INVITRO SUSCEPTIBILITY PROFILE OF *Plasmodiumfalciparum* ISOLATES TO ANTIMALARIAL AGENTS COMMONLY USED IN DALHATU ARAF SPECIALIST HOSPITAL LAFIA NIGERIA

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BY

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DEPARTMENT OF PHARMACEUTICAL MICROBIOLOGY,
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ZARIA

AUGUST, 2021

DECLARATION

I declare that the work reported in this thesis entitled “IN VITROSUSCEPTIBILITY PROFILE OF *Plasmodiumfalciparum* ISOLATES TO ANTIMALARIAL AGENTS COMMONLY USED IN DALHATU ARAF SPECIALIST HOSPITAL LAFIA NIGERIA” was conducted by me in the Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, under the supervision of Prof. J. O. Ehinmidu and Dr. R. O.Bolaji.

The information derived from the literature review has been duly acknowledged in the text and a list of the references provided. No part of this thesis has been presented in any previous application for another degree or diploma at any university.

Name	Signature	Date

CERTIFICATION

This thesis entitled "IN *VITRO* SUSCEPTIBILITY PROFILE OF *Plasmodiumfalciparum* ISOLATES TO ANTIMALARIAL AGENTS COMMONLY USED IN DALHATU ARAF SPECIALIST HOSPITAL LAFIA NIGERIA" by FaizahOseze SANI meets the regulations governing the award of the Degree, Masters of Science of Ahmadu Bello University, Zaria, Nigeria and is approved for its contribution to knowledge and literary presentation.

Prof. J.O. Ehinmidu	_____	_____
Chairman, Supervisory Committee	Signature	Date
Dr. R.O. Bolaji	_____	_____
Member, Supervisory Committee	Signature	Date
Prof. J.O. Ehinmidu	_____	_____
Head of Department	Signature	Date
Prof. S.A. Abdullahi	_____	_____
Dean Postgraduate School	Signature	Date

DEDICATION

I dedicate this work to my Mother and the bid for Malaria eradication.

ACKNOWLEDGEMENT

In the name of Allah, the beneficient and most Merciful. All thanks to Allah for the guidance, strength and knowledge to carry out this research. I applaud the dedication of my supervisor, Prof. J.O. Ehinmidu for his guidance, patience and efforts towards this work, may all his endeavours be fruitful. I also appreciate the effort of my second Supervisor, Dr. R. O. Bolaji for her efforts and support towards this work.

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ABSTRACT

Malaria is an infectious parasitic disease of public health importance. Several efforts have been made in the fight against malaria. This fight has however been prolonged due to several challenges, among which is the emergence of resistant strains to single and combined antimalarial drugs. This study was designed to determine the incidence of malaria and the susceptibility of *Plasmodium falciparum* isolates from patients of Dalhatu Araf Specialist Hospital Lafia to commonly prescribed antimalarials in Lafia. A retrospective study was carried out to determine the prevalence and to know the pattern of antimalarial drug prescriptions, age and gender of patients treated with malaria from 2014 - 2016. Susceptibility profile of *Plasmodium falciparum*isolates to test antimalarials was carried out*In vitro,* DNA of the resistant *P. falciparum* isolates were extracted and amplified to detect the presence of *Pf*mdr1, *Pf*crt and K13P genes.The observed Malaria prevalence was 5.90%, 4.40% and 4.80% for 2014, 2015 and 2016 respectively. The incidence of malaria among different age groups were 19.08%, 15.88%, and 65.04% for 0-5 years, 6-18 years and 19 years above respectively. Malaria was observed to be higher in females 58.87%, than male 41.11%. Antimalarial drug prescription was basically Artemisinin combination therapy 88.67%. Out of the 91 *Plasmodium falciparum* isolates evaluated, 32 (35.16%) were resistant to piperaquine, 26 (28.57%) were resistant to artesunate, 25 (27.00%) were resistant to chloroquine, 16 (17.58%) were resistant to Lumefantrine and 10 (10.99%) were resistant to artemether. Study on the presence of *Pf*mdr1 gene on eight multidrug resistant *P. falciparum* isolates showed that 6(75.00%) of the isolates harboured the *Plasmodium falciparum* multidrug resistance gene, none of the isolates harboured the Kelch 13 Propeller (K13P) gene and *Plasmodium falciparum* chloroquine resistant transporter gene. This study gives current information on the incidence of reported malaria and antimalarial susceptibility pattern of *Plasmodium falciparum*Isolates in Dalhatu Araf Specialist Hospital Lafia, Nigeria.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

CHAPTER 1

1.0 INTRODUCTION

Malaria is an infectious parasitic disease of public health importance and a major health problem in tropical countries (W.H.O, 2015). The disease is caused by hemoprotozoa of the genus *Plasmodium*. These parasites are transmitted by the bites of infected female anopheles mosquitoes. Six malaria species are known to cause human malaria: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale curtisi, Plasmodium ovale wallikeri, Plasmodium malariae,* and *Plasmodium knowlesi* (Sutherland *et al.,* 2010). Malaria caused by *P. falciparum* which is referred to as *falciparum* malaria, formerly known as subtertian or malignant tertian malaria is the most widespread and pathogenic of the human species with untreated infections causing severe disease and death, particularly in young children, pregnant women and non immune adults (Olowe *et al.,*2015).

The burden of malaria is huge and resources in Nigeria to tackle this problem are inadequate (PMI, 2018). Analysis shows that 10 of the 11 high-burden countries that accounted for 70% malaria burden in 2017 are sub-Saharan African countries, Nigeria inclusive. This countries were reported to not be on track to meet the Global Technical Strategy targets as at 2017 (WHO 2018).

Several efforts have been made in the fight against malaria, the fight is however being prolonged and in some places slowed down, by interconnected challenges, These are;

a. lack of robust, predictable and sustained international and domestic financing. This is compounded by the difficulty in maintaining political commitment and ensuring regional collaboration at the highest levels and

b. Emergence of parasite resistance to antimalarial medicines and of mosquito resistance to insecticides (GTSM, 2016).

To overcome the widespread and increasing level of parasite resistance to antimalarial drugs, the World Health Organization (WHO) recommended the use of artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated *P. falciparum*malaria. Base on this recommendation, most malaria endemic countries worldwide changed their antimalarial treatment protocols to the use of artemether (ATH) lumefantrine (LUM) and artesunate (AS)-amodiaquine (AQ) as first-line treatment of uncomplicated malaria (WHO, 2010).

Health education to the people and the community on malaria control measures (Hall, 2006).Treatment measures include early diagnosis and administration of appropriate drugs. Measures necessary for the prevention and control of malaria include efforts directed against the breeding of mosquito larva such as flushing or draining of breeding sites, clearing vegetation and spraying breeding sites with oil or chemicals and efforts directed against mosquito bites include bed netting treated with insecticides, wearing protective clothing, use of mosquito repellant and insecticides. Despite these efforts, progress in rolling back malaria is stalling (WHO 2018).

1.1 Statement of the Research Problem

Malaria is an important public health concern that threatens the lives and wellbeing of millions of people particularly in the tropics and place enormous burden on national health services. In the midst of this scourge, antimalarial drug resistance is one of the greatest challenges facing malarial control(Peletiri *et al.,* 2010).The World Health Organization (W.H.O 2019), reported 228 million cases and 405 000 deaths due to malaria globally in 2018, most of the cases in 2018 were in the WHO African Region (85%). The WHO African region also accounted for 94%

death in 2018. Among countries, Nigeria accounted for the highest proportion of malaria cases globally (25%), followed by the Democratic Republic of the Congo (12%), Uganda (5%), and Côte d'Ivoire, Mozambique and Niger (4% each). Nigeria also accounted for 24% deaths of the global estimate (WHO 2017).

In 2016, an estimated 323,892 malaria cases were reported in Nassarawa State. Being among the states reporting high malaria incidence, it was selected as one of the President Malaria Initiative(PMI) focus states when Nigeria began implementation as a President Malaria Initiative focus country in fiscal year (FY) 2011 along side two other states; Cross River and Zamfara and will still remain among the focal states in 2019 (PMI 2018).

This therefore necessitates the study of the disease pattern and resistance factors which will give a better understanding of its management through improved methods that give way to its prevention, better treatment and breaking the walls of malaria and Malarial drug resistance in the locality.

1.2 Justification

Emergence of resistant strains of malaria parasites to single and combined antimalarials has worsened the health and economic burden of Malaria globally and therefore made it imperative to verify the efficacy of commonly used antimalarial drugs to treat malaria in Nigeria.

Monitoring of drug resistance in malaria-endemic countries like Nigeria, with research into key contributing factors to resistance will enable effective prevention of drug resistance from spreading.

1.3 Null Hypothesis

The *Plasmodiumfalciparum* isolates obtained from patients attending Dalhatu Araf Specialist Hospital Lafia, Nassarawa State, Nigeria are not susceptible to the commonly prescribed antimalarial drugs in the hospital.

1.4 Alternate Hypothesis

The *Plasmodiumfalciparum* isolates obtained from patients attending Dalhatu Araf Specialist Hospital Lafia, Nassarawa State, Nigeria are susceptible to the commonly prescribed antimalarial drugs in the hospital.

1.5 Aim of the Research

The aim of this study is to determine the susceptibility profile of *Plasmodium falciparum* isolates from patients of Dalhatu Araf Specialist Hospital, Lafia to the commonly prescribed antimalarial drugs.

1.6 Research Objectives

The research objectives are to:

a. determine the incidence of malaria at Dalhatu Araf Specialist hospital Lafia for three consecutive years.

b. determine the antimalarial drugs commonly prescribed at Dalhatu Araf Specialist Hospital Lafia Nassarawa State, Nigeria.

c. identify the prevailing *Plasmodium* species in Lafia.

d. determine the susceptibility of *Plasmodiumfalciparum*isolates to some antimalarial drugs commonly prescribed in the selected hospital.

e. characterize the resistance gene present among the resistant *Plasmodium falciparum* isolates from the selected hospital.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 History of Malaria

Malaria originated in Africa and accompanied human migration to the Mediterranean shores, India and South East Asia. In the past it used to be common in the marshy areas around Rome. Malaria is a disease mostly of tropical and subtropical areas, it is particularly prevalent in sub-Saharan Africa, but also common throughout other tropical regions of China, India, Southeast Asia , South and Central America (Arora and Arora; (2008).

Ancient accounts of malaria date back to Vedic writings of 1600 B.C.E. in India and to the fifth century B.C.E. in Greece, when the Greek physician Hippocrates, described the characteristics of the disease and related them to seasons and location. The discovery of an association of malaria with stagnant waters led the Romans to develop drainage programs, which were among the first documented preventions against malaria. In seventh-century Italy, the disease was prevalent in foul-smelling swamps near Rome and was named mal' aria Italian for “bad air,” and was also known as Roman fever, ague, marsh fever, periodic fever, paludism (Martin, 2003).

The organism was first seen by Lavern in 1880 at a military hospital in Constantine, Algeria, when he discovered a microgametocyte exflagellating. In 1885, similar organisms were discovered within the blood of birds in Russia. There was brief speculation that birds might be involved in the transmission of malaria; in 1894 Patrick Manson hypothesized that mosquito could transmit malaria. The work of Giovanni Battista Grassi in Italy 1899 further confirmed that human malaria could only be transmitted by Anopheles mosquito. The British physician

Ronald Ross in India demonstrated the existence of *Plasmodium* in the wall of the midgut and salivary glands of a Culex mosquito using bird species as the vertebrate host in 1898 (Akanbi *et al.*, 2009).

2.2 Signs and Symptoms of Malaria

All the manifestations of malarial illness are caused by the infection of the red blood cells by the asexual forms of the malaria parasite and the involvement of the red cells makes malaria a potentially multisystem disease, as every organ of the body is reached by the blood (Srabasti *et al.*, 2008). All types of malaria manifest with common symptoms such as fever, some patients may progress into severe malaria. Although severe malaria is more often seen in cases of *P. falciparum* infection, complications and even deaths have been reported in non-*falciparum* malaria as well.

At the completion of the schizogony within the red cells, each cycle lasting 24-72 hours depending on the species of the infecting parasite, newly developed merozoites are released by the lysis of infected erythrocytes and along with them, numerous known and unknown waste substances, such as red cell membrane products, hemozoin pigment, and other toxic factors such as glycosylphosphatidylinositol (GPI) are also released into the blood. These products, particularly the GPI, activate macrophages and endothelial cells to secrete cytokines and inflammatory mediators such as tumor necrosis factor, interferon-γ, interleukin-1, IL-6, IL-8, macrophage colony-stimulating factor, and lymphotoxin, as well as superoxide and nitric oxide (NO) (Srabasti *et al.*, 2008).

The systemic manifestations of malaria such as headache, fever and rigors, nausea and vomiting, diarrhoea, enorexia, tiredness, achingjoints and muscles, thrombocytopenia, immunosuppression,

coagulopathy, and central nervous system manifestations have been largely attributed to the various cytokines released in response to these parasite and red cell membrane products (Cheesemanet *al.,* 2012). In addition to these factors, the plasmodial DNA is also highly proinflammatory and can induce cytokinemia and fever. The plasmodial DNA is presented by hemozoin (produced during the parasite development within the red cell) to interact intracellularly with the Toll-like receptor-9, leading to the release of proinflammatory cytokines that in turn induce COX-2-upregulating prostaglandins leading to the induction of fever (Peggy *et al.,* 2007).Hemozoin has also been linked to the induction of apoptosis in developing erythroid cells in the bone marrow, thereby causing anemia (Lamikanra *et al.,* 2006; Gordon *et al.,*2007). More severe complications can be associated with infections caused by *P. falciparum*, where erythrocytes infected with parasites adhere to the vascular endothelium of post-capillary venules, particularly in the brain. Vascular obstruction and/or immune reactions against the parasites can result in impaired consciousness, delirium, seizures, coma and death (White *et al*, 2014; Breman, 2015).

2.3 Causative Agent

The disease is caused by hemoprotozoa of the genus *Plasmodium*. These parasites are transmitted by the bites of infected female anopheles mosquitoes. Six *Plasmodium* species are known to cause human malaria: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale curtisi, Plasmodium ovale wallikeri, Plasmodium malariae,* and *Plasmodium knowlesi* (Sutherland *et al.,* 2010).

2.4 Life Cycle of Human Malaria Parasite

The life cycle of malaria parasite involve alternating cycles of asexual division (Schizogony) occuring in man (Intermediate host) and sexual development (sporogony) occuring in female

anopheles mosquito which serves as a definitive host (Olowe *et al.*, 2015).The infective form of the parasite (sporozoites), are present in the salivary glands of female anopheles mosquito and are injected directly into the blood stream when it bites a human host. After injection, all the sporozoites leave the blood stream and enter into liver parenchyma cells where they undergo development into primary exoerythrocytic schizont (primary exoerythrocytic schizogony).

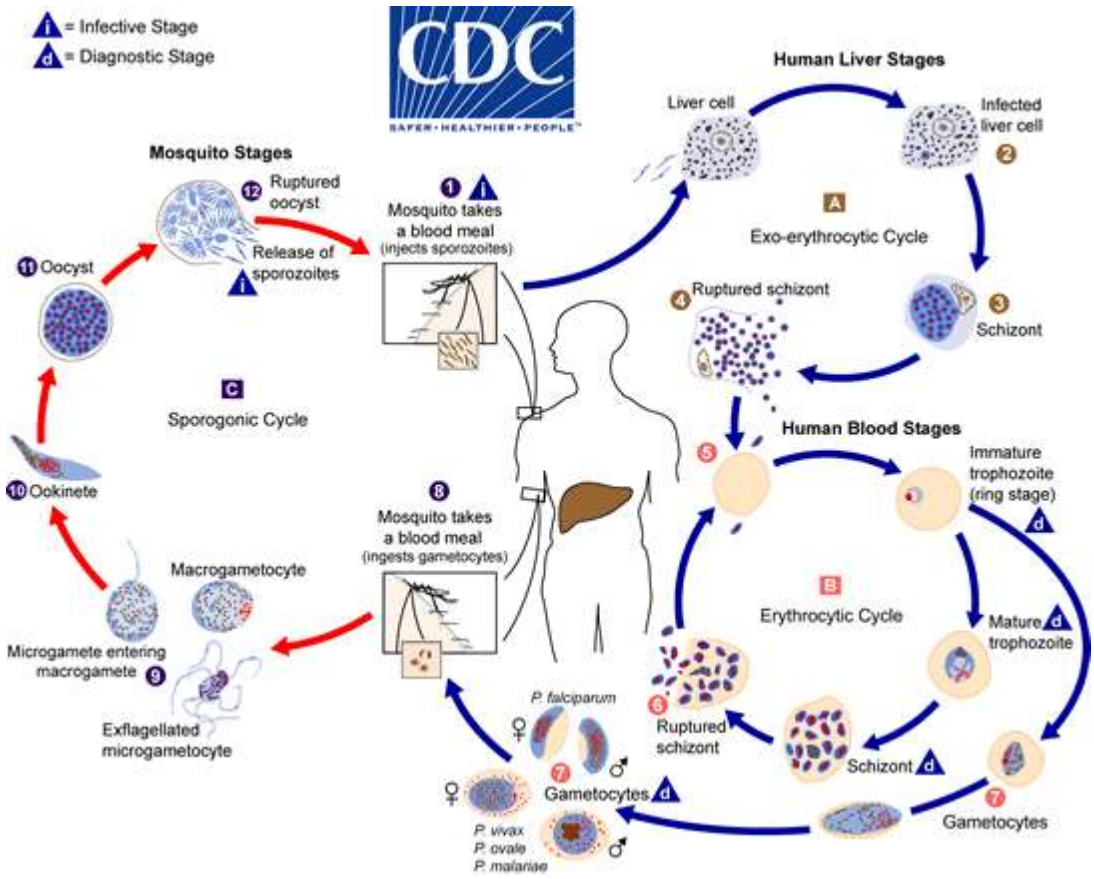

Figure I: Life Cycle of Malaria Parasite, *Plasmodium* species (Source: CDC, 2012)

The malaria parasite life cycle involves the human host and female anopheles mosquito. Infection begins when a malaria infected female anopheles mosquito inoculates sporozoites into the human host. The sporozoites infect liver cells, multiply asexually, mature into schizonts which rupture to release merozoites (Exoerythroytic Schizogony). In the bloodstream, the

parasite undergoes erythrocytic schizogony, merozoites infect red blood cells. The ring stage trophozoites mature into schizonts, rupture to release merozoites that invade other red blood cells. Some parasites differentiate into sexual erythrocytic stages (Gametocytes). The male (microgametocyte) and female (macrogametocytes) are ingested by the female anophelese mosquito during blood meal. The gametocytes generates zygote, which develop to motile ookinates that invade the mosquito's midgut wall where they develop into oocysts. The oocysts grow, rupture and release sporozoites which travel to the mosquito's salivary glands. The parasites multiplication in the mosquito is known as Sporogonic cycle. The cycle of infection begins again when the mosquito inoculate the sporozoites into another human host.

