0	100.0
16	0.0	32.4	67.6	86.5	97.3	100.0	100.0	100.0
17	0.0	44.3	67.0	75.3	87.6	100.0	100.0	100.0
18	0.0	43.2	73.0	86.5	97.3	100.0	100.0	100.0
19	0.0	17.5	36.3	70.0	91.3	98.8	100.0	100.0
20	0.0	45.0	87.0	87.0	94.0	100.0	100.0	100.0

Key: A is control, B to H represent concentration of test drug

Table 4.4: Degree of Amodiaquine inhibition of schizont maturation (%) at different antimalarial concentrations (μM)

Parasitemia blood samples	A control	B 0.04	C 0.08	D 0.16	E 0.32	F 0.64	G 1.28	H 2.56
1	0.0	21.4	41.1	60.7	73.2	92.9	100.0	100.0
2	0.0	17.1	42.9	70.0	81.4	95.7	100.0	100.0
3	0.0	35.8	62.7	77.6	86.6	94.0	100.0	100.0
4	0.0	17.6	34.1	62.4	77.6	90.6	96.5	100.0
5	0.0	48.6	68.1	84.7	91.7	95.8	100.0	100.0
6	0.0	30.6	61.3	75.8	85.5	93.5	100.0	100.0
7	0.0	43.4	60.4	67.9	81.1	88.7	94.3	100.0
8	0.0	26.2	44.6	66.2	83.1	87.7	95.4	100.0
9	0.0	25.5	45.5	52.7	65.5	80.0	90.9	100.0
10	0.0	19.6	46.7	67.4	80.4	91.3	96.7	100.0
11	0.0	27.5	58.0	72.5	85.5	95.7	100.0	100.0
12	0.0	25.0	52.9	67.6	73.5	86.8	95.6	100.0
13	0.0	56.8	78.1	93.2	100.0	100.0	100.0	100.0
14	0.0	39.5	58.1	86.0	97.7	100.0	100.0	100.0
15	0.0	69.7	80.9	92.1	97.8	100.0	100.0	100.0
16	0.0	29.7	73.0	89.2	97.3	100.0	100.0	100.0
17	0.0	39.6	68.8	77.1	93.8	100.0	100.0	100.0
18	0.0	42.9	68.6	85.7	100.0	100.0	100.0	100.0
19	0.0	24.2	66.1	85.5	96.8	100.0	100.0	100.0
20	0.0	42.6	85.1	85.1	95.0	100.0	100.0	100.0

Key: A is control, B to H represent concentration of test drug

Table 4.5: Degree of Artesunate inhibition of schizont maturation (%) at different antimalarial concentrations (μM)

Parasitemia blood samples	A Control	B 0.002	C 0.004	D 0.008	E 0.016	F 0.032	G 0.064	H 0.128
1	0.0	10.7	26.8	48.2	73.2	87.5	100.0	100.0
2	0.0	11.0	39.7	63.0	72.6	90.4	94.5	100.0
3	0.0	22.4	46.3	61.2	79.1	92.5	100.0	100.0
4	0.0	31.0	51.7	74.7	88.5	96.6	100.0	100.0
5	0.0	21.6	44.6	59.5	78.4	90.5	100.0	100.0
6	0.0	20.6	44.4	68.3	76.2	90.5	95.2	100.0
7	0.0	25.0	50.0	61.5	73.1	80.8	90.4	100.0
8	0.0	22.4	34.3	53.7	70.1	77.6	86.6	100.0
9	0.0	24.5	54.7	67.9	77.4	90.6	100.0	100.0
10	0.0	14.4	33.3	54.4	74.4	86.7	94.4	100.0
11	0.0	23.9	50.7	70.1	77.6	91.0	100.0	100.0
12	0.0	27.5	56.5	71.0	78.3	91.3	100.0	100.0
13	0.0	58.6	74.3	92.9	100.0	98.6	100.0	100.0
14	0.0	40.5	59.5	81.0	95.2	100.0	100.0	100.0
15	0.0	59.8	78.2	92.0	98.9	100.0	100.0	100.0
16	0.0	31.4	68.6	85.7	94.3	100.0	100.0	100.0
17	0.0	44.9	69.4	77.6	89.8	100.0	100.0	100.0
18	0.0	44.4	66.7	83.3	97.2	100.0	100.0	100.0
19	0.0	21.1	57.9	78.9	93.4	98.7	100.0	100.0
20	0.0	46.0	86.0	86.0	94.0	99.0	100.0	100.0

Key: A is control, B to H represent concentration of test drug

Table 4.6: Degree of Artesunate-Amodiaquine inhibition of schizont maturation (%) at different antimalarial concentrations (μM)

Parasitemia blood samples	A Control	B 0.021	C 0.042	D 0.084	E 0.168	F 0.336	G 0.672	H 1.344
1	0.0	9.3	38.9	50.0	77.8	92.6	100.0	100.0
2	0.0	27.8	58.3	73.6	86.1	95.8	100.0	100.0
3	0.0	27.3	54.5	71.2	81.8	95.5	100.0	100.0
4	0.0	20.0	37.6	65.9	83.5	94.1	100.0	100.0
5	0.0	32.9	58.9	75.3	90.4	95.9	100.0	100.0
6	0.0	49.2	68.3	81.0	87.3	95.2	100.0	100.0
7	0.0	21.2	46.2	61.5	67.3	78.8	88.5	100.0
8	0.0	23.1	36.9	56.9	73.8	81.5	90.8	100.0
9	0.0	22.2	53.7	64.8	81.5	88.9	96.3	100.0
10	0.0	14.0	31.2	54.8	74.2	81.7	93.5	100.0
11	0.0	29.0	53.6	71.0	79.7	92.8	100.0	100.0
12	0.0	24.6	59.4	71.0	84.1	92.8	100.0	100.0
13	0.0	68.5	79.5	91.8	97.3	100.0	100.0	100.0
14	0.0	34.9	62.8	83.7	97.7	100.0	100.0	100.0
15	0.0	64.8	80.7	93.2	100.0	100.0	100.0	100.0
16	0.0	27.8	66.7	88.9	100.0	100.0	100.0	100.0
17	0.0	51.0	67.3	75.5	91.8	100.0	100.0	100.0
18	0.0	38.9	69.4	88.9	100.0	100.0	100.0	100.0
19	0.0	27.2	66.7	84.0	95.1	100.0	100.0	100.0
20	0.0	46.5	86.9	86.9	92.9	99.0	100.0	100.0

Key: A is control, B to H represent concentration of test drug

The percentage schizont maturation inhibition was converted to probit and the respective LC_{50}, LC_{90}, LC_{95} and LC_{99} were computed for each *Plasmodium falciparum* isolate. The probit plots for the respective drugs are shown in Appendix VII. The results for each drug are presented herein. The inhibitory concentrations for Chloroquine, Amodiaquine, Artesunate and Artesunate-Amodiaquine are presented in Tables 4.7, 4.8, 4.9 and 4.10 respectively.

