CHAPTER ONE

INTRODUCTION

1.1 Background

The term malaria originated from Medieval Italian: *mala aria* meaning "bad air"; and the disease was formerly called ague or marsh fever due to its association with swamps. Malaria is the most prevalent tropical disease in the world today. It has infected humans for over fifty thousand years, and may have been a human pathogen for the entire history of our species. In the tropical and subtropical regions, during the first half of the twentieth century, malaria affected every walk of life so much so that one of the major problems in development of economy was the problem of malaria (Sacks and Malaney, 2002).

Malaria is a vector-borne infectious disease caused by protozoan parasites of the genus *Plasmodium* and is presently endemic in a broad band around the equator, in areas of the Americas, many parts of Asia and much of Africa, however, it is in sub-Saharan Africa that 85– 90% of malaria fatalities occur (Hay *et al*., 2004). It is estimated that up to 124 million people in Africa live in areas at risk of seasonal epidemic malaria, and many more in areas outside Africa where transmission is less intense (Hay and Snow, 2006). Each year, it is estimated to cause disease in approximately 650 million people and kills between one and three million, mostly young children in Sub-Saharan Africa (Hay *et al*., 2004). It is also a cause of poverty and a major hindrance to economic development (Sachs and Malaney, 2002). The economic impact includes costs of health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism (WHO, 2001). Moreover, it remains one of the leading causes of death in Sub-Sahara regions where Human Immunodeficient Virus (HIV) infection is endemic (Korenromp *et al.,* 2005).

Malaria causes about 500 million clinical cases each year (10% of the world population), and more than 1 million, mostly children, die as a result of this disease (Breman, 2001). This translates into a death from malaria every 30 seconds, rendering it an eminent disease in tropical countries and ranking it the third killer among communicable diseases behind HIV/AIDS and tuberculosis (Greenwood and Mutabingwa, 2002). Malaria has been a

common disease and it continues to be one of the most widely spread health hazards in tropical and subtropical regions. More than half of the world's population lives in the areas where they remain at risk of malarial infection. The vast majority of cases occur in children under the age of five years and pregnant women (Greenwood *et al*., 2005; Adefioye *et. al.*, 2007).

Despite efforts to reduce transmission and increase treatment, there has been little change in the areas that are at risk of this disease since 1992 (Hay *et al*., 2004). Indeed, if the prevalence of malaria stays on its present upwards course, death rate could double in the next twenty years (Sacks and Malaney, 2002). Precise statistics of morbidity and mortality are unknown because many cases occur in rural areas where people do not have access to hospitals or the means to afford health care. Consequently, the majority of cases of malaria are undocumented (Breman, 2001; Desai *et al*., 2007). The main cause of the worsened malaria situation recorded in recent years has been the spread of drug-resistant parasites, which has led to rising malaria-associated mortality, even though overall child mortality has fallen (Snow *et al.*, 2001).

Until recently, there has been a reliance on the cheap antimalarial drugs like Chloroquine and Sulphadoxine-Pyrimethamine. In 2001, the World Health Organization (WHO) recommended Artemisinin combination Therapies (ACTs) as the first line of treatment for uncomplicated malaria (WHO, 2001). The ACTs which include Artemether-lumefantrine (AL) and Amodiaquine (AQ) plus artesunate (AS) have been adopted for treatment of *P. falciparum* malaria in many African countries. To protect drugs from resistance, there is now clear evidence that combining them can improve their efficacy without increasing their toxicity (Olliaro and Taylor, 2002) and with the development of highly effective artemisinin derivatives, there is renewed hope for the treatment of malaria in the form of Artemisinin-Based Combination therapy (ACT).

The resistance of human malaria parasites to antimalarial compounds has become of considerable concern, particularly in view of the fast speed of emergence of resistant parasites, the fast spread of resistant parasites, and the shortage of novel classes of antimalarial drugs. Antimalarial drug resistance has emerged as one of the greatest challenges

facing malaria control today and has also been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated (Bloland, 2001). Drug resistance has also played a significant role in the occurrence and severity of epidemics in some parts of the world. Population movement has introduced resistant parasites to areas previously free of drug resistance. The emergence and spread of *P. falciparum* resistance to antimalarial drugs is now one of the greatest challenges facing the global effort to control malaria in Africa (WHO, 2003). Moreover, in recent years the situation has worsened due to malaria parasite becoming resistant to several antimalarial drugs. This resistance concerns numerous drugs, but is thought to be most serious with Chloroquine, the cheapest and most widely used drug to treat malaria (Sucharit *et al*., 1977). There have also been reports of resistance against new drugs such as Mefloquine-Lumefantrine (*Riamet*®), Mefloquine, Atovaquone-proguanil (*Malarone*®) and Cotrifazid Doxycycline and Mefloquine (*Lariam*®) (Mccollum *et al*., Hyde, 2002; 2006; Noedl *et al*., 2008).