2.5 Malaria parasites and vectors in Nigeria

The dominant species of malaria parasites in Nigeria is *Plasmodium falciparum* (≈95%); the most pathogenic of the human malaria parasites, which also occur as mixed species with other *Plasmodium* species. The other non-*falciparum* species are: *P.malaria* (9.8%) and *P. ovale* (5.8%) and mixed infection (10.4%) (NMEP, 2015).

Environmental, social and demographic factors such as climate change/variability drive the distribution of the dominant malaria vector species and their parasite transmission (Adepoju and Akpan 2017). They shift in response to changes in temperature and precipitation (Tolulope, 2014). *Anophelesgambiae* and *Anophelesarabiensis* are widespread across all ecological zones in Nigeria (Akpan *et al.*, 2018), where they co-exist in sympatric relationship (NMCP, 2013).Data from the 2017Nigeria Entomology Report showed that *Anopheles gambiae* was the most predominant varying from 48 percent in the arid/semi-arid Sahel to 89 percent in the mangrove swamps on the coast (PMI 2019).

Anophelesgambiae and *Anophelesarabiensis* breeds in sunlit, shallow, temporary bodies of fresh water such as puddles, pools and hoof prints. *An. gambiae s.s* feeds primarily on humans (anthropophagic) and rest indoors (endophilic) while *An. arabiensis* feeds on humans and other animals and mainly rests oudoors (exophilic) with sporozoite rates of 0-4.8%. Other are;

- *Anophelese funestus which* breeds in permanent or semi-parmanent body of fresh water with emergent vegetation: such as swamps, large ponds which sustain malaria transmission during dry season.Itfeeds on both humans, animals and rest indoors.
- *Anophelese melas,* another member of the *Anopheles gambiae* complex, breeds in the mangrove and coastal areas of the south-south and south western zones (NMEP, 2015).

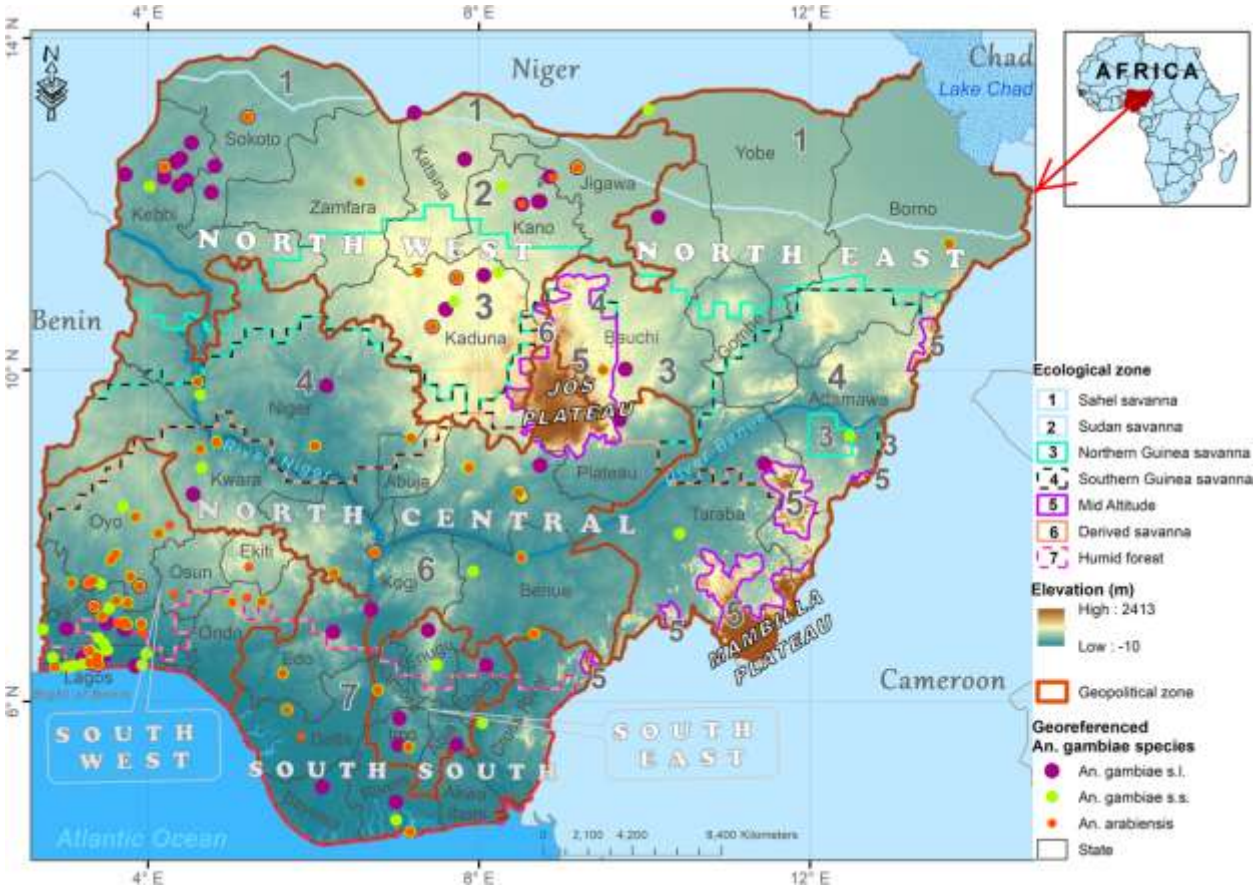

Fig I1. Map of Nigeria with georeferenced sampling points of *Anopheles gambiae* species, showing topographic relief, ecological, regional and state boundaries.
(Akpan *et al.*, 2019)

2.6 Malaria Transmission

The infection is transmitted by the bite of an infected female anopheles mosquito. The mosquito bites at dawn and at dusk, it gets infected by biting a patient infected with malaria, where it takes in the sexual forms of the parasite, the gametocyte continue the sexual phase of the cycle and the sporozoites fill the salivary glands of the infected mosquito. In rare cases, a person may contract malaria through contaminated blood. It has been reported that malaria also may be transmitted from a mother to her fetus before or during delivery "congenital" malaria. Malaria can also be transmitted through blood transfusion, organ transplant, or the shared use of needles or syringes contaminated with blood (Ridley, 2012).

2.6.1 Malaria transmission in Nigeria

Malaria transmission is high across the country; it was estimated that approximately 30% of the population lived in areas of high transmission intensity and 67% in the moderate transmission zone (NMEP, 2015). However, information has provided evidence of a progress divergence of in-country variation in malaria endemicity (NMCP, 2013). Bayesian model-based geo-statistical method were used to provide a prediction of malaria risk across Nigeria for the year 2000, 2005 and 2010 based on the effects of termperature, rainfall, distance to major rivers and urbanization (NMCP, 2013). As at 2010, 85% of Nigerians lived in areas supporting mesoendemic transmission, 15% lived under conditions ofhyper-holoendemicity and areas within FCT Abuja, Adamawa and Borno State support hypoendemicity.

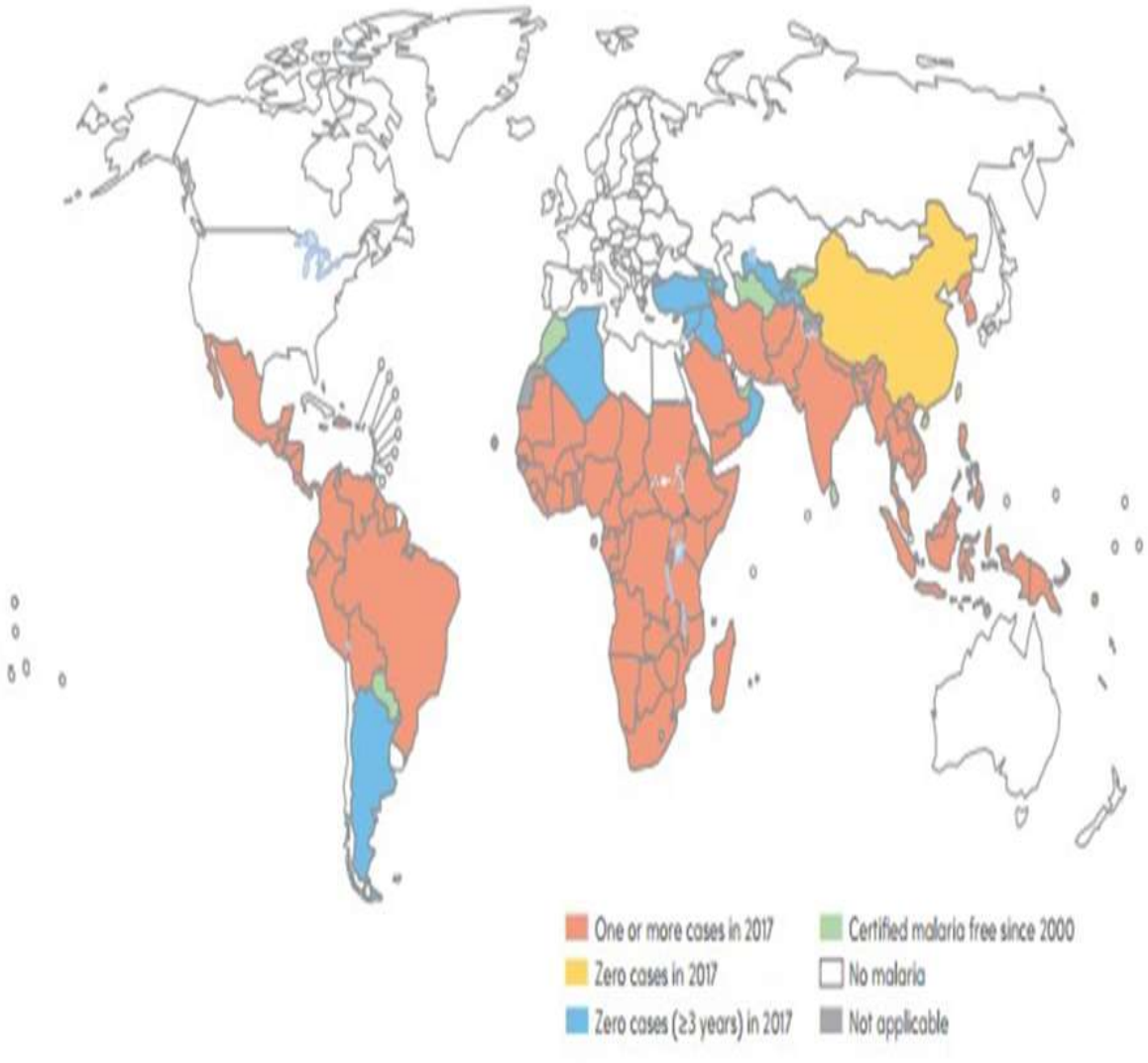

Figure III: 2017 Global distribution of Malaria (WHO, 2018)

2.7 Malaria Control in Nigeria

Nigeria's quest for effective control of malaria began well before the WHO global malaria eradication period betweeen 1955 and 1968 (Gilles *et al.*, 2007), involving series of activities ranging from the 1st sanitation campaign in schools (1889) to the western Sokoto pilot project in 1954.From 1955 however, the World Health Organization Global eradication programme conducted a pre-eradication pilot study in Kankiya District, which shows that malaria control was then more feasible than eradication (Olowe *et al.,* 2015). The Garki project was organized afterwards in 1969 to research on the epidemiology and control of malaria in the sudan savannah (WHO, 1980).

(archive.lshtm.ac.uk/www.linkmalaria.org/sites/link/files/content/attachments/2017-1129/NigeriaSingleBannerRGBDigitalVersion.pdf).

The National Malaria Control Committee (NMCC) produced the 3rd National Development Plan in 1975 with the set mandate to reduce the malaria burden by 25% . It produced a five year plan of action that terminated in 1980. It took another 8 years before progress was made when a health system reform was carried out in 1988, with the adoption of a Health Policy for the country (archive.lshtm.ac.uk/www.linkmalaria.org/sites/link/files/content/attachments/2017-11-29/NigeriaSingleBannerRGBDigitalVersion.pdf). Within this Policy, malaria was to be eradicated using the concept of Primary Health Care. The ministry of Health subsequently prepared guidelines for malaria control in 1989. Government finally came out with a National Malaria Control Plan of Action in 1996.

The Roll Back Malaria (RBM) partnership was initiated in 1998 with the aim to reduce malaria morbidity and mortality. In the year 2000, the world launched Millennium Development Goals (MDGs) and Goal 6C was to halt and reverse the incidence of malaria by

2015 (U.N. 2014A). Following the end of MDG, the World Health Organization member states, Nigeria inclusive, in May 2015, agreed to a new global malaria strategy for 2016-2030 (WHO 2015), which was built on the success of the 1st Global Malaria Action Plan (2008-2015) (AIM 2016-2030). The strategy aims to reduce the global disease burden by 40% by 2020, and by atleast 90% by 2030. Nevertheless, millions of people are still unable to access malaria prevention and treatment, and in most cases, deaths continue to go unregistered and unreported (Aribodor *et al.*, 2016). Nigeria has set out to achieve malaria elimination and has rebranded the National Malaria Control Programme (NMCP) to National Malaria Elimination Programme (NMEP) (Aribodor *et al.*, 2016).

2.8 Evaluation and diagnosis of malaria

Various methods for malaria diagnosis are:. Peripheral smear examination by light microscopy, Fluorescence microscopy techniques, Non-microscopic Rapid Diagnostic Tests (Immunochromatographic tests-detection of malaria antigen by HRP-2 and pLDH detection methods, Immunochromatographic dipstick assays used for diagnosis) and Molecular methods. (Raghuveer and Mangala 2012)

2.8.1. Microscopic Examination

Peripheral smear examination by light microscopy: Malaria is diagnosed microscopically by staining thick and thin blood films on a glass slide, to visualize malaria parasites (Chotivanich*et al.*, 2007).

Fluorescence microscopy techniques: The Quantitative buffy coat (QBC) technique was designed to enhance microscopic detection of parasites and simplify malaria diagnosis (Adeoye and Nga 2007). This method involves staining parasite deoxyribonucleic acid (DNA) in micro-hematocrit tubes with fluorescent dyes, e.g. acridine orange, and its subsequent detection by epi-fluorescent microscopy (Kocharekar *et al.*, 2014).

2.8.2 Rapid Diagnostic Test

RDTs detect malaria antigen in blood flowing along a membrane containing specific anti-malaria antibodies; they do not require laboratory equipment. Most products target a *P. falciparum*-specific protein, e.g. histidine-rich protein II (HRP-II) or lactate dehydrogenase (LDH) (Park *et al.*, 2006).. Some tests detect *P. falciparum* specific and pan-specific antigens (aldolase or pan-malaria pLDH), and distinguish non-*P. falciparum* infections from mixed malaria infections (Kim *et al.,* 2008).

2.8.3 Serological tests

Diagnosis of malaria using serological methods is usually based on the detection of antibodies against asexual blood stage malaria parasites (She *et al.,* 2007). For instance, the principle of Immuno Fluorescence Assay is that, following infection with any *Plasmodium* species, specific antibodies are produced within 2 weeks of initial infection, and persist for 3-6 months after parasite clearance. IFA uses specific antigen or crude antigen prepared on a slide, coated and kept at -30℃ until used, and quantifies both IgG and IgM antibodies in patient serum samples (Chotivanich*et al.,* 2007).

2.8.4 Molecular methods

Molecular biological technologies have permitted extensive characterization of the malaria parasite. This method include techniques such as Polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), microarray, mass spectrometry (MS), and flow cytometric (FCM) assay techniques among others. The PCR technique continues to be used extensively to confirm malaria infection, follow-up therapeutic response, and identify drug resistance (Chotivanich*et al.,* 2007; Rakotonirina*et al.,* 2008).The LAMP technique detects the conserved 18S ribosome RNA gene of *P. falciparum*(Poon*et al.,* 2006).

Flow cytometry technique is based on detection of hemozoin, which is produced when the intra-erythrocytic malaria parasites digest host hemoglobin and crystallize the released toxic heme into hemozoin in the acidic food vacuole. Hemozoin within phagocytotes can be detected by depolarization of laser light, as cells pass through a flow-cytometer channel (Padial*et al.*, 2005).

2.9 Treatment of malaria

Artemether-Lumefantrine (AL) is recommended as the first line treatment while Artesunate-Amodiaquine (AA) is the alternate treatment for uncomplicated malaria in Nigeria. Children weighing less than 5kg with uncomplicated malaria should be treated with Artemisinin combination therapy at the same mg/kg dose as recommended for children weighing 5kg. Quinine-Clindamycin should be administered in the first trimester while Artemisinin Combination Therapy is recommended in second and third trimester as well as lactating mothers. Injectable Artesunate is recommended for the treatment of severe malaria inclusive of first, second and third trimesters of pregnancy. Alternate treatments of choice are: Intramuscular artemether or intravaneous/intramuscular quinine. Atovaquine-Proguanil is recommended for Non- Immune visitors (NMSP, 2015).

2.10 Classification of antimalarial drugs

Anti malarial drugs can be classified according to their activity and structure.

- Based on mechanism of action, antimalarials are grouped into; 1) tissue schizonticides are active at the exoerythrocytic stage, such as proguanil, tetracycline and primaquine, 2) blood schizonticides are active on the schizont located inside of erythrocytes, such as quinine, chloroquine and artemisinin and 3) gametocides are active on the gametocytes in erythrocytes, such as primaquine **(**Sohsuebngarm*et al.*, 2014**)**

- According to their structure, antimalarial agents are classified into; Aryl amino alcohols, 4-aminoquinolines, Folate synthesis inhibitors, 8-aminoquinolines, Antimicrobials and Peroxides (Staines and Krishna 2012).

2.10.1Aryl Amino Alcohols: Quinine

Quinine is an arylamino recommended for the treatment of severe cases of malaria (WHO 2011). Quinine exact its action by interacting with heme detoxification.*Plasmodium falciparum* multidrug resistance transporter(*Pf*mdr-1) mutations are reported to be associated with quinine resistance (Saifi *et al.*, 2013).