In Table 4.7, the IC_{50} of 19 isolates (95%) exceeded the peak plasma level (PPL) break point of 114 nM for Chloroquine. In Table 4.8, the IC_{50} of 7 isolates (35%) exceeded the peak plasma level (PPL) break point of 80 nM for Amodiaquine. In Table 4.9, the IC_{50} of 15 isolates (75%) exceeded the peak plasma level (PPL) break point of 2.8nM for Artesunate. The reported PPL break point of Artesunate is 2.8 nM and that of Amodiaquine is 80 nM. Considering the upper limit (80 nM) as the PPL break point of Artesunate-Amodiaquine, only one (5%) isolate had IC_{50} greater than 80 nM.

The *in vitro* response of malaria parasites in various drug concentrations are expressed as the IC_{50} value (the 50% inhibitory dose, ID_{50}, or the 50% effective dose, ED_{50}). This is the concentration of an antimalarial drug that inhibits 50% of schizont maturation as compared with the development in drug-free control wells (Basco, 2007). The drug resistant *P. falciparum* parasites were identified as isolates with IC_{50}values greater than published threshold values for sensitive parasite isolates. The threshold values compared were Chloroquine: $IC_{50} > 114$ nM; Amodiaquine: $IC_{50} > 80$ nM; Artesunate: $IC_{50} > 2.8$ nM. The results are presented in Table 4.11.

The *in vitro* susceptibility of *P. falciparum* isolates to the selected test antimalarial drugs is in this order: Artesunate-Amodiaquine>Amodiaquine>Artesunate>Chloroquine.

Table 4.7: Inhibitory concentrations (nM) of Chloroquine against schizont maturation

	LC_{50}	LC_{90}	LC_{95}	LC_{99}	R^2
1	431.28	2271.61	3624.73	8876.66	0.995
2	510.29	2266.87	3447.99	7703.15	0.958
3	202.66	1098.27	1766.58	4393.14	0.978
4	310.62	1661.97	2663.68	6578.55	0.992
5	365.30	2310.74	3882.04	10493.10	0.999
6	160.69	1484.98	2775.48	9203.13	0.972
7	335.42	6270.72	14288.69	69268.62	0.815
8	502.67	2672.71	4276.07	10525.03	0.803
9	392.44	2560.87	4340.08	11929.56	0.980
10	520.93	3372.72	5703.37	15610.67	0.999
11	459.77	3567.95	6348.88	19160.07	0.974
12	425.62	3868.47	7196.50	23649.42	0.953
13	154.45	694.83	1060.63	2385.73	0.999
14	272.38	883.53	1230.13	2319.71	0.994
15	105.53	754.61	1312.22	3789.32	0.988
16	288.08	907.78	1253.64	2327.42	0.996
17	230.97	1969.08	3597.69	11421.76	0.978
18	232.08	870.73	1262.96	2576.00	0.993
19	494.57	1556.15	2148.14	3984.92	0.993
20	159.61	928.04	1522.59	3932.75	0.800
Average	328.77	2098.83	3685.11	11506.44	

Table 4.8: Inhibitory concentrations (nM) of Amodiaquine against schizont maturation

	LC_{50}	LC_{90}	LC_{95}	LC_{99}	R^2
1	113.21	590.74	940.17	2290.86	0.982
2	102.56	413.85	612.69	1299.68	0.990
3	58.34	396.41	679.51	1908.98	0.983
4	123.46	590.36	916.75	2131.03	0.995
5	38.53	281.70	492.91	1440.38	0.988
6	66.48	417.29	699.54	1883.01	0.974
7	54.09	735.19	1531.53	6251.96	0.992
8	94.82	628.72	1070.35	2967.57	0.983
9	128.61	1634.18	3340.45	13150.46	0.979
10	102.54	531.83	844.96	2052.12	0.983
11	75.10	379.12	597.77	1430.77	0.987
12	91.78	803.22	1478.41	4760.29	0.954
13	34.27	130.12	189.37	388.73	0.997
14	57.69	182.07	251.55	467.39	0.982
15	22.00	125.27	204.34	521.95	0.990
16	56.62	170.23	232.00	419.95	0.978
17	53.14	251.34	389.09	899.12	0.966
18	48.03	198.63	296.10	636.47	0.998
19	64.93	189.83	256.68	457.68	0.984
20	42.66	112.50	147.77	249.23	0.962
Average	71.44	438.13	758.60	2280.38	

Table 4.9: Inhibitory concentrations (nM) of Artesunate against schizont maturation

	LC_{50}	LC_{90}	LC_{95}	LC_{99}	R^2
1	8.26	35.98	54.42	120.29	0.999
2	6.93	35.06	55.31	132.51	0.969
3	4.98	27.62	44.72	112.57	0.994
4	3.70	16.96	26.02	59.10	1.000
5	5.48	30.89	50.25	127.66	0.995
6	5.27	32.78	54.81	146.81	0.983
7	5.27	60.01	118.92	441.12	0.975
8	7.50	77.62	149.75	527.68	0.988
9	4.65	28.95	48.43	129.84	0.957
10	7.34	39.65	63.72	158.18	0.997
11	4.60	28.78	48.20	129.48	0.975
12	3.98	28.47	49.51	142.97	0.965
13	1.44	8.13	13.22	33.57	0.976
14	2.83	11.45	16.98	36.10	0.989
15	1.68	6.23	9.01	18.23	0.986
16	2.88	10.62	15.34	31.03	0.974
17	2.20	17.03	30.26	91.08	0.976
18	2.46	9.90	14.64	30.98	0.987
19	3.82	12.61	17.64	33.58	0.993
20	2.72	10.16	14.72	29.96	0.852
Average	4.40	26.45	44.79	126.64	

Table 4.10: Inhibitory concentrations (nM) of Artesunate-Amodiaquine against schizont maturation

	LC_{50}	LC_{90}	LC_{95}	LC_{99}	R^2
1	70.83	282.08	416.07	876.40	0.946
2	39.06	174.82	266.48	597.75	0.984
3	42.38	218.48	346.52	838.80	0.984
4	55.25	224.92	333.81	711.47	0.989
5	34.37	176.12	278.86	672.85	0.996
6	11.04	481.50	1392.25	10654.65	0.469
7	65.13	739.61	1464.83	5427.81	0.957
8	68.70	607.25	1120.83	3628.31	0.991
9	49.17	313.45	527.73	1432.34	0.966
10	81.70	471.33	771.58	1984.53	0.989
11	34.27	265.98	473.29	1428.32	0.883
12	44.36	294.14	500.74	1388.32	0.909
13	11.37	71.61	120.18	324.15	0.988
14	31.14	99.75	138.41	259.26	0.993
15	13.59	67.23	105.40	249.51	0.990
16	32.01	84.86	111.63	188.82	0.995
17	21.48	164.32	291.23	872.22	0.958
18	23.47	90.94	133.11	276.27	0.996
19	33.07	111.32	156.61	301.28	0.982
20	17.00	94.24	152.57	384.09	0.888
Average	38.97	251.70	455.11	1624.86	

Table 4.11: *In vitro* Susceptibility of *P. falciparum* isolates to selected antimalarial drugs

Antimalarial drugs	Total number of *P. falciparum* isolates susceptible (%)
Chloroquine (n=20)	5 (1)
Amodiaquine (n=20)	65 (13)
Artesunate (n=20)	25 (5)
Artesunate-Amodiaquine (n=20)	95 (19)