Adverse effects of quinine are dose-related, one of which is Cinchonism which consists of tinnitus, headache, nausea and dizziness; most of these adverse effects are reversible. The drug has arrhythmogenic potential and can cause hypotension. In addition, it stimulates insulin secretion and in patients with G6PD deficiency may cause haemolytic anaemia (Schlitzer 2008). There is an increased risk of hypoglycaemia in pregnancy.

2.10.2 4-Aminoquinolines: Chloroquine, Amodiaquine

- Chloroquine

Chloroquine (CQ) was first synthesized in 1934 and became the most widely used antimalarial drug by the 1940s. It has antipyretic and anti-inflammatory properties (Kalia and Dutz 2007). The drug is effective against, *P. ovale*, *P. malariae*, *P. vivax* and sensitive *P. falciparum* parasites.Chloroquine inhibit heme polymerization, producing an environment that is toxic to the erythrocytic form of the parasite. Resistance to CQ is known to be associated with the CQ-resistance transporter, *Pf*CRT, the gene product, is a transporter that is found in the food vacuole membrane that regulates drug efflux and pH regulation. The *P. falciparum* resistance to chloroquine is due to the increased efflux of chlororquine out of the food vacuole caused by the mutation. This mutation stopschloroquine accumulation in the

parasites food vacuole, this blocks the inhibition of heme (Frosch *et al.*, 2011). Side effects such as nausea, dizziness and headache can occur . When administered orally, peak plasma concentrations is achieved within 3 h (Esperanca *et al.*,2010).

Despite the widespread resistance, CQ have been reported to remains effective for the treatment of *vivax* malaria (Awab *et al.*, 2010).

Amodiaquine

In 1960, amodiaquine (AQ) was developed to counteract resistance to chloroquine (Burrows *et al.*, 2011). Amodiaquine is a phenyl substituted analogue of Chloroquine recommended to be used in combination with artesunate for the treatment of uncomplicated malaria (WHO, 2010 B). Adverse reactions to AQ are generally similar to those of chloroquine, in addition, amodiaquine can induce toxic hepatitis and fatal agranulocytosis following its use for prophylaxis (Taylor and White, 2004). AQ is contraindicated for chemoprophylaxis and in persons with haematological and hepatic disorders.Studies suggest that AQ is safe during second and third trimester of pregnancy. Resistance to Amodiaquine is reported to be associated with mutations on *Pf*crt and *Pf*mdr1 (Holmgren *et al.*, 2007).

2.10.3 8-Aminoquinolines: Primaquine, Piperaquine and Tafenoquine

- Primaquine

Primaquine phosphate is recommended as a radical cure and prevention of relapse of malaria caused by *P. vivax*, and for use in areas of endemic malaria caused by *P. vivax*. Primaquine is administered along with chloroquine (WHO 2014).Primaquine's activity against the hepatic stage of malarial parasites results from the effects of an active metabolite resulting from metabolism by hepatic CYP2D6 (Pybus *et al*, 2013). Its active metabolite is responsible for inflicting extensive oxidative damage that interferes with mitochondrial electron transport in

parasites. primaquine is reported to increase the oxidative stress on human red blood cells, an effect that contributes to its hemolytic side effects (Butterworth et al, 2013). Because it is not effective against the erythrocytic stages of *P. falciparum*, primaquine is not used to treat *falciparum* malaria.

- Piperaquine

Piperaquine (PPQ) is a bisquinoline antimalarial drug developed in the 1960s in China in response to the increasing prevalence of CQ-resistant parasites in Southern China (Davis *et al.*,2005). Its use as monotherapy, resulted in the emergence of PPQ-resistant parasites, which diminished its use by the late 1980s (Eastman *et al.*, 2011). PPQ was subsequently combined as part of China-Vietnam 4 (known as CV4), an Artemisinin Combination Therapy that consisted of dihydroartemisinin (DHA), trimethoprim, piperaquine and primaquine (PQ). The World Health Organization now recommended the administration of piperaquine in combination with Dihydroxyartemisinin.

PPQ resistance is reported to be associated *Pfmdr1* (Eastman *et al.*, 2011). The 8-aminoquinoline is one of very few medications active against the liver stages of *Plasmodium* (Fernando *et al.*, 2011). It is mainly used to treat *vivax* or *ovale* malaria. PPQ still remains the only treatment against P. *vivax* liver infections.It is contraindicated in pregnant women and in glucose-6-phosphate dehydrogenase-deficient patients. Chloroquine combined with Piperaquine is reported to be the treatment of choice for CQ-sensitive *vivax* malaria (Burrows *et al.*, 2011).

- Tafenoquine

Tafenoquine is an 8-aminoquinoline antimalarial developed as a replacement for primaquine for radical cure of *P. vivax* malaria and as a potential prophylactic agent (Crockett and Kain,

2007). Modes of action of tefenoquine include drug-induced mitochondrial dysfunction, inhibition of receptor recycling by endosomes and inhibiting haematin polymerizationIt causes methaemoglobinaemia and haemolytic anaemia in individuals with deficiency of G6PD (Frampton, 2018).

2.10.4 4-Methanolquinolines: Mefloquine, Lumefantrine

- Mefloquine

Mefloquine (MQ) acts by inhibiting both the haemozoin formation and oxidative and glutathione dependent degradation of haeme. After haemoglobin digestion within the digestive vacoule, it releases a toxic free haememoiety (Kumar and Bandopadhyay, 2005). A major portion of the haeme moiety is converted in the acidic conditions in the digestive vacoule to form haemozoin or the malarial pigment (Kumar *et al.,* 2007). The rest of the free haeme moves out into the cytosol and gets detoxified by interacting with either glutathione or glutathione reductase or hydrogen peroxide (Bhattacharjee and Shivaprakash 2016). MQ can cause mild gastrointestinal and neurological adverse effects. Resistance to mefloquine has been primarily attributed to amplification in *Pf*mdr-1 gene (Rohrbach *et al.,* 2006). MQ is recommended to be used in combination with artesunate for the treatment of uncomplicated *falciparum* malaria (WHO 2011).

- Lumefantrine

Lumefantrine also named benflumetol is an aryl alcohol, first synthesized in the 1970s in China (WHO 2013). It is the only compound of its class approved for the treatment of malaria. In contrast to most other ACT partner drugs, lumefantrine has never been used or recommended as monotherapy. It is used in combination with artemether as the first-line treatment for uncomplicated malaria. (Makoah and Gabriel2013). Side effects include nausea, abdominal pain, diarrhoea, pruritus and skin rashes.

2.10.5 Antifolates

Antifolate agents used for the treatment of malarial infection act on the folate metabolism of the parasite based on the enzyme they inhibit.The antifolates are grouped into two classes: inhibitors of dihydrofolate reductase (DHFR) and inhibitors of dihydropteroate synthase (DHPS). The combination of DHFR and DHPS inhibitors is synergistic, hence their use in combination in the treatment of malaria (Nzila, 2006). The antifolates used against malaria are the DHFR inhibitors pyrimethamine and proguanil (metabolized in vivo to the active form cycloguanil) and the DHPS inhibitors sulfadoxine and dapsone. The combination sulfadoxine/pyrimethamine (SP) was introduced as a synergistic antimalarial drug and replaced CQ as a first-line treatment of *P. falciparum* malaria in many parts of Africa (Eriksen*et al.,* 2008). Point mutations in the *Plasmodium falciparum* dihydroxy folate reductase and *Plasmodium falciparum* dihydroxypteroate synthase genes confer resistance to SP, with the decreasing susceptibility of *P. falciparum*to sulfadoxine/pyrimethamine combination, the WHO recommends the combination of SP and artesunate for the treatment of uncomplicated malaria (Barnes *et al.*, 2006).

2.10.6 Antibiotics: Clindamycin, Azithromycin

Clindamycin is a semi-synthetic antibiotic derived from lincomycin. It is a blood schizontocide with a relatively slow action (Achan *et al.,* 2011). It is usually given in combination with QN for the treatment of *P. falciparum* malaria when decreased susceptibility to quinine is reported (McGready *et al.*, 2001).

Azithromycin is a macrolide antibiotic, semi-synthetic derivative of erythromycin with activity against *P. falciparum* (Noedl *et al.*, 2006). Azithromycin acts in synergy with quinine, this combination is reported to be effective in drug-resistant *P. falciparum* malaria (Miller *et al.*, 2006). Almost all antibiotics with antimalarial activities target the prokaryotic ribosomes of the organelle, thus blocking the apicoplast´s translational machinery. Because

the apicoplast has essential metabolic functions for the parasite, such as fatty acid synthesis, lipoic acid metabolism and isoprenoid biosynthesis, its functional inhibition by the antibiotics results in death of the parasite (Pradel and Schlitzer 2010).

2.10.7 Artemisinins: Artemisinin, Dihydroartemisinin, Artemether and Artesunate

Artemisinin drugs belong to the sesquiterpene lactone endoperoxides. The parent compound of this class is artemisinin (quinghaosu), whereas dihydroartemisinin, artesunate, artemether, and β-arteether are the most common derivatives. Dihydroxyartemisinin is the main bioactive metabolite of all artemisinin derivatives (artesunate, artemether, β-arteether, etc.), and is also available as a drug itself (Schlitzer 2008; Wiesner, 2003). WHO has recommended the use of artemisinin-based combination therapies (ACTs) for treating *falciparum* malaria in all countries where resistance to monotherapies or non-artemisinin combination therapies (e.g., SP) is prevalent (WHO, 2010). The rationale for the use of ACTs is based on the facts that artemisinin derivatives are highly potent and fast acting, and that the partner drug in ACT have a long half-life, which allows killing the parasites that may have escaped the artemisinin inhibition (Bosman and Mendis, 2007).

2.11 Malaria Vector Control

Vector control is the key intervention for global malaria control and elimination efforts. Such control targets the mosquitoes capable of transmitting malaria parasites. It is critical for the reduction and, ultimately, for the interruption of malaria transmission. The two most common vector control interventions are long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS). Together, these account for almost 60% of global investment in malaria control (Meredith, 2012).

Vector-control strategies should be devised through an integrated vector management (IVM) approach. Such an approach seeks to improve the efficacy, cost-effectiveness, ecological

soundness and sustainability of controlling disease vectors. Key considerations for malaria vector control include vector abundance, behaviour and susceptibility to the insecticides used in LLINs and IRS (WHO, 2015).

Integrated Vector Management (IVM) as recommended by WHO/AFRO is the main vector control strategy in Nigeria. Vector monitoring and control falls under the preventive section of the NMSP 2014-2020. The prevention strategy for the NMSP 2014-2020 includes three strategies, namely: i) Integrated vector management ii) Prevention of MIP; and, iii) Seasonal malaria chemoprevention (SMC).

The NMSP 2014-2020 integrated vector management strategy includes universal access to ITNs; scaling up IRS in targeted areas to interrupt malaria transmission; expanding larval source management as complementary strategies for ITNs and IRS; and vector sentinel surveillance and resistance monitoring. The use of ITNs is the primary vector control method in Nigeria as IRS is not widely implemented. The goal of the NMSP 2014-2020 is to achieve universal coverage of ITNs to the population that is at-risk. Universal coverage is defined as one ITN for every two persons. From 2011-2013, PMI supported IRS in two LGAs in Nasarawa State to demonstrate how quality IRS can be implemented. The IRS was discontinued in 2013 and efforts refocused on entomological surveillance and insecticide resistance monitoring.

2.11.1 Environmental management

The World Health Organization (WHO) defines environmental management (EM) for vector control as "The planning, organization, carrying out and monitoring of activities for the modification and/or manipulation of environmental factors or their interaction with man with a view to preventing or minimizing vector propagation and reducing man-vector-pathogen contact". EM is not a replacement of other interventions, but one of several optional

components that will make up an integrated vector management (IVM) approach in a vector control programme (Castro *et al.,* 2009).If such measures result in long-lasting or permanent changes in land, water or vegetation, they are often referred to as environmental modification. When such measures have a temporary effect and need to be repeated, they are known as environmental manipulation**.**

Environmental management is a key element of IVM. Environmental management can include environmental modification, environmental manipulation, and strategies that reduce contacts between vectors and humans (WHO 2004).

2.11.2 Larviciding

Larval source management (LSM) is the management of water bodies that are potential breeding sites for malaria vectors. It includes habitat modification or the addition of chemicals to water bodies to prevent the development of adult mosquitoes (larviciding). Larviciding has been recognized as a valuable addition to malaria vector control in specific settings. (WHO 2013B).

The Economic Union of West African States (ECOWAS) showed interest in scaling-up larviciding in West Africa, which led to a tripartite agreement between ECOWAS, Venezuela and Cuba in 2009 to provide financial and technical support to scale-up larviciding in the region with a view to eliminating malaria. Technology transfer for the establishment of microbial larvicide factories in Ghana, Nigeria and Cote d'Ivoire forms part of the agreement, in a bid to create jobs and make larvicides readily available in the region (ECOWAS, 2013).

Microbial larvicides have been shown to be protective against malaria (Geissbuhler *et al.,* 2009), but only one strain (*Bacillusthuringiensis* subsp. *israelensis*, strain AM65-52, WG)

has been approved for larviciding by the WHO's Pesticide Evaluation Scheme (WHOPES) (WHO, 2013B).

Knowledge of local conditions, the vectors and their biology, the type of water and accessibility of larval sources must be available to plan successful programmes. It is feasible only in areas where most breeding sites are relatively fixed so that they are consistently identifiable and, as such, can be fully covered by the intervention. It often entails high costs and intensive operational input, as most larvicides must be applied at 1–8-week intervals. Studies reports that larval control can be successful in appropriate settings: it reduced malaria transmission by 30% in targeted areas in Dar-es-Salaam, United Republic of Tanzania (Fillinger *et al.,* 2008), and had a significant complementary effect to LLINs in western Kenya (Fillinger *et al.,* 2009).

2.11.3 Biological Control of Malaria Vector

- Entomopathogenic Fungi

The Entomopathogenic fungi act by producing infective spores (conidia) that attach to and penetrate the cuticle of mosquitoes, releasing toxins that result in mosquito death ((Scholte *et al.,* 2004)). Studies have shown the pathogenic effect on malaria mosquito vectors (Knols *et al.,* 2010) and on Ae. aegypti (Paula *et al.*, 2011; Dabro *et al,* 2012). As entomopathogenic fungi are mostly targeted towards adult mosquitoes, and because several different toxins produced during fungal infection are lethal to mosquitoes, selection pressure for resistance is likely to be less intense when compared to rapid-killing insecticides (Scholte *et al.,* 2005). Therefore, the evolution of fungus resistance is predicted to be much slower than the evolution of insecticide resistance (Blandford *et al.,* 2005).

Fungal species of choice include those belonging to the genera Coelomomyces, Culicinomyces, Beauveria, Metarhizium, Lagenidium, and Entomophthora (Scholte *et al.,*

2004). Unlike other infectious agents, fungus does not require host ingestion; external contact with the insect's cuticle is all that is needed to promote an infection. This way of launching an infection is not only practical and easily applied in the field, but also resembles many currently used chemical insecticide delivering strategies. Fungal spores can be applied in outdoor attracting odor traps, on indoor house surfaces, on cotton pieces hanging from ceilings, bed nets, and curtains, and can persist for a couple of months on many of these surfaces (Okumu *et al.*, 2010) . Fungal infections can either act alone or in synergy with various insecticides, and its effectiveness against both insecticide resistant and insecticide susceptible mosquitoes was another major reason behind incorporating fungus in integrated vector management or in insecticide-resistant management approaches (Farenhorst and knols, 2010). It has also been shown that fungal pathogens influence the feeding habits of mosquitoes, affecting their survival (Scholte *et al.*, 2006). Even the survival rates of malaria parasites within the mosquito were shown to be affected (Charles and Nelson,2004) .

- Bacterial Agents

Bacillus thuringiensis (Bti) and *Bacillus sphaericus* (Bs) are the most promising bacterial larvicidal strains in malaria vector control. Bacillus strains are cheap, can be locally cultured and easily handled (Charles and Nelson 2004).

- Larvivorous Fish

Fish species, like those belonging to the family Cyprinodontidae, are used however, the use of *Gambusiaaffinis* predominated (Walker *et al.*, 2006).

- The Sterile Insect Technique

The Sterile Insect Technique (SIT) is a genetic suppression strategy that involves rearing large numbers of males of the target species and either irradiating or treating them with

chemosterilizing agents to generate chromosomal aberrations and dominant lethal mutations in sperm. These sterilized male insects are released and when they mate with wild females produce no progeny (Devine *et al.,* 2010). A sustained SIT programme results in an increasing ratio of released sterile males to wild males (as the population decreases) eventually leading to population elimination. Major interventions over the past 50 years using SIT against agricultural pests have proved very successful, including the eradication of the New World screwworm, *Cochliomyiahominivorax,* from North and Central America, and the eradication of Glossina austeni tsetse flies from Unguja Island, Zanzibar (WHO, 2020).

2.11. 4 Chemical Control of Malaria Vector

- Insecticide treated nets

WHO recommended that Insecticide Treated Nets should be made available to all people at risk, i.e. "universal access" (WHO, 2007). In order to meet the target of universal access, one long lasting insecticide treated net (LLIN) should be distributed for every two persons and rounding up in households with an odd number of members (e.g. 3 LLINs for a household with 5 members, etc) (WHO, 2006).

Only LLINs recommended by the WHO Pesticide Evaluation Scheme (WHOPES) should be procured by national malaria control programmes and partners for malaria control (WHO 2011B). Behaviour change interventions including information, education, communication campaigns and post-distribution "hang-up campaigns" are strongly recommended to ensure the use and not just ownership of LLINs (WHO, 2007).

International technical norms for net products have been developed by WHO and are therefore recommended as minimum standards for production. The national standards have been reviewed by the Standard Organization of Nigeria to be in line with the international standards (NMIS, 2015).

- Indoor residual spraying (IRS)

IRS is the application of a long-lasting, residual insecticide to potential malaria vector resting surfaces such as internal walls, eaves and ceilings of all houses or structures (including domestic animal shelters) where such vectors might come into contact with the insecticide. When carried out correctly, IRS is a powerful intervention to rapidly reduce adult mosquito vector density and longevity and, therefore, to reduce malaria transmission. (Pluess *et al.*, 2010).