4.4 Molecular Analysis

The DNA of sixteen (16) *Plasmodium falciparum* parasite isolates were extracted and displayed in Appendix VIII, Plate I. Twelve (12) isolates have high concentration of DNA bands. Chloroquine Transporter (*Pfcrt*- RA) (1.6 kb) and Chloroquine (76 bp) is presented in Appendix VIII, Plate II. Eleven (11) *Plasmodium falciparum* parasite isolates were observed and displayed, had Chloroquine resistant gene which amplified around 76 bp. Fifteen (15) *Plasmodium falciparum* parasite isolates were observed to possess resistant *Pfcrt*/FB (76T) gene (Appendix VIII, Plate III) which amplified around 76 bp. Thirteen (13) *Plasmodium falciparum* parasite isolates were observed to possess multi-antimalarial drug resistant *Pfcrt*/FB (76K) gene which amplified around 76 bp (Appendix VIII, Plate VI). The multi-antimalarial drug resistance Gene *Pf*ATPase for Artemisinin derivatives is presented in Appendix VIII, Plate V. Twelve (12) *Plasmodium falciparum* parasite isolates were observed to possess resistant *Pf*ATPase gene which amplified around 432 bp. The base pair (bP) for the resistant Gene is presented in Table 4.12.

Table 4.12: Molecular analysis of the base pairs of resistant amplicons from the test *Plasmodium* species

DNA serial no	Blood *Isolate no*	Chloroquine *Pfcrt*-RA	multidrug *Pfcrt*/FB (76K)	multidrug *Pfcrt*/FB (76T)	PfATPase
2	1		150	109	
3	2	126	132	57	479
4	3		106	68	479
5	4	81	106	41	479
6	5	108	150	91	479
7	6	89	150	83	479
8	7	79	287	79	479
9	9	92	150	72	479
10	10	131	132	87	479
11	14	121	132	20.4	
12	15	139	132	26	479
13	16	139	132	100	479
14	17			100	
15	18			109	479
16	19		140	109	479
17	20				

Nine (9) malarial patient *P. falciparum* isolates were observed with multiple antimalarial drugs resistance across the test drugs with molecular size (DNA size) ranging from 26, 41, 57, 72, 79, 83, 87, 91100 and 479 bp. Three (3) malarial patient *P. falciparum parasite* isolates demonstrated resistant to two drugs with DNA size ranging from 20.4 and 132 for one patient, and from 68, 140 and 479 bp for the other two patients. Two (2) malarial patient *P. falciparum* isolates demonstrated resistant to two drugs with DNA size ranging from 109 and 150 bp, and 109 and 479 bp.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Nigeria has been reported with the largest population at risk of malaria in Africa (WHO, 2012). Malaria transmission in Nigeria takes place during both rainy and dry seasons in the southern part but have been reported to be more pronounced during rainy season in the northern region (WHO, 2012). Approximately 50% of the Nigerian population has been reported to experience at least one episode per year with an official estimate of an average of four malaria attack per person per year (WHO, 1995 and WHO, 2002).

The results of the retrospective study in this work showed that malaria patients visit hospital more during the rainy season (April to October). This rainy season avail good environmental condition for the vector of malarial parasites in Sabon-Wuse, Niger State, Nigeria.

The study of the blood samples of volunteered malarial patients showed that 54.2% of the patients that attended UMYMH had *Plasmodium* species in their blood. The results further showed that female (54.8%) was more infected than male (45.2%). These results point to the fact that female are more accessible to malaria parasite vector than male in the locality. The investigation further showed that 70% of sampled malarial patients were in the age group 31-70 years with *Plasmodium species* malarial parasite. *Plasmodium* species were not detected in the stained blood smears in a high proportion of children brought to hospital with the symptom of fever. The high proportion of adult who visited UMYMH for medical advice with fever had *Plasmodium* species. Ramadhan *et al*. (2000) stated that the frequency of malaria attack depends more on the immunity of the individual and the rate of exposure to the

parasites. The high incidence of *P. falciparum* with adult could be attributed to the high rate of exposure of this adult age group to the *P. falciparum* vector in Sabon-Wuse, Niger State, Nigeria.

This study also showed that the *Plasmodium falciparum* was 85.7% of the positive sample population, while 14.3% had mixed infection with *Plasmodium malariae*. The result showed that *Plasmodium falciparum* was the predominant *Plasmodium* species in Sabon-Wuse, Niger State, Nigeria.

Observation from this study also showed that 95% (19/20) of the parasites were resistant to test antimalarial drugs, while 5% (1/20) were sensitive *in vitro* to Chloroquine respectively. This observation is similar to Folarin *et al*. (2008) report that 85% (71/84) of malaria parasites in Ibadan, southwestern Nigeria were resistant to Chloroquine. Thirty-five per cent (7/20) were resistant to Amodiaquine, while 65% (13/20) of the *Plasmodium falciparum* parasites isolates were sensitive *in vitro* to Amodiaquine. This is similar to the earlier report in Southwest, Nigeria (Oyedeji *et al*., 2005), where resistant of *Plasmodium falciparum* isolate to Amodiaquine was found to be 39% (14/36). However, the result contradicts Falaki (2012) who reported that *Plasmodium falciparum* isolates in Kano were 100% sensitive to Amodiaquine. It was found that 25% (4/20) of the *Plasmodium falciparum* isolates were sensitive to Artesunate. This is similar to Na-Bangchang *et al*. (2013) who reported 36.7% declining in sensitivity to artesunate in Thai-Myanmar border.

One of the factors to be considered in the prophylaxis, treatment, and control of *Plasmodium falciparum* malaria is the resistance of parasite strains that may arise against virtually every drug available. Identification of *Pfcrt* as the central determinant of chloroquine-resistant *P.*

falciparum malaria provides a molecular marker that can be used for surveillance of resistance and to evaluate drug treatment and prophylaxis policies (Olasehinde *et al.*, 2014). This work shows that 58.8% (11 samples) had Chloroquine resistant gene which amplified around 76 bp. This investigation also shows that all the samples analysed had multi-antimalarial drug resistant gene *Pfcrt*/FB (76T) which amplified around 76 bp. Furthermore, 87.5% (14) of the *Plasmodium falciparum* isolate samples analysed had the multi-antimalarial drug resistant gene *Pfcrt*/FB (76K) around 76bp. PCR analysis finding corroborates the *in vitro* susceptibility result in this work. In addition, it agreed with earlier findings where molecular markers of resistance were found in samples that gave *in vivo* resistance/drug failure (Djimde *et al*., 2001; Chen *et al*., 2001; Folarin *et al*., 2008; Olasehinde *et al*., 2014).