The active ingredients of all WHO-recommended products for IRS come from only four classes of insecticide: pyrethroids, organochlorines (dichlorodiphenyltrichloroethane, DDT), organophosphates and carbamates (W.H.O, 2011B). IRS is a method for community protection and, to achieve its full effect, IRS requires a high level of coverage, in space and time, of all the surfaces where the vector is likely to rest, with an effective dose of insecticide. The selection of the insecticide has to take into account the susceptibility status of local vectors and duration of the residual effect in relation with the length of the transmission season.

However, spraying may be added in certain situations in order to either prevent or mitigate resistance in areas where nets are routinely used. When those two interventions are deployed together, an insecticide with a different mode of action to that used on nets should be used for spraying. Supplementary methods may be appropriate in specific settings, for instance larval source management where mosquitoes' aquatic habitats are few, fixed and findable (WHO, 2012).

2.11.5 Insecticides used for malaria vector control

The most prominent classes of insecticides are organochlorines (OCs), organophosphates (OPs), carbamates (Cs), and pyrethroids (PYs). In general, they act by poisoning the nervous

system of insects, which is fundamentally similar to that of mammals. A small amount of pesticide can be fatal for an insect, primarily because of its small size and high rate of metabolism. Such an amount is not fatal for humans (Toxipedia, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Materials

Microtitre Plate, 12 X 8 Wells, Micropipettes 0-50µl, 0-100µl, and 100- 1000µl Pipette Tips, Alluminium Foil,Microscope Slides, Capillary Tubes, Polymerase Chain Reaction tubes, silica membrane, eppendorff tubes (0.2ml), Cotton Swabs, Gloves, Syringe.

Equipment

Autoclave, Anearobic jar, Weighing Balance,Forceps, Racks, PCR machine, Gel documentation machine, biosafety cabinet, electrophoresis unit, refrigerator.

Reagents

Giemsa stain, RPMI 1640 Liquid medium, Disinfectant, Buffer, antimalarial drugs, PCR Master mix, pfcrt primer, *Pfmdr1* primer, K13P primer, Agarose gel and Candles.

3.2 Methods

3.2.1 Study Centre

The study samples were collected from outpatients of Dalhatu Araf Specialist Hospital. The hospital is located in Lafia and have 403 bed spaces. Lafia is a town in North Central Nigeria. It is the capital city of Nasarawa State and has a population of 330,712 inhabitants according to the 2006 census results.It is located at latitude 8.49 and longitude 8.52 and it is situated at elevation of 179 meters above sea level. It has an average annual temperature and rainfall of 26°C and 674mm respectively (Salihu *et al.*, 2012).

3.2.2 Sample Collection

Blood samples were collected with the assistance of a certified medical laboratory technologist in the study hospital asceptically. 2mls of venous blood was collected with a sterile disposable syringe into Ethylenediaminetetraacetic acid (EDTA) bottles. The bottles contained 5ul gentamicin to minimize bacterial contamination. The blood samples were transported in a sterile ice packed air tight box within 24 hours of collection to the Pharmaceutical Microbiology Laboratory of Ahmadu Bello University Zaria.

- Inclusion Criteria

  This study involved all patients of all ages and gender who have given consent and are diagnosed with malaria monoinfections (*P. falciparum*). All blood samples with mixed infections were excluded.

3.2.3 Ethical consideration

Ethical clearance was obtained from Nassarawa state Ministry of Health. Written informed consent was obtained from patients prior to recruitment into this study. Consent for the children was provided by the parents/guardians (Appendix 1).

3.2.3 Retrospective analysis

A retrospective study was carried out to know the pattern of antimalarial prescription, gender and age distribution of patients presenting with malaria at Dalhatu Araf Specialist Hospital Lafia. The medical records of reported malaria cases for 2014, 2015 and 2016 were extracted from the medical records office of the hospital. The required data include Age, Gender, prescribed antimalarial drugs, occupation, total number of malaria cases and total number of patient's attendance for the years 2014, 2015 and 2016.

3.2.5 Determination of sample size

The sample size was determined by the formular adopted from Kadani and Bhalero (2010)

Thus, $n = P (1- P) z^2 /d^2$

Where;

n = Number of Sample

Z = Standard normal deviation at 95% confidence limit = 1.96

P = Prevalence = Mean of prevalence for the year 2014, 2015 and 2016.

d = Allowable Error of 5% = 0.05

Z = 1.96

P = 5.9 + 4.4 + 4.8/ 3 = 5

d= 0.05

$n = 0.05 \times (1- 0.05) \times 1.96^2 / 0.05^2 = 73$

3.2.6 Microscopic examination

Thick and thin films were prepared to determine the parasite densities species respectively. Thick blood film was prepared by adding 2 drops of blood to the centre of a labeled greese free microscopeslide. The blood was spread using the corner of another clean slide to a 1-2cm size and left to dry. Thin film was prepared by spreading a drop of blood placed on the centre of a clean grease free slide. A clean cover slip was held towards the drop of blood and allowed to spread along the width of the slip, the slip was pushed forwad smoothly. The blood film was dried and fixed with 70% (vol/vol) ethanol for 20minutes. The blood films

were both stained with 10% Giemsa and were examined microscopically using 100 x (oil immersion) objectives.

3.2.7 Counting parasite numbers

Parasites were counted by estimating the parasite's number/µl of blood from the thick film. Thick film was prepared with 8ul of blood, the number of parasite/ul of blood was estimated by multiplying the average number of parasites from ten high power field (100 x objective) by 500 (Peletiri *et al.*, 2010).

3.2.8 Preparation of drugs

The antimalarial drugs used for this study are; Artemether (Emzor), Lumefantrine (Sigma Aldrisch), Piperaquine (Sigma Aldrisch), Chloroquine (Sigma Aldrisch) and Artesunate (Emzor). Stock solutions of these antimalarial drugs were prepared in appropriate solvents; Artemether, Artesunate and chloroquine were dissolved in ethanol, Piperaquine was dissolved in Sterile Distilled Water while Lumefantrine was dissolved in 100% DMSO. Further dilutions of each drug was made to obtain a working solution of desired concentration (Appendix 10).

3.2.9 Performance of the *in vitro* micro test

The cultivation of *Plasmodium falciparum* and antimalarial drug susceptibility and was carried out by the method adopted from Gurjeet and Urhekar(2015).From the working solutions of the drugs, 10µl of each drug concentration was used to dose a well of a 96-well flat bottom culture plates and left to dry for 20minutes. The 96-well culture plate consists of rows A-H; row A was drug-free (control) while rows B-F represent the drug concentrations in an decreasing order of doubling dilutions.90µl of a complete medium (RPMI 1640, blood serum and sodium bicarbonate) was added to each well, followed by the addition of 10µl parasitized blood. The plates were incubated at 37°c for 30 hours using the candle jar method

(5% CO_2) (Procedure for media preparation is shown in appendix 2). After incubation, The supernatant was removed, erythrocytes re-suspended in the remaining fluid, and a thick blood film was made from each well in duplicate. The blood smears were air-dried for 24 hours and stained with 10% Giemsa stain for 10 minutes. The stained thick films were examined with the oil immersion objective (100x).

3.2.10 Molecular characterization of resistant strain

- Genomic dna extraction

Genomic DNA from blood samples was extracted using the QiagenDNeasy Blood and Tissue Kit. This was carried out following the manufacturers instructions.

200ul of qiagen protease (proteinase k) was added to a 1.5ml microtube, 200ul of the blood sample and 200ul buffer AL was added. The mixture was centrifuged at 3000rpm for 30seconds. 200ul of 100% ethanol was added to the mixture and vortex for 15sec after which it was Centrifuge for 30sec at 3000rpm.The mixture was transferred into a Qiagen mini spin column placed on a 2ml collection tube. 500ul of buffer AW1 was added without wetting the rim and Centrifuged at 8000rpm for 1min, the flow through and spin column was discarded. 500ul of buffer AW2 was added and centrifuged at 14000rpm for 3min. The flow through was discarded and the spin column was transfered to a new collection tube and centrifuged at 14000rpm for 1min.

For DNA elution. AE buffer (Elution buffer) was added to the membrane of the spin column, incubated for 5min at room temperature (maximize yield) and centrifuged at 8000rpm for 1min. Elution was done twice. The final volumn of extracted DNA was 100ul, the DNA was stored at -20^{o}c until use.

DNA gel was runned by loading 5ul of the extracted DNA on agarose gel at 100V for 15 minutes and viewed using a uv transilluminate in a biorad gel doc system.

- PCR for detection of *Pf*crt gene

The *pf*crt gene was amplified by Nested PCR using the method of Olasehinde *et al.,* 2014. Pfcrt-Out-F-5'CCGTTAATAATAAATACAGGC3'and *pf*crt –OUT-R-5'CTTTTAAAAATGGAAGGGTGT3', pair of primer was used for the first reaction. The second reaction primer pair was *pf*crt-IN-F 5'CCGTTAATAATAAATACAGGC3' and *pf*crt-IN-R 5'CTTTTAAAATGGAAGGGTGT-3'. The outer region of the gene was amplified in a mixture of 20µl standard PCR mixture (12.5ul master mix, 5.5ul Molecular grade water,1ul of each primer) and 5ul DNA template. The cycling protocol was as follows: 95°C for 5 min for initial denaturation; 40 cycles of 94°C for 30 s, 52°C for 40 s and 72°C for 30 s; and a final extension of 72°C for 5 min. A second, nested amplification from this segment was performed at the following cycling conditions: 94°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds, 52°C for 40 seconds, and 72°C for 45 seconds and a final extension of 72°C for 5 minutes. using 5µl of the product solution. All PCR reaction were carried out in a thermal cycler (Gene Amp Applied Biosystems 9700). PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

- PCR for detection of *Pfmdr1* gene

The *Pfmdr1* gene was amplified by Nested PCR using the method of Olasehinde *et al.,* 2014. MDR1-Out-F- 5'-ATGGGTAAA GAGCAGAAAGA-3' and MDR2- OUT-R-5'AACGCAAGTAATACATAAAGTCA-3' pair of primer was used for the first reaction. The second reaction primer pair was MDR3 –IN-F-5'TGGTAACCTCAGTATCAAAGAA-3' and MDR4 –IN- R- 5'ATAAACCTAAAAAGGAACTGG3'. The outer region of the gene was amplified in a mixture of 20µl standard PCR mixture (12.5ul master mix, 5.5ul Molecular

grade water,1ul of each primer) and 5ul DNA template. The PCR amplification stages were at 94°C for 2 minutes, followed by 35 cycles at 94°C for 20 seconds, 52°C for 10 seconds, 48°C for 10 seconds, and 60°C for 1.5 minutes. A second, nested amplification from this segment was performed under the same PCR conditions using 5µl of the product solution. All PCR reaction were carried out in a thermal cycler(Gene Amp Applied Biosystems 9700). PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

- PCR for detection of K13P gene

The *Pfmdr1* gene was amplified by Nested PCR using the method of Ariey *et al.,* 2014. The K13 propeller domain was amplified by nested PCR using the following primers: for the primary PCR (kelch-out-f 5′-gggaatctggtggtaacagc-3′ and kelch-out-r 5′-cggagtgaccaaatctggga3′) and the nested PCR (kelch-in-f 5′-gccttgttgaaagaagcaga-3′ and kelch-in-r 5′gccaagctgccattcatttg-3′). The outer region of the gene was amplified in a mixture of 20µl standard PCR mixture (12.5ul master mix, 5.5ul Molecular grade water,1ul of each primer) and 5ul DNA template. Cycling conditions were 95°C for 1 min, followed by 35 cycles at 95°C for 20 sec, 57°C for 20 sec, and 60°C for 150 sec, with an extension at 60°C for 3 min. A second, nested amplification from this segment was performed at the following cycling conditions: 95°C for 1 min, followed by 35 cycles at 95°C for 20 sec, 55°C for 20 sec, and 60°C for 1 min, with an extension at 60°C for 3 min. using 5µl of the product solution. All PCR reaction were carried out in a thermal cycler (Gene Amp Applied Biosystems 9700). PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

3.2.11 Statistical analysis

Data were analysed using Excel 2010, Non linear regression analysis was used to determine the IC_{50} of individual *Plasmodium* isolates susceptibility to each drug. Test for significance at 95% confidence limit was performed.

CHAPTER 4

4.0 RESULTS

4.1 RETROSPECTIVE STUDY RESULTS

The total number of reported malaria cases at Dalhatu Araf Specialist Hospital Lafia for 2014, 2015 and 2016 were 61,936, 95,287 and 53,026 respectively. The malaria prevalence rate for the three years was 5.90%, 4.40% and 4.80% respectively (Average 5.03%).

4.1.1 Age Group of Patients Presenting with Malaria in 2014, 2015 and 2016

Using the age groups of 0-5.9, 6-18.9 and 19 and above, the highest number of cases in 2014 was in the older group;19 above (62.21%), followed by the minors; 0-5years (24.22%)and the least was observed in those within the 6-18years (13.57%) .

In 2015, the highest number of cases was in the older group;19 and above (68.30%), followed by the minors; 0-5.9 years (16.29%) and the least was observed in those within the 6-18,9 years (15.34%).

The highest number of cases in 2016 was in the older group; 19 and above (64.60%), followed by those within the 6-18years group (18.74%) and the least was observed in the minors; 0-5.9 years (16.66%).

Figure 4.1 shows the age distribution of patients presenting with malaria for the years 2014, 2015 and 2016.

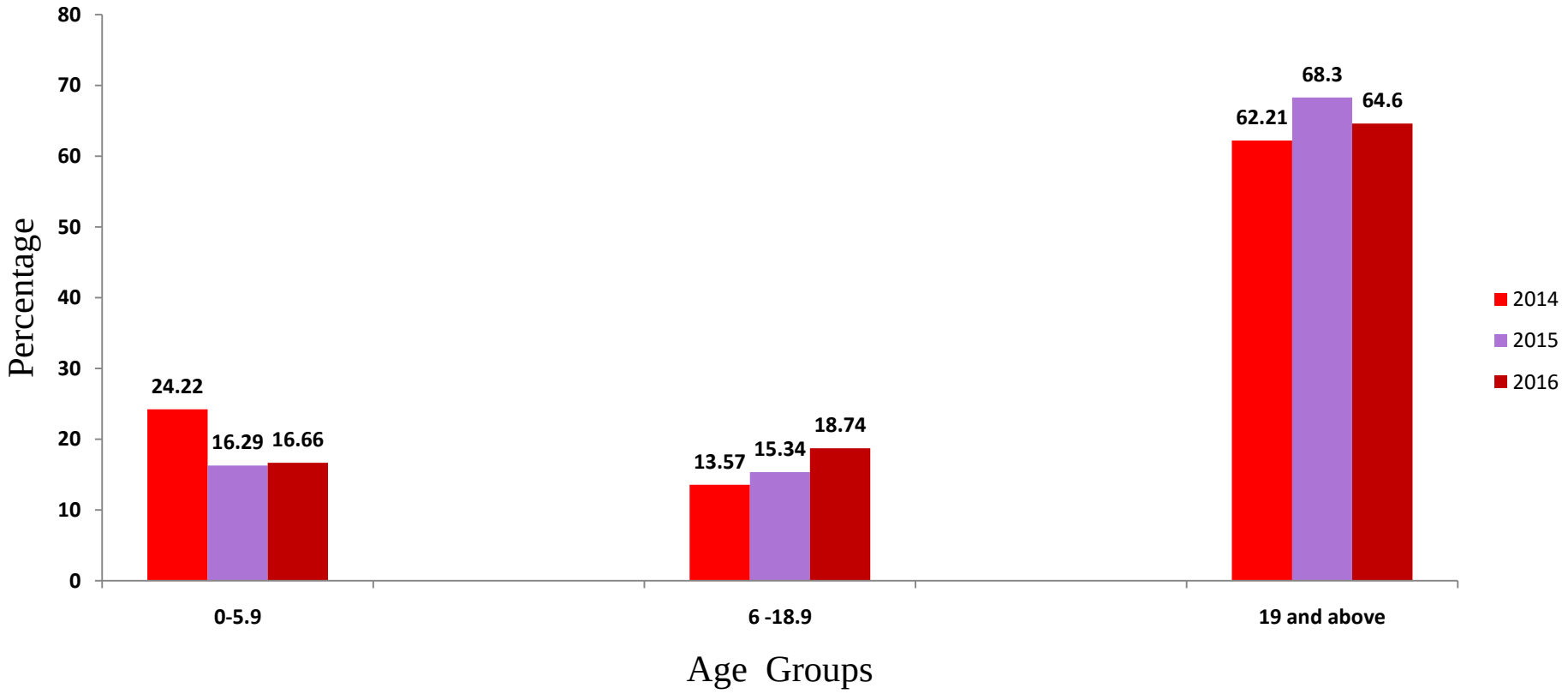

Figure 4.1: Age distribution of patients presenting with Malaria at Dalhatu Araf Specialist Hospital Lafia Nigeria for the years 2014, 2015 and 2016

4.1.2 Profile of Gender of Reported Malaria Patients in 2014, 2015 and 2016

Data from the retrospective study showed that reported malaria cases was higher in the females (59.77%, 61%, and 53.83%) for 2014, 2015 and 2016 as compared to the male (40.23%, 38.93% and 44.17%) for the three years respectively.Figure 4.2 shows the Gender distribution of reported malaria patients at Dalhatu Araf Specialist Hospital Lafia Nigeria for the three years.

4.1.3 Antimalarial Drug Prescription for 2014, 2015 and 2016

Antimalarial Drugs prescription reported for patients in the three years were basically Artemisinin combination therapy; 90%, 90% and 86% for 2014, 2015 and 2016. Fansidar^{Rx} (Sulphadoxine/pyrimethamine) usage was 10%, 10% and 14% respectively. The Artemisinin Combination reported in use were Arthemeter/Lumefantrine (Coartem or Lonart) for the three years while Dihydroxyartemisinin/Piperaquine (Artequick^{Rx}) was prescribed in 2014 only (Figure 4.3).

4.1.4 Profile of Malaria cases per month at Dalhatu Araf Specialist Hospital Lafia

The average monthly distribution of malaria from 2014-2016 is shown in figure 4.4. Cases were more in October (Average: 18%), the test was observed in January (Average: 6.2%).