This work showed that 75% (12) of the *Plasmodium falciparum* isolates analysed had *PfATPase* enzyme which amplified at 432 bp. This appears to indicate that the 12 samples had *PfATPase* gene, which codes for Artemisinin derivatives resistance. This agrees with artemisinin resistance reported in the Thai-Cambodia border (Noedl *et al*., 2009, Na-Bangchang *et al*., 2013) and the western border of Thailand (Aung *et al*., 2012). However, the resistance to Artemisinin derivatives in the study area contradicts earlier study in South-west Nigeria by Olasehinde *et al*. (2014), where 100% isolates carried the artemisinin sensitive wild type allele, S769. This calls for concern because Artemisinin and its derivatives are currently used as first line of treatment in Sabon-Wuse, Niger State, Nigeria; just like other African countries (Basco and Ringwald, 2001; Djimde *et al*., 2001; Mayor *et al*., 2001; Adagu and Warhurst, 2001; Babiker *et al*., 2001; Kyosiimire-Lugemwa *et al*., 2002; Olasehinde *et al*., 2014). From the retrospective study, malarial patients were prescribed with either single or combination of Artemisinin derivatives to the tune of 3,213

(97.2%) and 3,751 (97.9%) in 2011 and 2012 respectively. Resistant development to this antimalarial agent (Artesunate and its combinations) as observed in this study is a serious health hazard in Sabon-Wuse, Niger State Nigeria.

5.2 Summary

The observations made in this study are as follows:-

a) The total number of reported malaria cases increased from 3,350 in 2011 to 3,837 in 2012 (14.5% increase)

b) Artemesinin combination therapy (ACT) are the current first line drug commonly prescribed in Umaru Musa Yardua Memorial Hospital, Sabon-Wuse, Niger State, Nigeria.

c) *Plasmodium falciparum* is the predominant malaria parasite isolated from malaria patient in Sabon-Wuse.

d) There is a high resistant to Chloroquine drug based on the *in vitro* study, which is also the least prescribed antimalarial drugs in the hospital. DNA analysis indicates the presence of *Pfcrt*-Ra Gene in the study area, which further confirms resistance to Chloroquine drug.

e) *In vitro* study shows resistant of *P. falciparum* isolate to Artesunate drug in the study area. DNA analysis indicates the presence of *PfATPase* Gene in the study area, which indicate resistance to Arthemisinin based drugs like Artesunate .

f) Thirty-five percent of the *P. falciparum* isolates were found to be resistant to Amodiaquine drug.

g) Artesunate-Amodiaquine combination shows a high sensitivity to *P. falciparum* isolates in the study area.

5.3 Conclusion

This study showed the presence of MDR, ATPase and Chloroquine resistant genes in *P. falciparum* isolated in Sabon-Wuse, Niger State. These genes might be responsible for resistant to some of the antimalarial drugs used in Sabon-Wuse, Niger State. Based on the findings from this study, possibility of eradicating malaria is still a problem in our environment.

5.4 Recommendation

There is the need for periodic antimalarial surveillance in order to curb the wide spread of resistance associated with antimalarial drugs in Sabon-Wuse, Nigeria.

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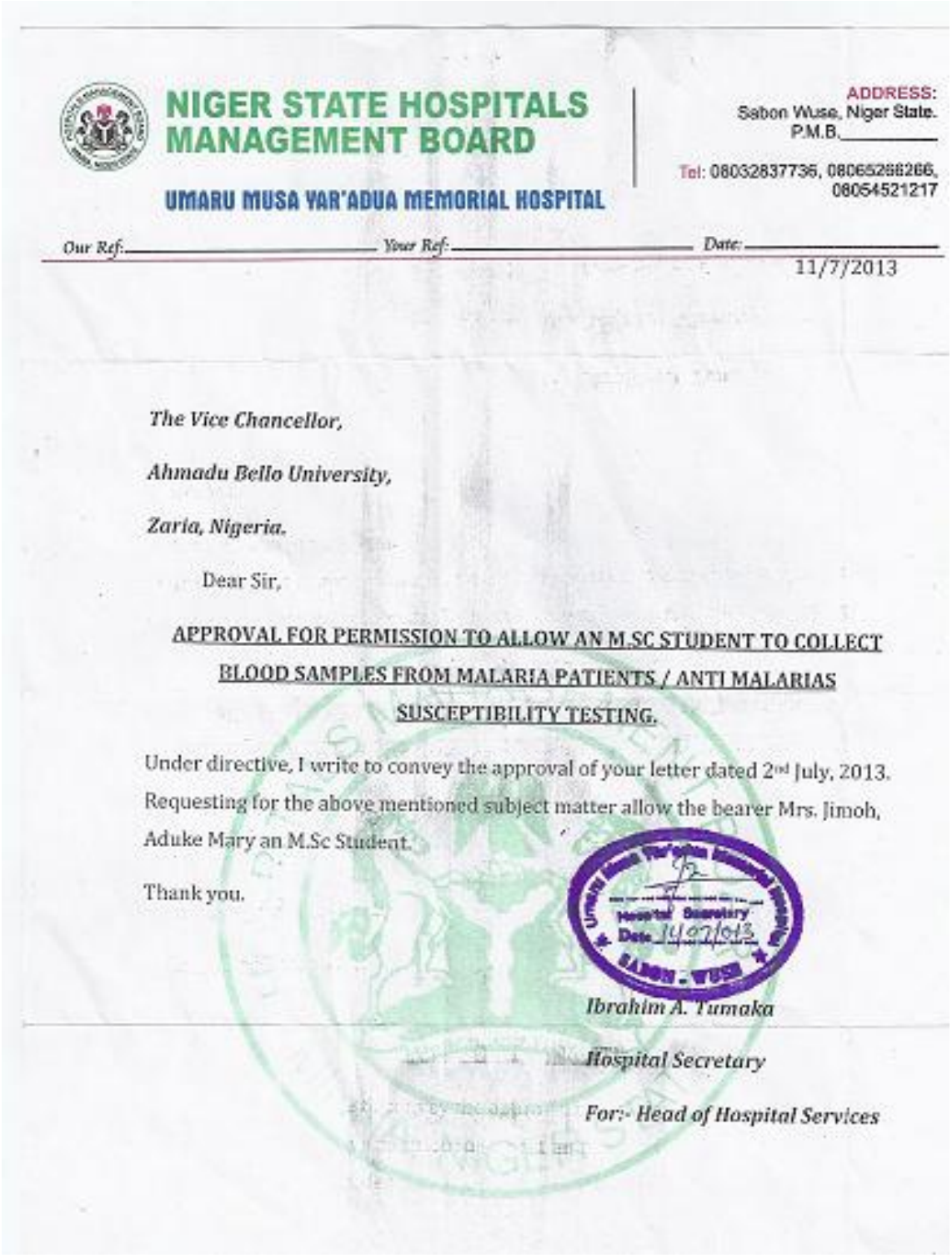
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APPENDIX I

ETHICAL APPROVAL



NIGER STATE HOSPITALS MANAGEMENT BOARD

UMARU MUSA YAR'ADUA MEMORIAL HOSPITAL

ADDRESS:
Sabon Wuse, Niger State.
P.M.B._____
Tel: 08032837736, 08065266266, 08054521217

*Our Ref:*_____ *Your Ref:*_____ *Date:*_____
11/7/2013

The Vice Chancellor,

Ahmadu Bello University,

Zaria, Nigeria.

Dear Sir,

APPROVAL FOR PERMISSION TO ALLOW AN M.SC STUDENT TO COLLECT BLOOD SAMPLES FROM MALARIA PATIENTS / ANTI MALARIAS SUSCEPTIBILITY TESTING.

Under directive, I write to convey the approval of your letter dated 2nd July, 2013. Requesting for the above mentioned subject matter allow the bearer Mrs. Jimoh, Aduke Mary an M.Sc Student.

Thank you.

Hospital Secretary
Date 14/7/2013
SABON - WUSE

Ibrahim A. Tumaka

Hospital Secretary

For:- Head of Hospital Services

APPENDIX II

REVIEW OF MALARIA TREATMENT RECORDS

Table IIa: Number of cases by age, sex and occupation

No of cases	Age group			Sex			Occupation					
	Children	Young adult	Adult	M	F		A	B	C	D	E	F
					I	II						

Key:

A=Students	B=Civil Servants	C=Business
D=Housewife	E=Farmer	F=Unknown
I=Pregnant female	II=Non-pregnant female	
Children= 0-5years	Young Adult= 6-18 years	Adult = above 18 years

Table IIb: Number of cases per drug prescribed

Drugs								
A	B	C	D	E	F	G	H	I

Key:

A= Chloroquine	B= Coartal forte or Coartem	C= Paluther + Fansidar
D= Paluther + Artesunate	E= Artesunate + Fansidar	F= Paluther + Quinine
G= Paluther	H= Paluta + p-alaxin or Solatep	I= Amodaquinine + Artesunate

APPENDIX III

PREPARATION OF SOLUTIONS

(a) Preparation of Chloroquine

The stock solution of chloroquine was prepared by dissolving 66.0mg of Chloroquine diphosphate (Formula Weight 515.9) in 100ml sterile distil water. The molar concentration of the stock solution was calculated and the working solution was obtained by diluting the stock solution 1/100 in distilled water.

(b) Preparation of Amodiaquine

The stock solution of Amodiaquine was prepared by dissolving 11.9mg of Amodiaquine dihydrochloride (Formula Weight 464.8) in 100ml sterile distil water. The molar concentration of the stock solution was calculated and the working solution was obtained by diluting the stock solution 1/100 in distilled water.

(c) Preparation of Artesunate

The stock solution of Artesunate was obtained by dissolving 49.2 mg of Artesunate (Formula Weight 384.4) in 100ml of pure methanol. The molar concentration of the stock solution was calculated. An intermediate solution was prepared by diluting the stock solution by 1/100 in pure methanol. The working solution was obtained by further diluting the intermediate solution by 1/100 in pure methanol.

APPENDIX IV

Layout of the micro-culture plate

Well	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Well A is the control. Well B to H represent drug concentrations, e.g. Chloroquine: 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 µmol per l blood.

APPENDIX V

BUFFER AW1, AW2, AL and AE SOLUTIONS

Buffer AW1 and Buffer AW2 are supplied as concentrates. Buffer AW1 is supplied as 242 ml Wash Buffer (1) Concentrate and Buffer AW2 as 324 ml Wash Buffer (2) Concentrate 1. Buffer AW1 is used to denature proteins. Buffer AW2 is 70% ethanol to wash salts out. Buffer AW1 contains guanidine hydrochloride. Buffer AW2 contains a qiagen concentrate.

Buffer AL (216 ml Lysis Buffer) contains guanidine hydrochloride, which can form highly reactive compounds when combined with bleach.

Buffer AE is 10 mMTris·Cl, 0.5 mM EDTA, pH 9.0. Elution with Buffer AE guaranteesoptimal recovery and stability of eluted DNA. For long-term storage of DNA, elution in Buffer AE is stronglyrecommended since DNA stored in water is subject to acid hydrolysis. Buffer AE shouldbe used at room temperature (15–25°C). Heating Buffer AE before elution is notnecessary.

A **lysis buffer** is a buffer solution used for the purpose of lysing cells for use in molecular biology experiments that analyze the compounds of the cells (e.g. western blot). Most lysis buffers contain salts (e.g.Tris-HCl or EDTA) to regulate the acidity and osmolarity of the lysate, while detergents (such as Triton X-100 or SDS) are added to break up membrane structures. In studies like DNA fingerprinting the lysis buffer is used for DNA isolation. Dish soap can be used in a pinch to break down the cell and nuclear membranes, allowing the DNA to be released.

APPENDIX VI

SCHIZONT MATURATION COUNTS

Table IV.1: Chloroquine

	Schizont counts (per 200 parasites) in well								
	A	B	C	D	E	F	G	H	
Test Number	Control	0.2	0.4	0.8	1.6	3.2	6.4	12.8	µmol/l
1	55	40	28	17	10	3	0	0	
2	71	60	34	22	15	4	0	0	
3	65	35	21	10	4	0	0	0	
4	85	53	39	17	9	3	0	0	
5	74	56	36	21	11	5	0	0	
6	64	31	16	11	7	2	0	0	
7	51	35	20	18	14	10	4	0	
8	67	42	30	20	9	6	3	0	
9	54	38	23	18	10	4	0	0	
10	98	73	56	39	20	10	4	0	
11	69	50	36	21	18	7	3	0	
12	67	49	30	21	16	9	0	0	
13	148	60	32	12	0	0	0	0	
14	86	52	34	12	2	0	0	0	
15	176	60	32	18	6	0	0	0	
16	74	50	24	10	2	0	0	0	
17	97	54	32	24	12	0	0	0	
18	74	42	20	10	2	0	0	0	
19	160	132	102	48	14	2	0	0	
20	200	110	26	26	12	0	0	0	

Table IV.2: Amodiaquine

	Schizont counts (per 200 parasites) in well								
	A	B	C	D	E	F	G	H	
Test Number	Control	0.04	0.08	0.16	0.32	0.64	1.28	2.56	µmol/l
1	56	44	33	22	15	4	0	0	
2	70	58	40	21	13	3	0	0	
3	67	43	25	15	9	4	0	0	
4	85	70	56	32	19	8	3	0	
5	72	37	23	11	6	3	0	0	
6	62	43	24	15	9	4	0	0	
7	53	30	21	17	10	6	3	0	
8	65	48	36	22	11	8	3	0	
9	55	41	30	26	19	11	5	0	
10	92	74	49	30	18	8	3	0	
11	69	50	29	19	10	3	0	0	
12	68	51	32	22	18	9	3	0	
13	146	63	32	10	0	0	0	0	
14	86	52	36	12	2	0	0	0	
15	178	54	34	14	4	0	0	0	
16	74	52	20	8	2	0	0	0	
17	96	58	30	22	6	0	0	0	
18	70	40	22	10	0	0	0	0	
19	124	94	42	18	4	0	0	0	
20	202	116	30	30	10	0	0	0	