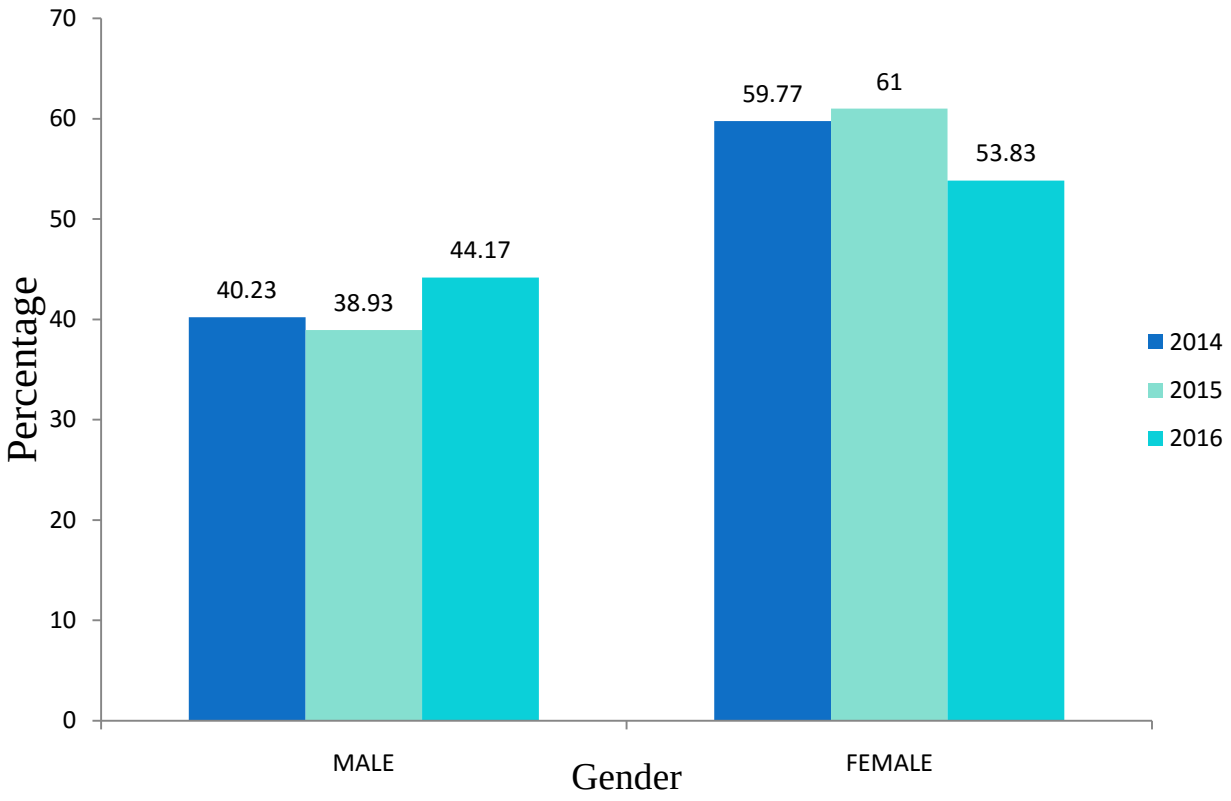

Figure 4.2: Gender distribution of Reported Malaria patients at Dalhatu Araf Specialist Hospital Lafia Nigeria for the years 2014, 2015 and 2016

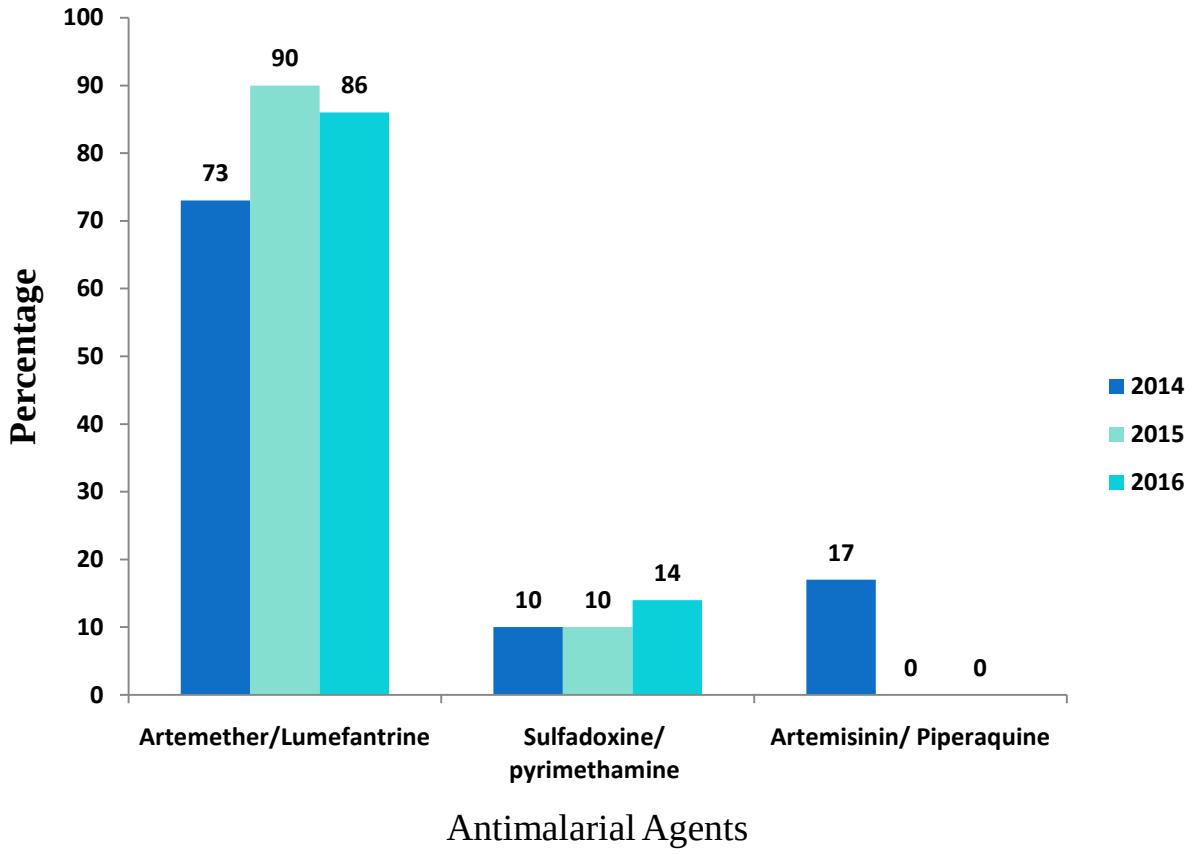

Figure 4.3: Reported Antimalarial Drugs Prescription at Dalhatu Araf Specialist Hospital Lafia Nigeria for the years 2014, 2015 and 2016

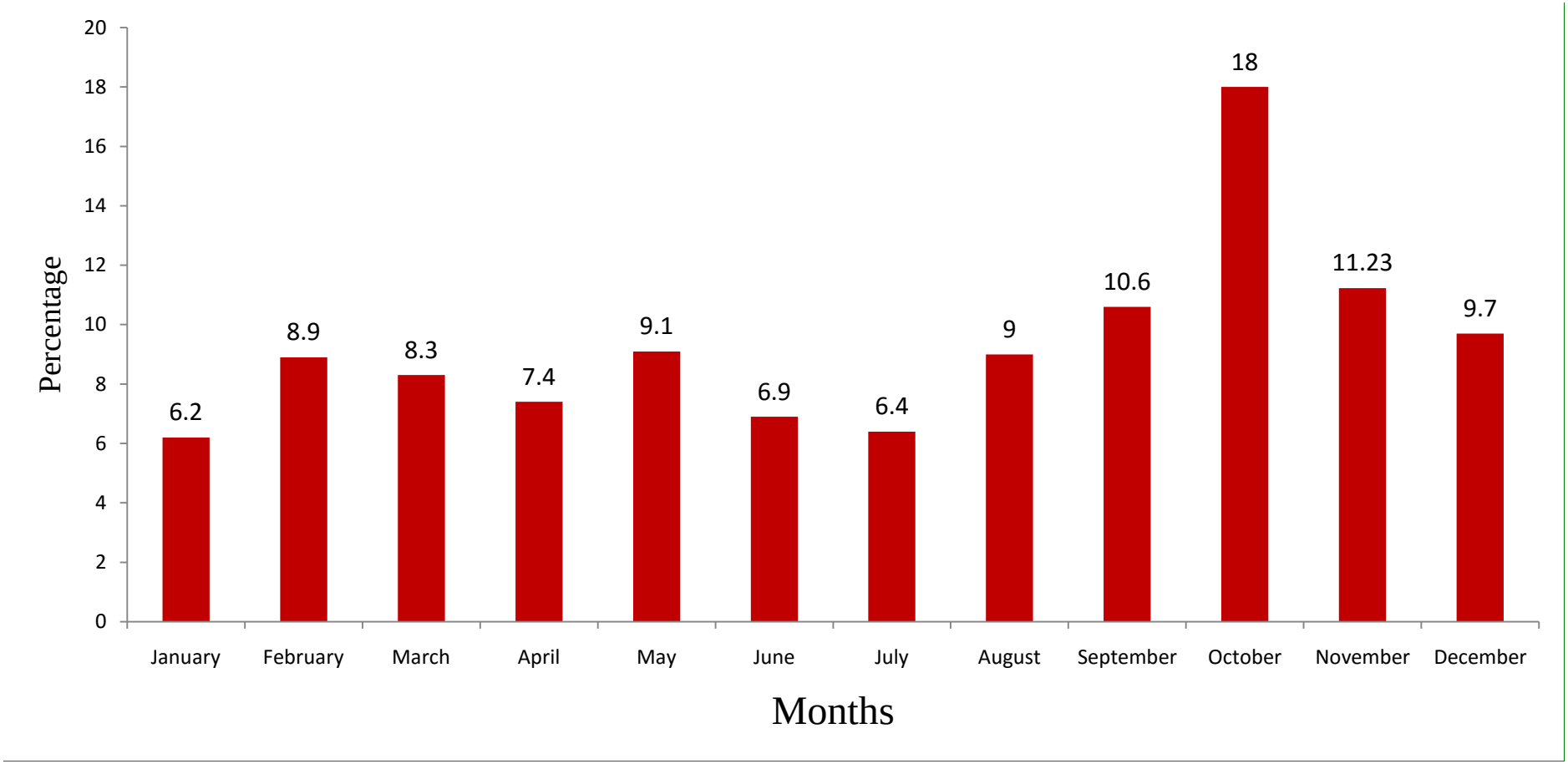

Figure 4.4: Profile of Malaria patients cases per Month at Dalhatu Araf Specialist Hospital Lafia, Nigeria

4.2 PROSPECTIVE STUDY RESULT

4.2.*1* *In vitro*antimalarial susceptibility test

A total of 112 samples were collected from patients with uncomplicated malaria at Dalhatu Araf Specialist Hospital Lafia Nigeria, only 91 were successfully analyzed due to contamination and lack of schizont maturation. The study subject was composed of 45 males and 67 females.Microscopic analysis showed that 91(81.25%) were *P. falciparum infection*, 8(7.14%) was a mixed infection of *P. falciparum and P. ovale* while 13(11.61%) were mixed infection of *P. falciparum/malariae*. The age group of the population was 0-5years (17.86%), 6-18 (35.71%) and 19 above (46.43%). The antimalarial susceptibility test was carried out using five antimalarial agents viz; Chloroquine, Piperaquine, Artemether, Artesunate and Lumefantrine.

4.2.2*In vitro* susceptibility of *Plasmodium falciparum* isolates to chloroquine

Some test isolates of *Plasmodium falciparum* (25 (27%)) displayed*in vitro*resistance to chloroquine. At a break point of 4.47µM, the geometric IC_{50} mean was 3.68µM and the IC50 range was observed to be 1.29- 12.23µM. Figure 4.5 shows the IC_{50} values of individual *Plasmodium falciparum* isolates to chloroquine while Figure 4.6 shows the percentage of*Plasmodium falciparum* isolates susceptible to the test antimalarial agents as observed in this study.

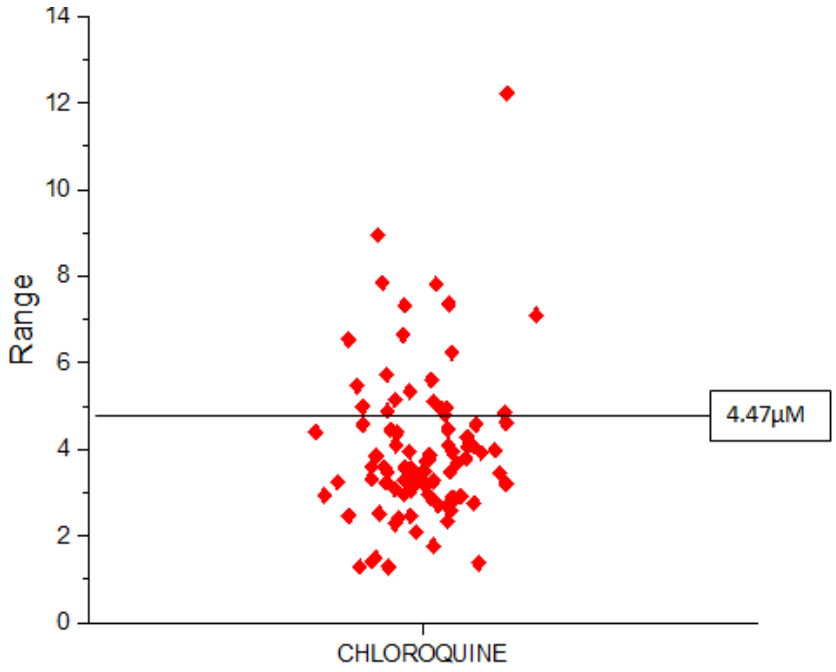

Figure 4.5: Inhibitory Concentration (50%) Values of test chloroquine against *Plasmodium falciparum* Isolates

Key

- Peak plasma concentration of test chloroquine 4.47µM as break point values.
- All *P. falciparum* isolates with chloroquine IC_{50} values above the horizontal line are resistant

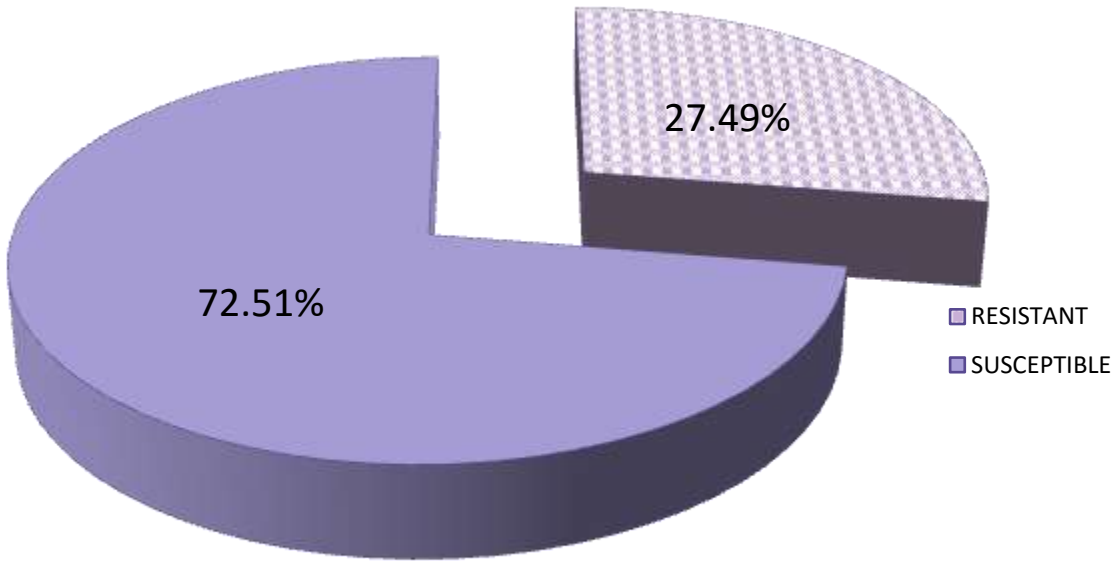

Figure 4.6: Pattern of *Plasmodium falciparum* isolates Susceptibility to Test Chloroquine

4.2.3*In vitro* susceptibility of *Plasmodium falciparum* isolates to piperaquine

The result of *in vitro* antimalarial susceptibility showed that 32(35.16%) of the 91 isolates wereresistant to piperaquine. At a break point of 1.4µM, the geometric IC_{50} mean was 1.12µM and the IC_{50} range was 0.07- 6.48µM. Figure 4.7 shows the IC_{50} values of individual *Plasmodium falciparum* isolates to test piperaquine while Figure 4.8 shows the percentage of test *Plasmodium falciparum* susceptibility to the test piperaquine as observed in this study.

4.2.4*In vitro* susceptibility of *Plasmodium falciparum* isolates to artemether

The result of *in vitro* susceptibility test showed that 10 (10.99%) of the 91 isolates were resistant to test Artemether. At a break point of 1.81µM, the geometric IC_{50} mean was 1.05µM and the IC_{50} range was 0.29- 3.62µM. Figure 4.9 shows the IC_{50} values of individual *Plasmodium falciparum* isolates to Artemether while Figure 4.10 shows the percentage of test P. falciparum susceptibility to the test Artemether as observed in this study.

4.2.5*In vitro* Susceptibility of *Plasmodium falciparum* Isolates to Artesunate

The result of *in vitro* susceptibility test showed that 26 (28.57%) of the 91 isolates wereresistant to Artesunate. At a break point of 77.11µM, the geometric IC_{50} mean was 49.08µM and the IC_{50} range was 10.04- 157.74µM. Figure 4.11 shows the IC_{50} values of individual *Plasmodium falciparum* isolates to Artesunate while Figure 4.12 shows the percentage of test *plasmodium falciparum* isolates susceptibility to the test Artesunate as observed in this study.

4.2.6*In vitro* susceptibility of *Plasmodium falciparum* isolates to lumefantrine

Observation from the *in vitro* susceptibility result indicated that 16(17.58%) of the 91 isolates displayed *in vitro*resistance to Lumefantrine. At a break point of 53.51µM, the geometric IC_{50} mean was 27.77µM and the IC_{50} range was 1.32- 100.76µM. Figure 4.13 shows the IC_{50} values of individual *Plasmodium falciparum* isolates to Lumefantrine while Figure 4.14 shows the percentage of *P. falciparum* susceptibile to Lumefantrine as observed in this study.

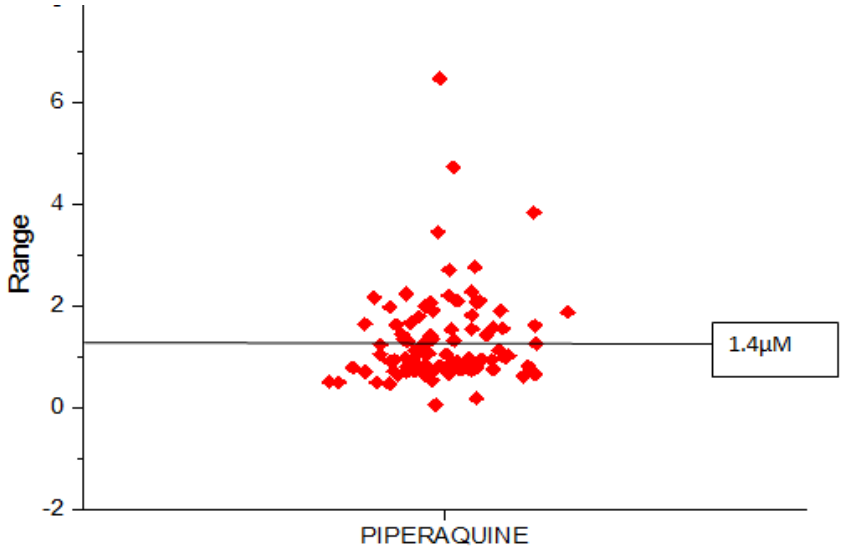

Figure 4.7: Inhibitory Concentration (50%) Values of test piperaquine agaist *Plasmodium falciparum* Isolates

Key

- Peak plasma concentration of test Piperaquine is1.4µM as break point value
- All *P. falciparum* isolates with Piperaquine IC_{50} values above the horizontal line are resistant

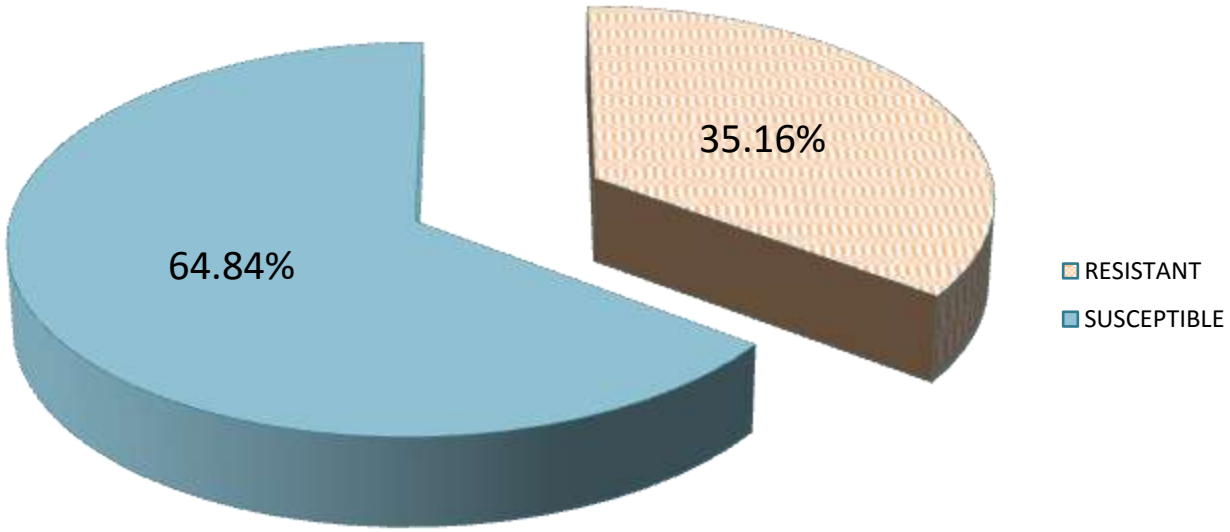

Figure 4.8: Pattern of *Plasmodium falciparum* isolates susceptibility to test Piperaquine

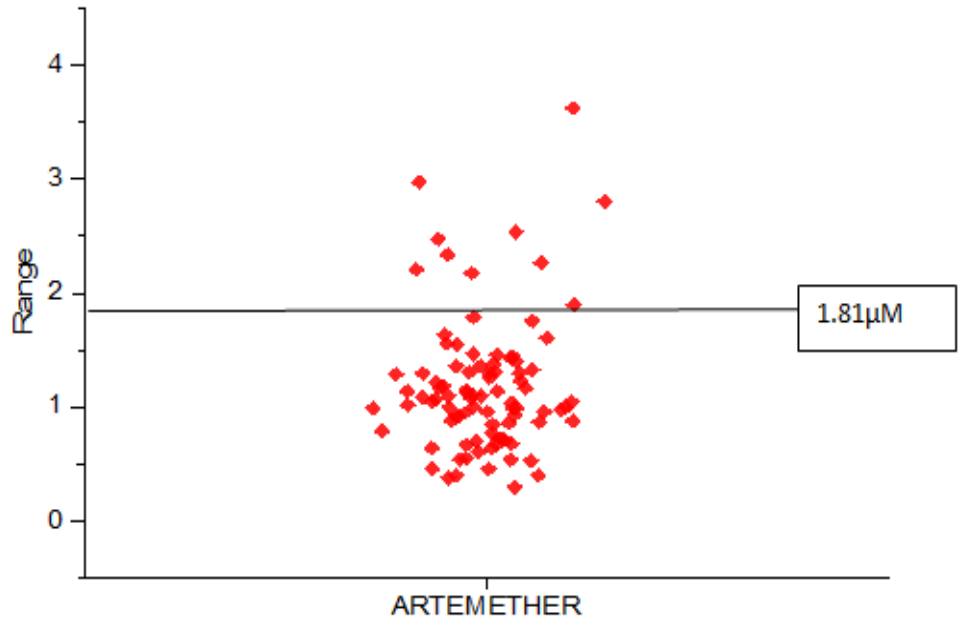

Figure 4.9: Inhibitory Concentration (50%) Values of artemether agaist test *P. falciparum* Isolates susceptibility to Artemether

Key

- Peak plasma concentration of test artemether is 1.81µM as break point value
- All *P. falciparum* isolates with artemether IC_{50} values above the horizontal line are resistant

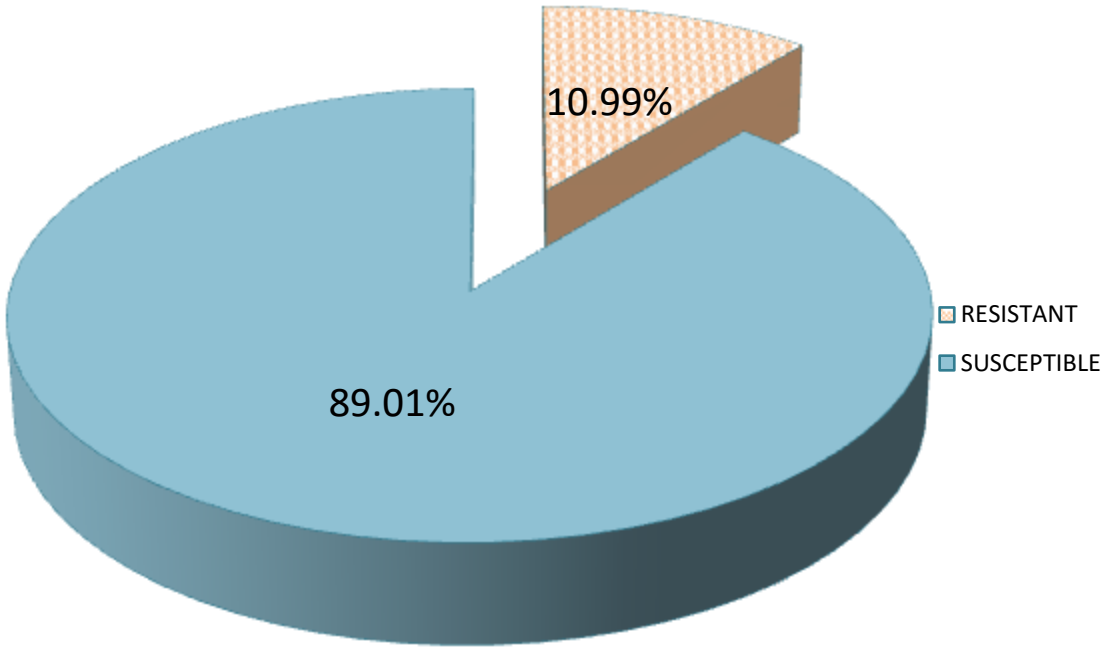

Figure 4.10: Pattern of *Plasmodium falciparum* isolates susceptibility to test Artemether

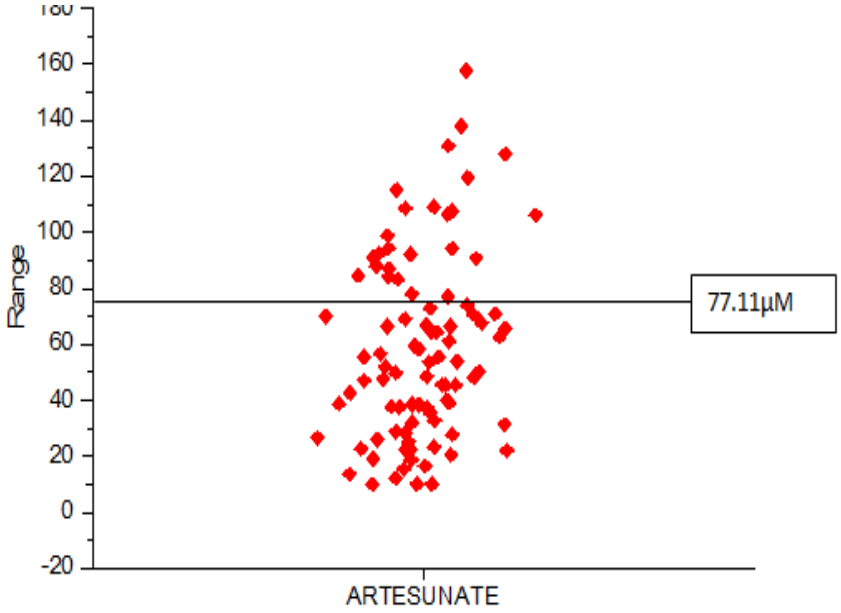

Figure 4.11: Inhibitory Concentration (50%) Values of test artesunate against *Plasmodium falciparum* Isolates

Key

- Peak plasma concentration of test artesunate is 77.11µM as break point value
- All *P. falciparum* isolates with artesunate IC_{50} values above the horizontal line are resistant

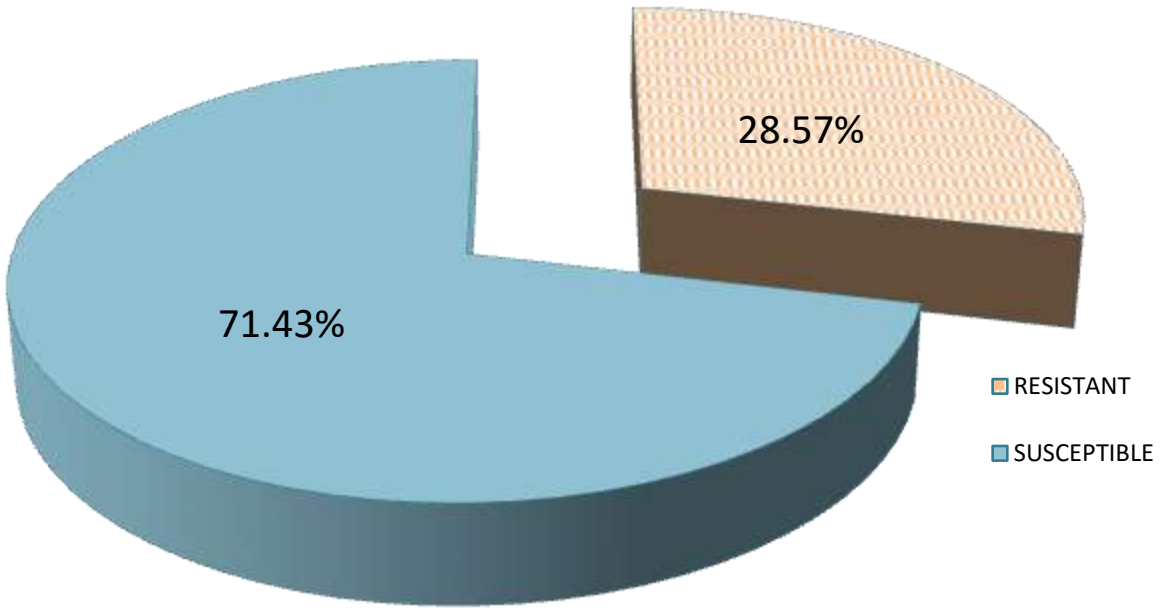

Figure 4.12: Pattern of *Plasmodium falciparum* isolates susceptibility to test Artesunate

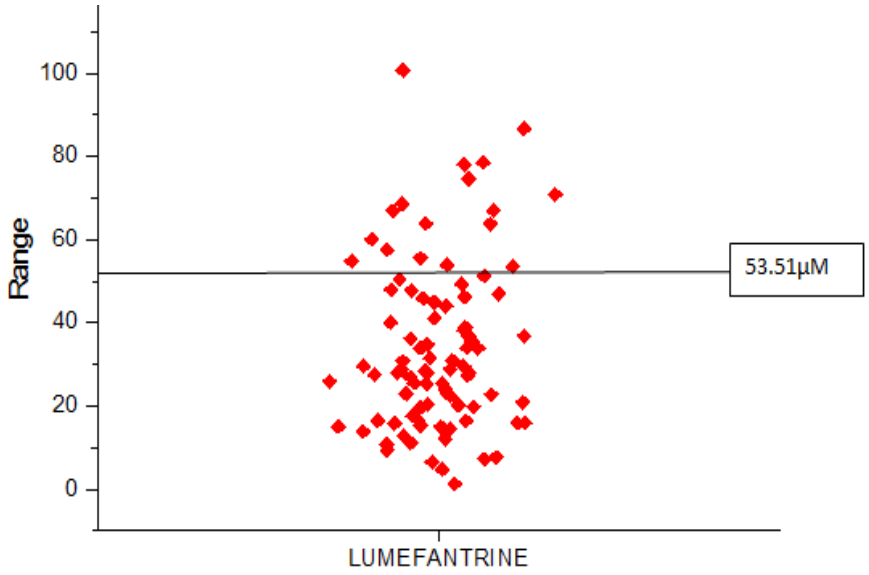

Figure 4.13: Inhibitory Concentration (50%) Values of test Lumefantrine against *Plasmodium falciparum* Isolates

Key

- Peak plasma concentration of lumefantrine is 53.51µM as break point value
- All *P. falciparum* isolates with lumefantrine IC_{50} values above the horizontal line are resistant

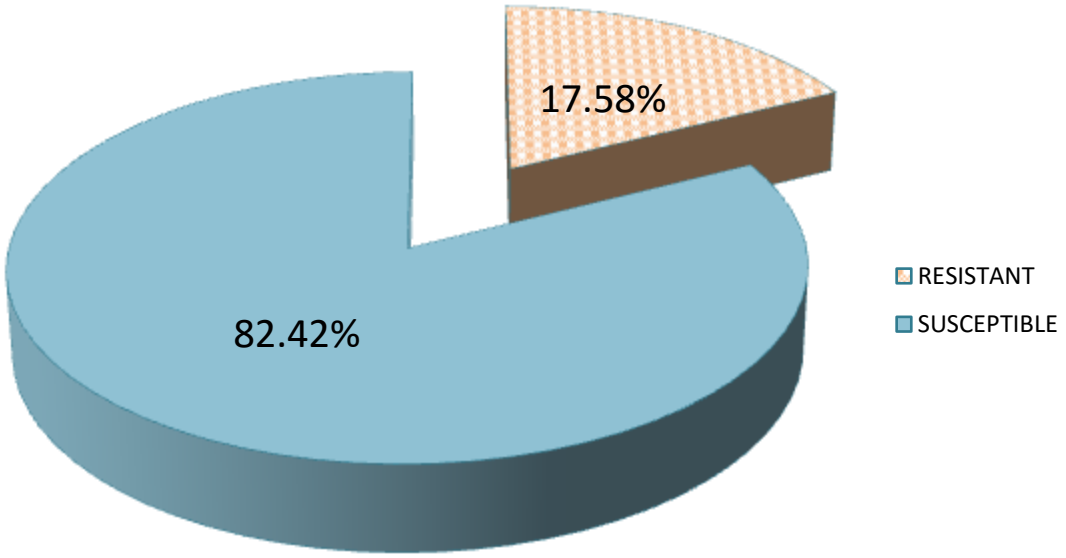

Figure 4.14: Pattern of *Plasmodium falciparum* isolates susceptibility to test Lumefantrine

Table 4.1: Profile of *Plasmodium falciparum* Isolates Susceptibility to Five test Antimalarial Agents in Lafia, Nigeria

Test Antimalarial Agents	Peak Plasma Concentration as Break Point Value (µM)	Resistance Profile of Test *P. falciparum* (%) (n=91)
Chloroquine	4.47	25 (27.49)
Piperaquine	1.40	32 (35.16)
Artemether	1.81	10(10.99)
Artesunate	77.11	26(28.57)
Lumefantrine	53.51	16(17.58)

4.3: Molecular Characterization of Resistant Strains

A total of 8 *Plasmodium falciparum* isolates were observed to be resistant to the five antimalarial agents tested.The result of PCR of the resistant *P. falciparum* isolatesshowed that*Plasmodium falciparum* chloroquine resistance transporter (Pfcrt) and Kelch 13 Proppeler (K13P) genes were absent (Plate4.1. and 4.2respectively), however, theresults obtained showedthe presence of *Plasmodium falciparum*multidrug resistance (*Pf*mdr1) gene with base pair ranging from 165bp-548bp (Plate4.3).