Table IV.3: Artesunate

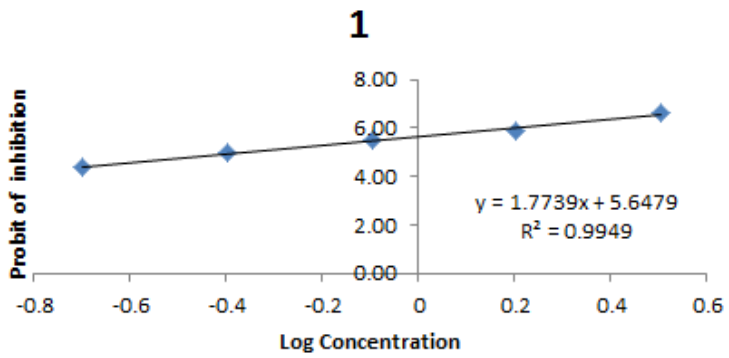
	Schizont counts (per 200 parasites) in well								
	A	B	C	D	E	F	G	H	
Test Number	Control	0.002	0.004	0.008	0.016	0.032	0.064	0.128	µmol/l
1	56	50	41	29	15	7	0	0	
2	73	65	44	27	20	7	4	0	
3	67	52	36	26	14	5	0	0	
4	87	60	42	22	10	3	0	0	
5	74	58	41	30	16	7	0	0	
6	63	50	35	20	15	6	3	0	
7	52	39	26	20	14	10	5	0	
8	67	52	44	31	20	15	9	0	
9	53	40	24	17	12	5	0	0	
10	90	77	60	41	23	12	5	0	
11	67	51	33	20	15	6	0	0	
12	69	50	30	20	15	6	0	0	
13	140	58	36	10	0	2	0	0	
14	84	50	34	16	4	0	0	0	
15	174	70	38	14	2	0	0	0	
16	70	48	22	10	4	0	0	0	
17	98	54	30	22	10	0	0	0	
18	72	40	24	12	2	0	0	0	
19	152	120	64	32	10	2	0	0	
20	200	108	28	28	12	2	0	0	

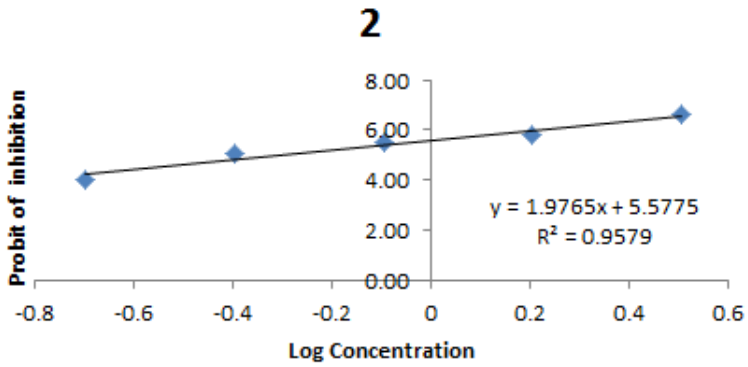
Table IV.4: Artesunate_amodiaquine

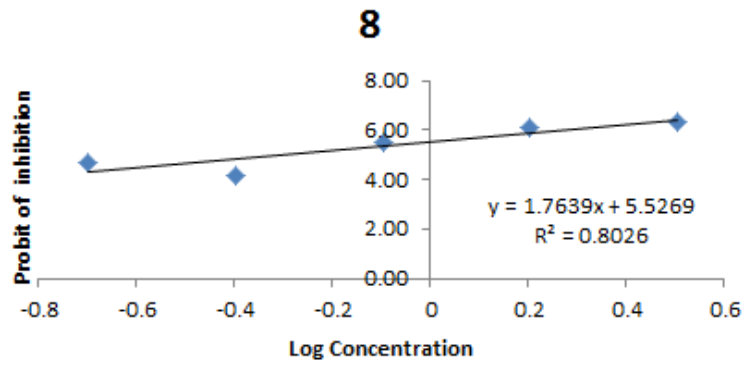
	Schizont counts (per 200 parasites) in well								
	A	B	C	D	E	F	G	H	
Test Number	Control	0.021	0.042	0.084	0.168	0.336	0.672	1.344	µmol/l
1	54	49	33	27	12	4	0	0	
2	72	52	30	19	10	3	0	0	
3	66	48	30	19	12	3	0	0	
4	85	68	53	29	14	5	0	0	
5	73	49	30	18	7	3	0	0	
6	63	32	20	12	8	3	0	0	
7	52	41	28	20	17	11	6	0	
8	65	50	41	28	17	12	6	0	
9	54	42	25	19	10	6	2	0	
10	93	80	64	42	24	17	6	0	
11	69	49	32	20	14	5	0	0	
12	69	52	28	20	11	5	0	0	
13	146	46	30	12	4	0	0	0	
14	86	56	32	14	2	0	0	0	
15	176	62	34	12	0	0	0	0	
16	72	52	24	8	0	0	0	0	
17	98	48	32	24	8	0	0	0	
18	72	44	22	8	0	0	0	0	
19	162	118	54	26	8	0	0	0	
20	198	106	26	26	14	2	0	0	