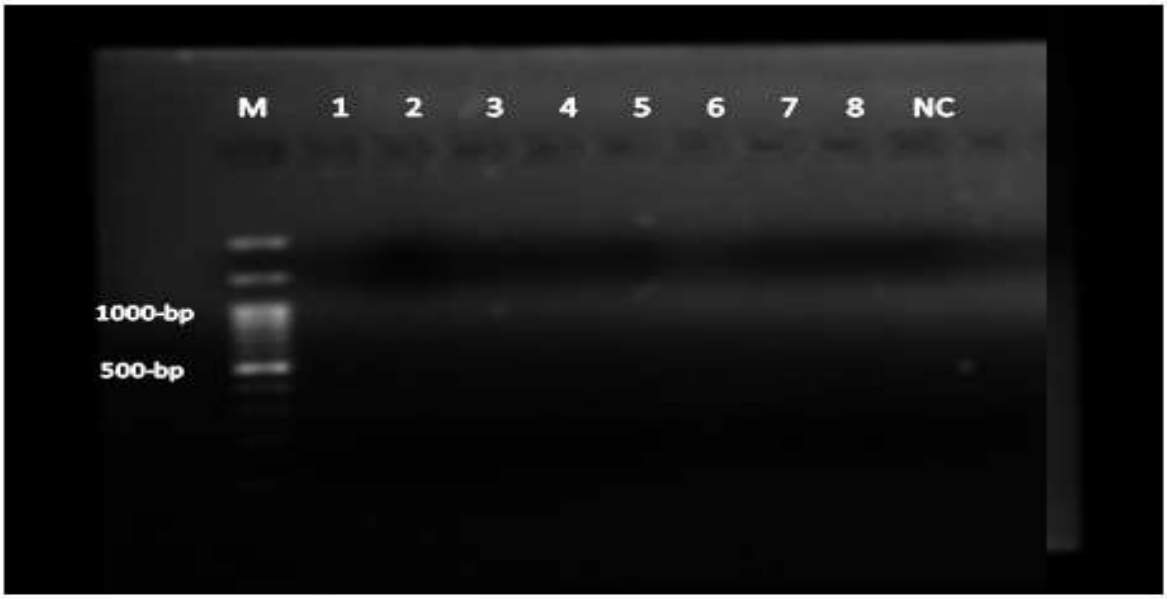

Plate 4.1: Results of the nested PCR-amplified product for the Pfcrt gene

KEY:

M (Marker) ------ 1kb Ladder

Lane 1-8 --- Samples

Lane NC --- Negative Control

BP --- Base Pairs

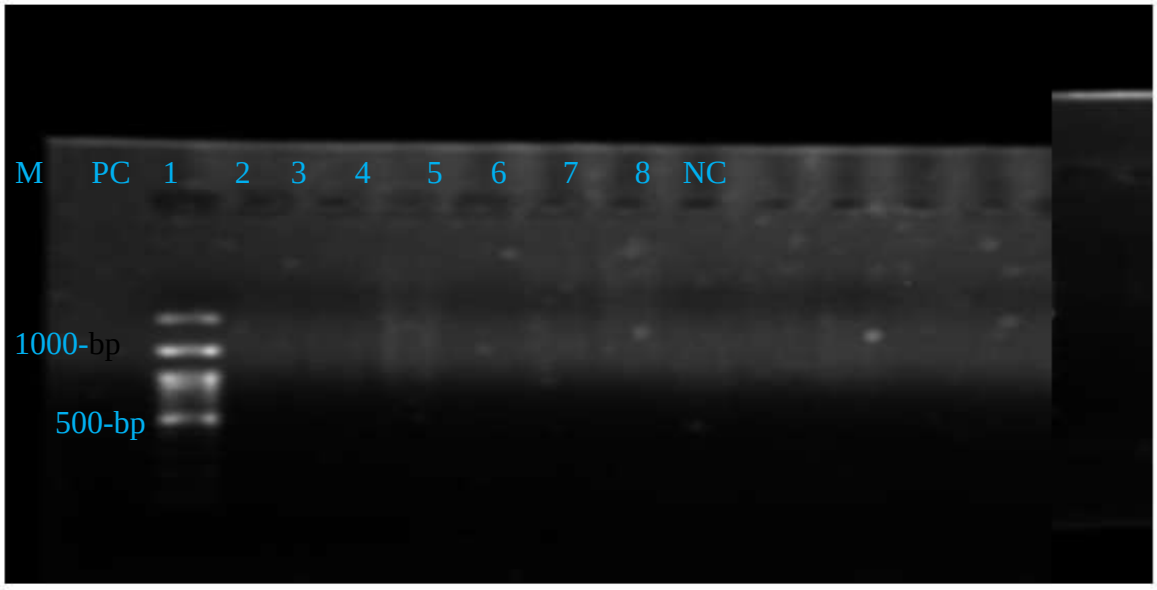

Plate4.2: Results of nested PCR-amplified product for the K13P gene

KEY:

Lane M (Marker) ---- 1kb Ladder

Lane PC --- Positive Control

Lane 1-8 --- Samples

Lane NC --- Negative Control

BP --- Base Pairs

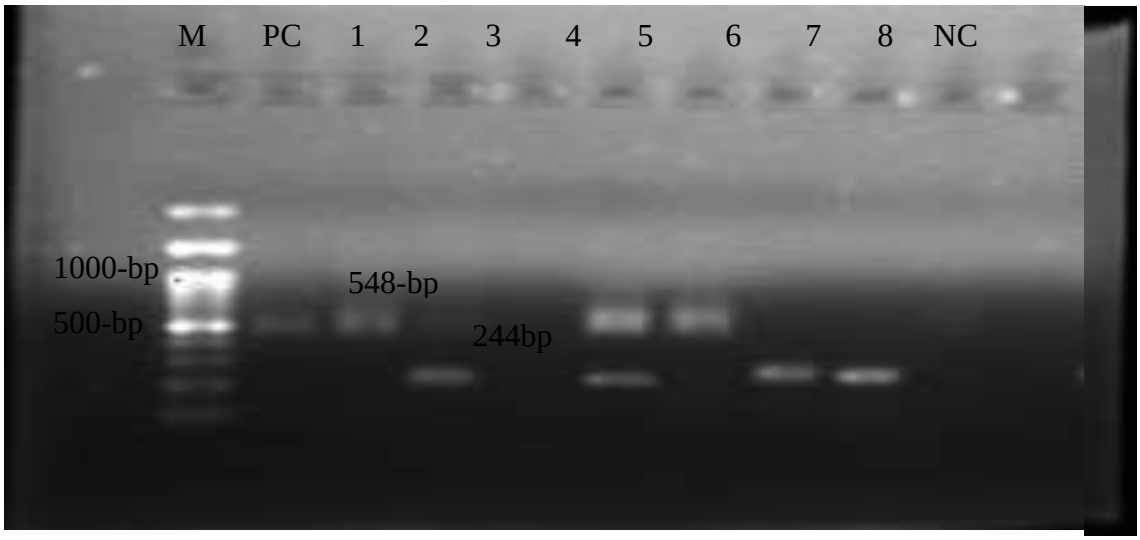

Plate 4.3: Results of nested PCR-amplified product for Pfmdr1 gene

KEY

Lane M (Marker) -- 1kb Ladder

Lane PC -- Positive Control amplified at 548bp

Lane 1and 5 -- *Pf*mdr1 amplified at 548bp

Lane 4 -- *Pf*mdr1 amplified at 548bp and 244bp

Lane 2,6 and7 -- *Pf*mdr1 amplified at 244bp

Lane NC -- Negative Control

BP -- Base Pairs

Table 4.2: Molecular Characterization of Resistant Strains

Resistant *P.Falciparum* isolates	***PF*CRT**	**K13P**	**PFMDR1**
1	–	–	+
2	–	–	+
3	–	–	–
4	–	–	+
5	–	–	+
6	–	–	+
7	–	–	+
8	–	–	–

CHAPTER 5

5.0 DISCUSSION

Data obtained from the retrospective study showed that malaria was higher among the adults (62.2%, 68.3% and 64.6%) for 2014, 2015 and 2016 respectively followed by the minors (13.57% and 16.3%) for 2014 and 2015. The least was observed in the 6-18 years group (13.57% and 15.3%) for 2014 and 2015 respectively. In 2016, cases were higher in the 6-18 age group (18.7%) than the minors (16.7%). Although the minors are more vulnerable to malaria, the low prevalence observed might be due to the various interventions in play, such as the President's Malaria Initiateve's (PMI) support in this locality. This is similar to the work of Nas *et al.,* (2017) who reported 34.1% cases in adults as compared to 28.3% among the 10-18years. This however contradicts the results of Singh *et al.*,(2014) and Nmadu *et al.,* (2015) who reported higher cases in children 60% and 58% respectively.

Gender wise, Malaria was observed higher in female patients (59.8%, 61% and 53.8%) than the male (40.2%, 38.9% and 47.2%) for the three years respectively.The difference observed might be due to the varying predisposing factors of each gender to the infection. This range from differences in immune response resulting in differential vulnerability of both gender to malaria. During pregnancy, menstruation, ovulation e.t.c, female immunity is weakened thus increasing vulnerability to malaria. Women also wake up before dawn to perform household chores, which expose them to mosquitoes and consequently malaria infection. This finding agrees with the work of Nas *et al.,* (2017) who reported 54% cases in female and 46% in Male. The result does not agree with the

work of Nwaorgu and Orajaka (2011) who reported no significant difference in prevalence among male and female.

The antimalarial drugs observed prescribed at the Dalhatu Araf Specialist Hospital Lafia was basically Artemisinin combination therapy. This indicates the hospitals compliance with the National antimalarial treatment guideline on the use Artemisinin Combination Therapy for the treatment of uncomplicated malaria in Nigeria. This result agrees with the survey of Shorinwa and Ebong (2012) who revealed that Artemisinin combination therapy drugs were the most frequently purchased antimalarial drugs in Port Harcourt and the study of Okoro and Jamiu (2018) who reported high usage of Artemisinin Combination Therapy (95.8%) for malaria treatment at the University of Nigeria Teaching Hospital (UNTH).

There were report of malaria cases throughout the year. Result on monthly distribution of malaria showed the highest occurrence from May to October. This might be because the period corresponds to the rainy season when conditions for the multiplication of malaria vector is favourable.

The *in vitro* test is a useful tool in assessing the intrinsic *in vitro* sensitivity of the *Plasmodium falciparum* parasite to antimalarial drugs without the cofounding factor from the host that influence test *in vivo*. It is thus a more objective approach to parasite resistance as it is base on direct contact between the parasite and increasing drug concentration.

The *in vitro* test result showed that *P. falciparum* isolates were susceptibile to Artemether (89%). This agrees with the work of Bustamante *et al.,* (2012) who also reported a high

susceptibility of test *P. falciparum* to Artemether. Result on Artesunate activity showed that 71% of the test *P. falciparum* were susceptible. Aminu and Mukhtar, (2012) and Olasehinde *et al.,* (2014) reported that 100% of test *P. falciparum* isolates were susceptibile to artesunate *in vitro*. The *in vitro* results from this study showed reduced susceptibility of test *P. falciparum* against the Artemisinin derivatives tested such as Artesunate 71% and Artemether 89.01%. The reduced susceptibility of the test isolates to the artemisinin derivatives observed in this study may be due to the misuse or use of substandard Artemisinin monotherapy for the treatment of uncomplicated malaria. The report of Uzochukwu *et al.,* (2010) in Nigeria showed that the prescription of Artemisinin monotherapy was 18.2% and 15.8% in the southwest and southeast part of Nigeria. The misuse or substandard Artemisinin monotherapy for the treatment of uncomplicated malaria might result to emergence of resistant *P. falciparum* (Bustamante *et al.,* 2012).

The *in vitro* antimalarial activity of Lumefantrine and Piperaquine was evaluated and observations from the results showed that test *P. falciparum* was 82% and 65% susceptible respectively . The reduced *invitro* susceptibility of test *P. falciparum* to Piperaquine might be because of its previous use . The reduced *in vitro* effectiveness of Lumefantrine might be because of the presence of *Pf*mdr1 gene found in the resistant isolates.

In vitro test has been reported useful for monitoring changes overtime in susceptibility of *P.falciparum* to antimalarial drug that have been withdrawn. This study reveals a 73% susceptibility of test *P.falciparum* to chloroquine. The low resistance observed might be as a result of withdrawal of chloroquine for the treatment of uncomplicated malaria. Over the time of chloroquine withdrawal, new generations of *Plasmodium* might lose the

resistance trait to chloroquine. This study agrees with the work of Olasehinde *et al.,* (2014) who reported 80% *in vitro* susceptibility to chloroquine at Ijebu (Southwest). This however does not agree with the work of Balogun *et al.,* (2016) who reported that 67% of test *P. falciparum* were observed to be resistant in Azare (Northwest) and 84% were resistant in Maiduguri (NorthEast).

Observations from polymerase chain reaction with appropriate primers as indicated in this report showed that *Pf*crt and K13P resistant genes were absent in the 8 multidrug resistant *P. falciparum* isolates.

It has been reported that *Pf*mdr1 resistant gene can effect drastic reduction in the antimalarial activity of agents such as chloroquine, artesunate, artemether, lumefantrine and piperaquine (Frosch *et al.,* 2014; Olasehinde *et al.,* 2014 and Some *et al.,* 2016). The studied 8 multidrugs resistant *P. falciparum* isolates showed that 6(75%) displayed *pf*mdr1 genes with base pair ranging from 244-548bp. Four out of the seven multidrugs resistant *P. falciparum* isolated harboured multidrug resistant gene of 244bp while three displayed multidrug resistant gene of 548bp. This study has shown that Pfmdr1 gene of *P. falciparum* with 244bp and 548bp encodes resistance to chloroquine, artesunate, artemether, lumefantrine and piperaquine. Olasehinde *et al.,*(2014) reported the presence of multidrug resistance gene base pair of 244bp and 548bp in resistant *P. falciparum* isolates. Several studies suggest that Arthemeter/Lumefantrine contributes to the selection of *pf*mdr1 gene among parasite isolates in Africa (Thomsen *et al.*, 2013). The prevalence of *pf*mdr1 gene in this study was 75%. This may likely be due to the widespread use of Arthemeter/Lumefantrine as first line treatment in Lafia, Nigeria which selects the wild type sequence of the gene (Some *et al.,* 2010).

Data from this study supports the work of Lawal (2018) who reported 75% prevalence of pfmdr1 gene in test *P. falciparum*from pregnant women in Minna. Simon Oke *et al.,* (2018) reported 51% prevalence in Akure, Olasehinde *et al.*,(2014) reported 50% in Ijebu, 69% in Yewa, 48% multidrug resistant *P. falciparum* isolates in Egba Ogun state. However, the work of Okungbowa and Mordi (2013) reported 18.9% multidrug *P. falciparum* in Edo state, Some *et al.,*(2014) reported 19.58%, Wurtz *et al.,* (2014) reported 14.9% and Balogun *et al.,* (2016)who reported 0% prevalence in Maiduguri.

CHAPTER 6

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

- Malaria prevalence rate in this study was 5.03%, observations from this study showed that malaria incidence was more with older age group (19 and above years) and females.
- Artemisinin combination therapy prescription was (88.67%), there was no chloroquine prescription at the study site.
- *Invitro* study results indicate emergence of *P. falciparum* resistance to Artesunate (71%), arthemeter (89%) Lumefantrine (82%) and Piperaquine (65%).
- This study showed that the resistant *P. falciparum* isolates harboured *Pf*mdr1 genes with base pair ranging from 244bp to 548bp.

6.2 CONCLUSION

Malaria prevalence rate in this study was 5.03%, observation from the study also showed that ACT were highly prescribed. 91% of the blood samples screened had *Plasmodium falciparum* infections. *Invitro* susceptibility of *P. falciparum* isolates to tested antimalarial agents are: Artesunate (71%), arthemeter (89%) Lumefantrine (82%) and Piperaquine (65%).*In vitro*susceptibility study displayed emergence of resistant *P. falciparum* isolates, 75.00% with *Pf*mdr1 gene of 244bp- 548bp.

6.3 RECOMMENDATION

1. More research are required to provide evidence for the stratification of the country's malaria epidemiology.
2. There should be increased surveillance of molecular markers of drug resistance and particularly, potential markers of resistance to Artemisinin Combination Therapies.

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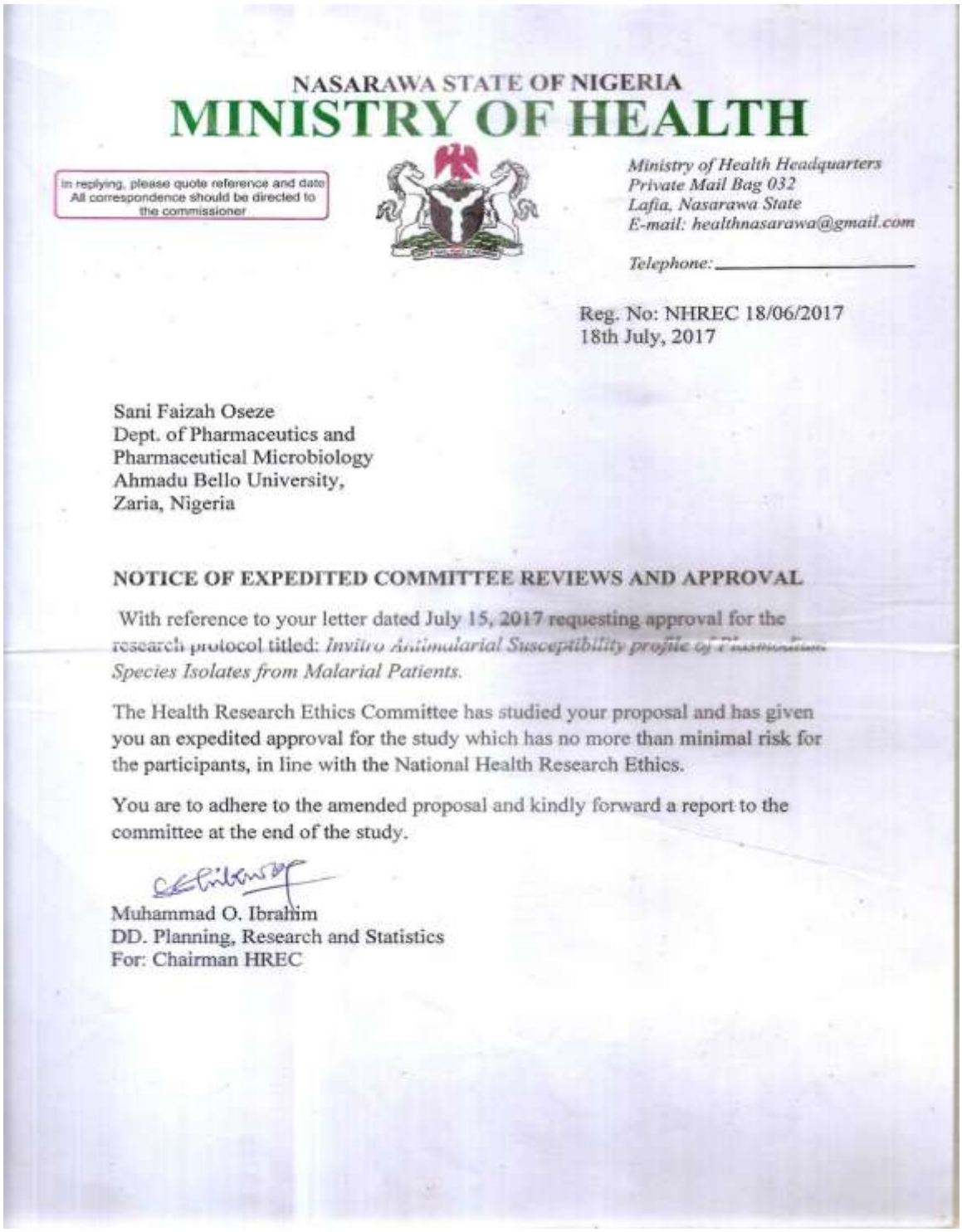
APPENDIX

Appendix 1 ETHICAL CLEARANCE FORM



NASARAWA STATE OF NIGERIA

MINISTRY OF HEALTH

In replying, please quote reference and date
All correspondence should be directed to
the commissioner

Ministry of Health Headquarters
Private Mail Bag 032
Lafia, Nasarawa State
E-mail: healthnasarawa@gmail.com

*Telephone:*_____

Reg. No: NHREC 18/06/2017
18th July, 2017

Sani Faizah Oseze
Dept. of Pharmaceutics and
Pharmaceutical Microbiology
Ahmadu Bello University,
Zaria, Nigeria

NOTICE OF EXPEDITED COMMITTEE REVIEWS AND APPROVAL

With reference to your letter dated July 15, 2017 requesting approval for the research protocol titled: *Invitro Antimalarial Susceptibility profile of Plasmodium Species Isolates from Malarial Patients.*

The Health Research Ethics Committee has studied your proposal and has given you an expedited approval for the study which has no more than minimal risk for the participants, in line with the National Health Research Ethics.