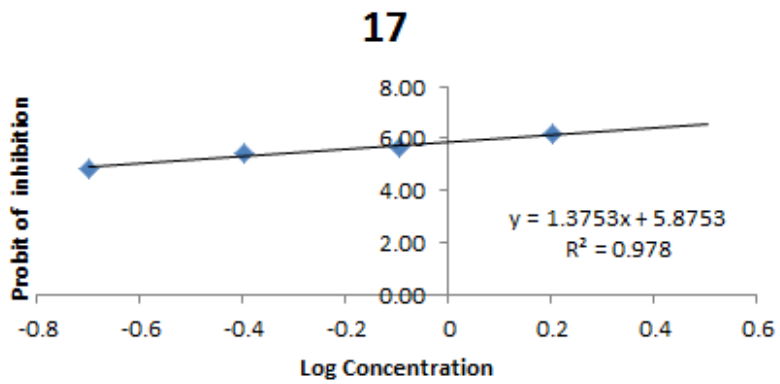
APPENDIX VII

PROBIT OF SMI VERSUS LOG CONCENTRATION FOR CHLOROQUINE









APPENDIX VIII

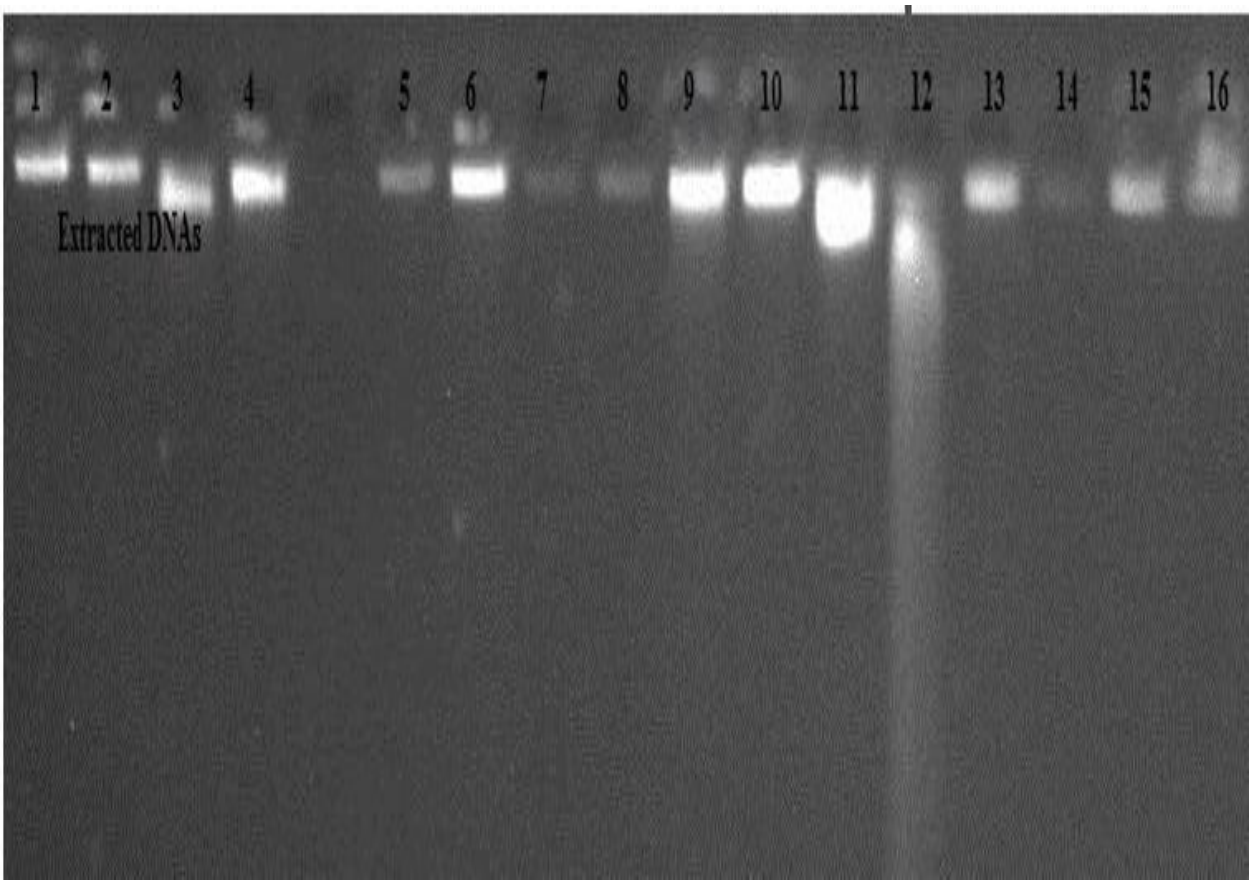


Plate I: DNA Extraction

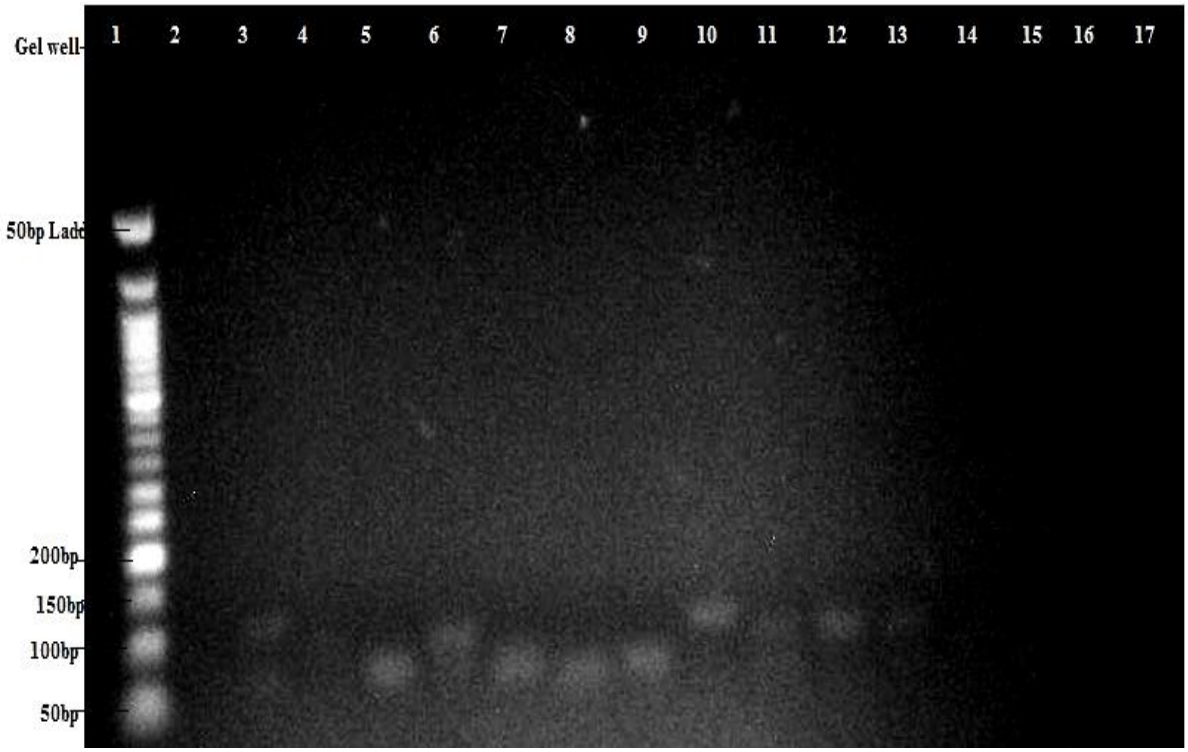


Plate II: Chloroquine Transporter (*Pfcrt*- RA) (1.6kb) and Chloroquine (76bp)

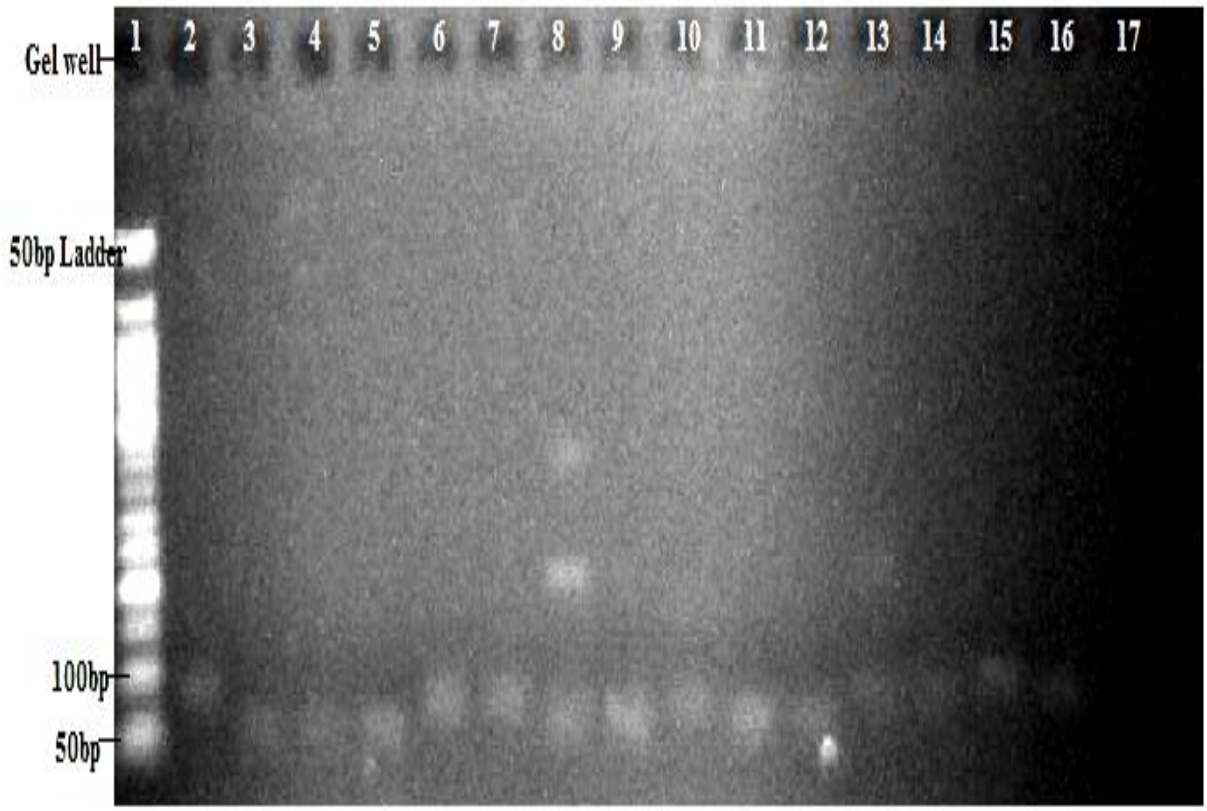


Plate III: Multidrug Resistance Gene *Pfcrt*/FB (76T)

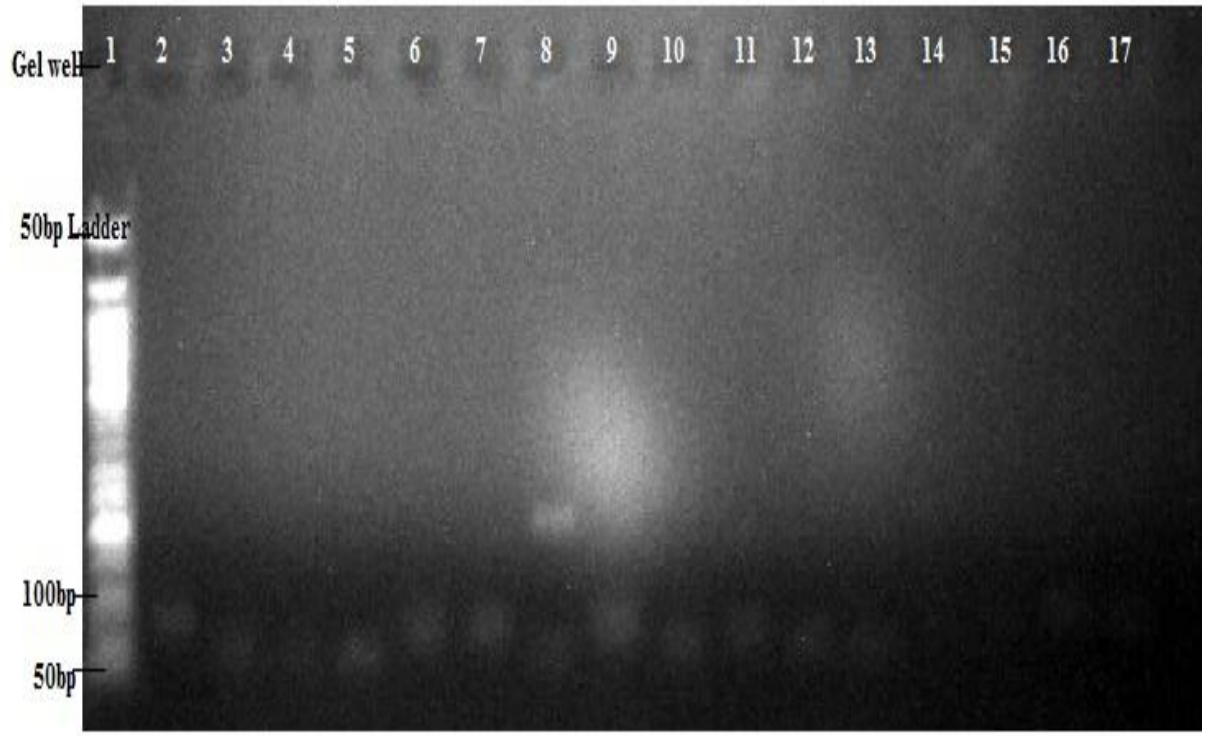


Plate IV: Multi-antimalarial drug Resistance Gene *Pfcrt*/FB (76K)

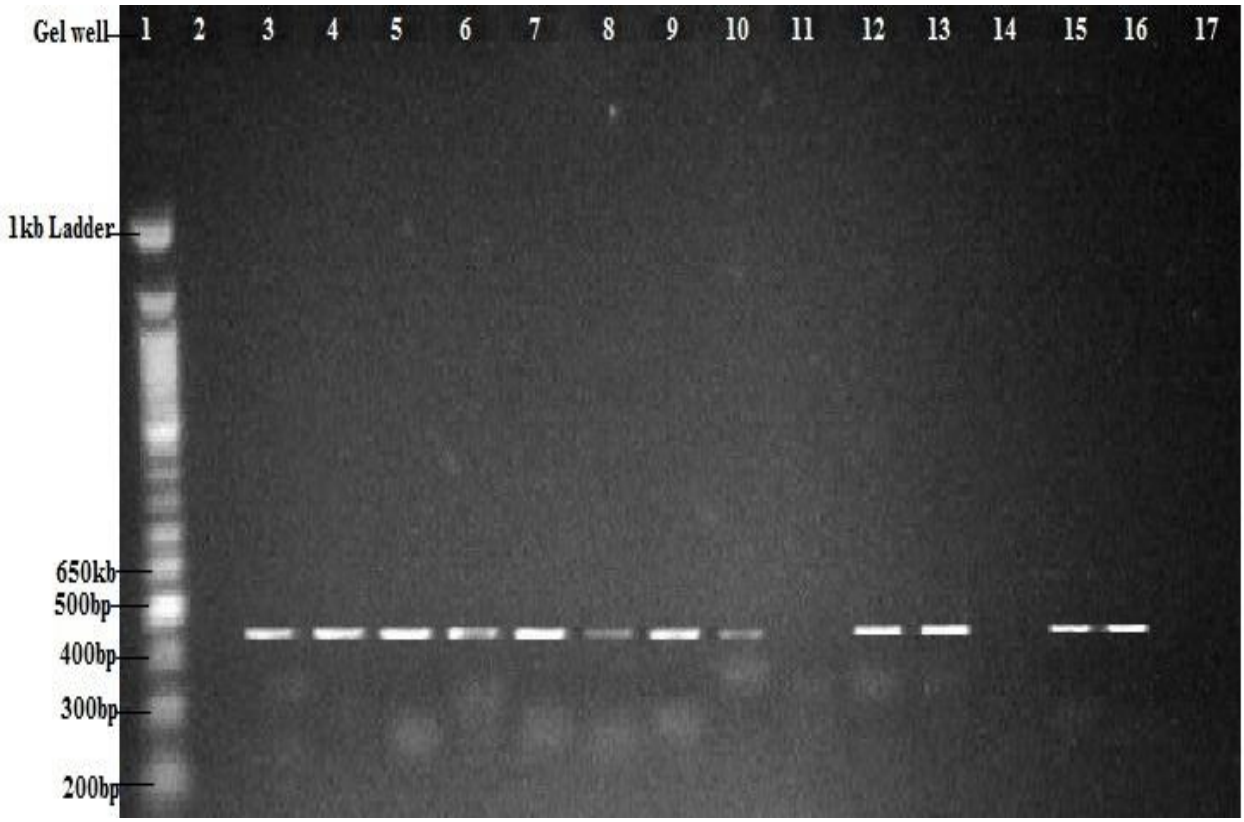


Plate V: *Pf*ATPase for Artemisinin derivatives