You are to adhere to the amended proposal and kindly forward a report to the committee at the end of the study.

Muhammad O. Ibrahim
DD. Planning, Research and Statistics
For: Chairman HREC

Appendix 2 Media preparation

Preparation of culture medium

One liter of Malaria culture medium was prepared by dissolving 10.43g RPMI 1640 powder (Sigma Aldrich) and 5.94g of HEPES (Sigma Aldrich) in 900mls of distilled-deionised water. Zero point six two five milliliter (0.625ml) of 40mg/ml gentamicin was added and sterile water was added to 960ml. The medium was filtered using 0.22 µm pore size membrane filter and stored at 4 ºC in aliquots of 100ml.

Preparation of incomplete media

4.2ml of 5% sodium Bicarbonate was added to the 100ml RPMI 1640 aliquout.

Preparation of complete media

To prepare 20ml of the complete culture medium, 10% O positive Human serum was added to the incomplete media (i.e 2ml of the O positive human serum into 18ml of the incomplete culture media).

Appendix 3: Age distribution of patients presenting with Malaria at Dalhatu Araf Specialist Hospital Lafia for the years 2014, 2015 and 2016

AGE GROUP	2014 (%)	2015 (%)	2016 (%)	Average (%)
0-5	885 (24.22)	683 (16.29)	424 (16.66)	19.06
6-18	496(13.57)	643 (15.34)	477 (18.74)	15.88
19above	2273 (62.21)	2863 (68.30)	1644 (64.60)	65.04

KEY:

- 2014, 2015 and 2016 total cases recorded are 61,936, 95,287 and 53,026 respectively
- Prevalence for 2014, 2015 and 2016 are 5.90%, 4.40% and 4.80% respectively

Appendix 4: Gender profile of patients presenting with Malaria at Dalhatu Araf Specialist Hospital Lafia for the years 2014, 2015 and 2016

GENDER	2014 (%)	2015 (%)	2016(%)	Average
MALE	1470 (40.23)	1632 (38.93)	1124 (44.17)	41.11
FEMALE	2184(59.77)	2557 (61)	1421 (55.83)	58.87

KEY:

- 2014, 2015 and 2016 total cases recorded are 61,936, 95,287 and 53,026 respectively
- Prevalence for 2014, 2015 and 2016 are 5.90%, 4.40% and 4.80% respectively

Appendix 5: Antimalarial Drug Prescription at Dalhatu Araf Specialist Hospital Lafia for the years 2014, 2015 and 2016

PRESCRIBED AGENT	2014 (%)	2015 (%)	2016 (%)	AVERAGE%
Artemether/Lumefantrine	2667 (73)	3770 (90)	2191 (86)	88.67
Sulfadoxine/ Pyrimethamine	366 (10)	419 (10)	357 (14)	11.33
Artemisinin/ Piperaquine	622 (17)	__	__	

KEY:

- 2014, 2015 and 2016 total cases recorded are 61,936, 95,287 and 53,026 respectively
- Prevalence for 2014, 2015 and 2016 are 5.90%, 4.40% and 4.80% respectively

Appendix 6: Number of Malaria Cases Recorded per Month of 2014, 2015 and 2016 at DASHL

MONTHS	2014 (%)	2015 (%)	2016 (%)	AVERAGE%
January	323 (8.84)	17 (0.41)	236 (9.27)	6.17
February	515 (14.09)	441 (10.52)	54 (2.12)	8.91
March	253 (6.92)	287 (6.84)	283 (11.11)	8.29
April	267 (7.31)	79 (1.88)	337 (13.24)	7.46
May	567 (15.52)	115 (2.74)	232 (9.12)	9.13
June	398 (10.89)	358 (8.54)	31 (1.22)	6.88
July	4 (0.11)	534 (12.74)	__	6.42
August	90 (2.46)	650 (15.51)	__	8.99
September	505 (13.82)	311 (7.42)	__	10.62
October	424 (11.60)	758 (18.08)	619 (24.32)	18.00
November	308 (8.43)	315 (7.51)	455 (17.88)	11.27
December	__	324(7.73)	298 (11.71)	9.72

KEY:

- 2014, 2015 and 2016 total cases recorded are 61,936, 95,287 and 53,026 respectively
- Prevalence for 2014, 2015 and 2016 are 5.90%, 4.40% and 4.80% respectively

Appendix 7: IC50 VALUES OF ANTIMALARIAL DRUGS AGAINST P. Falciparum species FROM LAFIA

SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
1	3.88	0,87	35.50	0.84	24. 18
2	7.82	0.77	64.40	0.72	30.93
3	2.41	1.80	37.62	0.53	25.61
4	7.33	2.01	28.36	0.66	19.67
5	1.29	1.32	87.08	0.99	12.83
6	3.17	0.07	59.48	0.69	31.57
7	3.32	0.48	10.04	0.63	10.84

SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
8	2.97	0.66	69.11	0.54	15.35
9	3.61	1.99	19.29	0.45	9.40
10	1.38	0.99	50.22	0.95	7.78
11	2.09	3.46	10.14	0.60	6.58
12	2.59	2.78	20.55	0.29	27.31
13	2.96	1.55	53.71	0.63	12.14
14	2.76	1.92	48.15	0.86	22.81
15	4.46	1.67	37.76	0.87	22.99

SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
16	6.54	1.66	13.72	1.13	13.92
17	12.23	1.27	22.15	1.89	16.00
18	5.10	2.11	32.79	1.13	22.49
19	4.99	1.25	55.49	1.29	16.52
20	3.56	0.72	32.12	1.78	20.41
21	4.93	0.84	45.67	0.70	20.26
22	3.95	1.44	22.44	1.07	28.55
23	6.65	1.27	15.62	0.94	16.47
24	3.50	1.06	16.61	0.95	15.06
25	3.28	0.93	23.41	1.45	28.90

SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
26	7.36	0.77	39.16	0.98	16.47
27	2.94	0.51	70.08	0.78	15.06
28	1.42	0.93	91.01	1.04	57.57
29	3.98	0.63	70.92	0.97	53.62
30	3.25	0.8	38.88	1.28	54.89
31	3.09	0.78	49.91	0.91	26.96
32	4.1	0.83	61.12	1.44	38.93
33	2.92	1.44	138.00	1.16	33.94
34	3.48	0.86	66.50	0.92	34.03
35	3.07	1.36	78.02	1.46	27.82

SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
36	4.39	1.06	83.31	0.91	17.61
37	3.21	0.67	65.82	0.87	36.88
38	2.89	4.74	64.57	1.37	53.94
39	3.68	0.96	53.96	1.22	19.86
40	2.72	0.77	55.49	0.72	1.32
41	3.24	0.99	66.50	1.55	28.83
42	5.61	1.33	10.14	1.30	23.04
43	3.48	0.81	84.16	1.09	100.76
44	3.46	0.83	62.69	0.99	16.01
45	4.81	0.99	45.39	0.85	49.32

SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
46	3.28	0.84	58.39	1.09	41.16
47	3.77	0.82	72.96	0.76	44.05
48	4.58	1.06	47.14	1.08	16.49
49	3.80	0.95	157.74	0.52	78.58
50	1.50	0.73	87.81	1.06	40.10
51	3.29	0.85	108.61	1.12	55.67
52	4.89	0.72	94.40	0.37	30.86
53	3.95	0.80	94.25	0.98	28.14
54	4.07	0.77	119.56	1.32	7.34
55	4.09	1.14	70.75	0.39	63.82

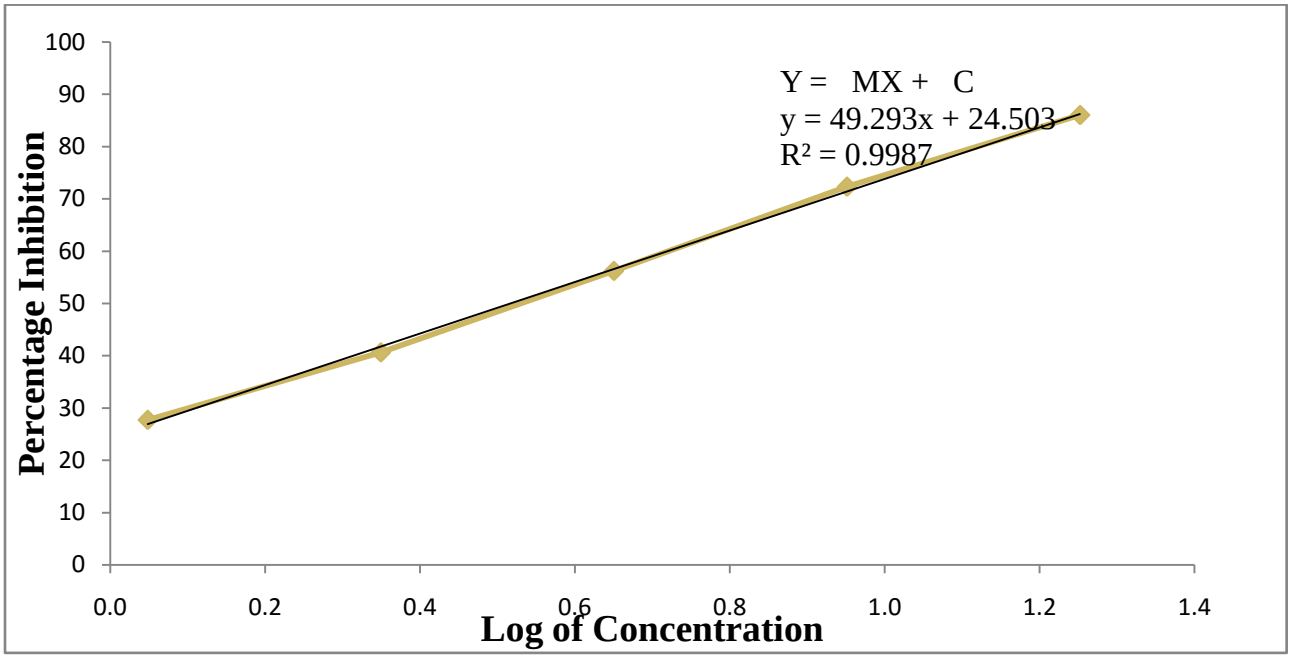
SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
56	5.34	2.08	92.17	2.17	63.92
57	3.93	1.04	67.74	1.60	47.03
58	6.24	2.09	107.65	2.53	74.65
59	4.29	1.58	73.99	1.75	51.33
60	5.73	2.25	98.86	2.33	68.66
61	2.47	0.72	42.68	1.01	29.60
62	3.59	1.06	22.44	1.14	33.95
63	2.90	0.20	27.89	1.39	36.90
64	5.15	0.73	28.91	0.39	11.23
65	4.40	0.52	26.67	0.98	25.97

SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
66	1.29	0.51	22.80	2.97	27.56
67	2.47	0.55	18.84	1.10	25.40
68	4.96	0.75	40.06	0.53	29.64
69	3.27	1.08	25.34	1.30	45.90
70	2.29	0.89	12.26	1.35	36.20
71	3.85	0.95	26.12	1.21	47.99
72	3.72	0.68	66.90	0.45	4.78
73	1.77	0.87	109.13	0.66	14.56
74	2.35	1.56	106.56	1.42	38.20
75	8.95	1.64	92.44	2.47	66.99

SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
76	3.59	1.36	51.92	1.63	50.53
77	3.25	1.92	38.55	0.99	34.86
78	7.10	1.89	106.27	2.80	70.88
79	4.10	1.14	115.18	1.54	47.83
80	3.21	2.72	37.36	1.25	14.73
81	4.58	1.57	90.79	2.26	67.02
82	4.85	3.85	31.45	1.04	20.99
83	2.71	2.29	77.14	0.67	78.09
84	5.47	2.18	84.57	2.20	60.10
85	2.87	2.11	45.54	1.29	35.52

SAMPLE NO.	CHLOROQUINE (Peak Plasma Concentration 4.47µM)	PIPERAQUINE (Peak Plasma Concentration 1.4µM)	ARTESUNATE (Peak Plasma Concentration 77.11µM)	ARTEMETHER (Peak Plasma Concentration 1.81µM)	LUMEFANTRINE (Peak Plasma Concentration 53.51µM)
86	2.52	0.66	56.69	1.16	15.91
87	7.85	1.46	47.63	1.18	28.12
88	3.16	2.22	48.57	1.29	25.37
89	3.41	6.48	38.57	1.35	45.01
90	4.47	1.83	130.89	1.03	46.27
91	4.62	1.63	128.00	3.62	86.69
Total Number Of Samples	91	91	91	91	91
Total Number Of Resistant Samples	25	32	26	10	16
Percentage OfResistant Samples	27.47	35.16	28.57	10.99	17.58

Appendix 8: GRAPH FOR THE ESTIMATION OF IC50 VALUES



Inhibitory concentration (50%) was calculated using a straight line equation (Y = MX + C) from a Scatter plot of Percentage Inhibition of drug concentrations (Y axis) over the Log of the Concentrations (X axis), Were M is the Gradient and C is the Intercept on the Y axis.

Thus, IC50 was calculated as

$$IC50 = Antilog((50 - C) / M)$$

Appendix 9: BAND SIZE MEASUREMENT FOR PFMDR1 GENE AMPLIFICATION

Base Pair	Distance (cm)	Log Base Pair
100	3.5000	2.0000
200	3.2	2.3010
300	2.9	2.4771
400	2.7	2.6021
500	2.5	2.6990

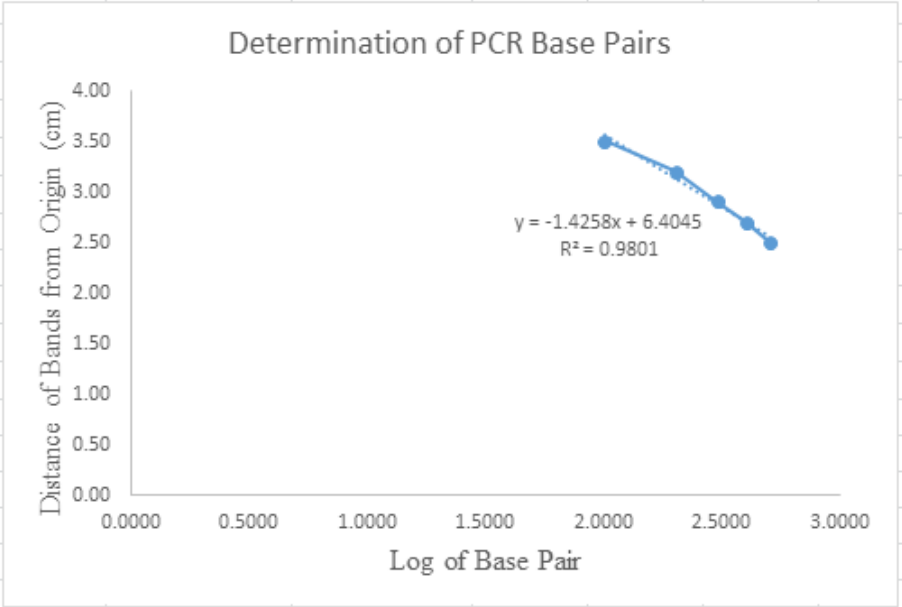
Log Base Pair	Distance (cm)
2.0000	3.50
2.3010	3.20
2.4771	2.90
2.6021	2.70
2.6990	2.50

Base Pair for PFmdr1 Outer Region

6.4045	-3.9045
-1.4258	2.738462617
Base Pair	**547.60**

Base Pair for PFmdr1 Inner Region

6.4045	-3.4045
-1.4258	2.387782298
Base Pair	**244.22**



Appendix 10: Concentrations of test antimalarial drugs in micro titre plates

Antimalarial Agents	Well A	Concentrations (µM) In Well B	Concentrations (µM) In Well C	Concentrations (µM) In Well D	Concentrations (µM) In Well E	Concentrations (µM) In Well F
Arthemeter	Control	0.46	0.91	1.81	3.62	7.24
Artesunate	Control	19.28	38.56	77.11	154.22	308.44
Chloroquine	Control	1.12	2.25	4.47	8.94	17.88
Piperaquine	Control	0.34	0.68	1.36	2.72	5.44
Lumefantrine	Control	13.38	26.76	53.51	107.02	214.04