Several medicinal plants have also been used locally to treat malaria infection. Some of such plants are *Enantia chloranta, Nauclea natifolia, Salacia nitida* (Ogbonna *et al*., 2008), *Acalypha fruticosa*, *Azadirachta indica*, *Cissus rotundifolia, Echium rauwalfii*, *Dendrosicyos socotrana* and *Boswellia elongate* (Merlin, 2004; Clarkson *et al*., 2004; Alshwash *et al*., 2007), *Cymbopogon giganteus and Morinda lucida* (Awe and Makinde, 1997; Azas *et al*., 2002).The use of these local herbs for the treatment of malaria has helped to reduce mortality and morbidity rates especially in the rural areas of the developing world where antimalarial drugs are not readily available. One way to prevent drug resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents (Azas *et al*., 2002). Traditional healers claim that some medicinal plants are more efficient to treat infectious diseases than synthetic antibiotics. Medicinal plants might represent an alternative treatment in non-severe cases of infectious diseases. They can also be a possible source for new potent antibiotics to which pathogenenic strains are not resistant (Elujoba *et al*., 2005; Ogbonnaa *et al*., 2008).

Malaria remains uncontrolled and requires newer drugs and vaccines. Untill the malaria vaccine and newer class of antimalarial drugs become available, the existing drugs need to be used cautiously. This is because the irrational use of antimalarial drugs can cause the drug resistant malaria. Effective usage of existing antimalarial drugs for malaria control strategies

requires continuous input of the drug resistance pattern in the field. Resistance to antimalarial drugs can be assessed *in vivo* and also *in vitro* by parasite susceptibility assays or by the use of molecular techniques including Polymerase Chain Reaction (PCR) methods to detect genetic markers. *In vivo* tests are traditionally the "gold standard" method for detecting drug resistance (WHO, 1996). This involves the assessment of clinical and parasitological outcomes of treatment over a certain period following the start of treatment, to check for the re-appearance of parasites in the blood. *In vitro* assays are based on the inhibition of the growth and development of malaria parasites by different concentrations of a given drug, in relation to drug-free controls (WHO, 1996). The WHO *in vitro* micro-test is based on counting the parasites developing into schizonts, while the isotopic micro-test is based on measurement of the quantity of radio-labeled hypoxanthine, a DNA precursor, incorporated into the parasites (Childs *et al.*, 1988). Molecular methods are now being used for the detection of malaria infection in both clinical and research laboratories using PCR method (Mens *et al*., 2006).

PCR is the most important new scientific technology to come along in the last hundred years (Beck, 1999; de Monbrison *et al*., 2003). This technique is more accurate than microscopy and its value lies in its sensitivity with the ability to detect ≤5 parasites/µl of blood with 100% sensitivity and equal specificity. PCR methods are particularly useful for studies on strain variation, mutations and studies of parasite genes involved in drug resistance. Rapid real-time assays (Real time PCR, Quantitative Nucleic Acid Sequence Based Amplification, QT-NASBA) based on the polymerase chain reaction are emerging as high-throughput genotyping platforms (Ojurongbe *et al.*, 2007). Molecular studies using various markers can provide the advance information on the emergence of drug resistance pattern in the field and such can be used to design malaria control strategies. Using molecular studies, point mutations on the *P. falciparum* Chloroquine Resistant Transporter (PfCRT) and *P. falciparum* Multi-drug Resistant1 (PfMDR1) genes have been reported to play an additional role for the chloroquine resistance in *P. falciparum* isolates while dhfr and dhps were associated with resistance to sulfadoxine-pyrimethamine (Ittarat *et al*., 1994; Adagu and Warhurst, 1999).

A major breakthrough in the search for the genetic basis of CQR in *P. falciparum* was the identification of PfCRT gene, which encodes a putative transporter or channel protein (Fidock

et al., 2000). A K76T change on the PfCRT gene appears necessary for the resistance phenotype, and is the most reliable molecular marker of resistance among the various fifteen polymorphic amino acid positions in PfCRT gene associated with CQR in field isolates (Djimde *et al*., 2001; Plowe, 2003; Ojurongbe *et al.*, 2007). PfMDR1, is a homologue of the mammalian multiple drug resistance gene encoding a P-glycoprotein on the chromosome 5 of the *P. falciparum*. Several field studies indicated the positive association between the asparagine to tyrosine change at position 86 (N86Y) and the chloroquine resistance both *in vitro* (Adagu and Warhurst, 1999; Basco and Ringwald, 2001; Pickard *et al*., 2003) and *in vivo* (Ringwald and Basco, 1999; Basco and Ringwald, 2001; Dorsey *et al*., 2001). However, other studies have cast doubts on this association (Pillai *et al*., 2001; Ojurongbe *et al*., 2007).

In vitro resistance of *P. falciparum* to pyrimethamine and to chlorcycloguanil is due to specific point mutations in *P. falciparum* Dihydrofolate reductase (DHFR), which is encoded by a bi-functional gene (*dhfr-ts*) also encoding thymidylate synthase (Kublin *et al*., 2002; Marks *et al*., 2005). A single point mutation causing a Serine (Ser) to Asparagine (Asn) change at codon 108 causes pyrimethamine resistance with only a moderate loss of susceptibility to chlorcycloguanil. The addition of Asn to Isoleucine (Ile) at position 51 and/or Cysteine (Cys) to Arginine (Arg) at position 59 confers higher levels of pyrimethamine resistance. Ile to Leucine **(**Leu) at position 164, when combined with Asn108 and Ile51 and/or Arg59, confers high-level resistance to both drugs. Point mutations in the gene encoding DHPS have similarly been associated with *in vitro* resistance to the sulfa drugs and sulfones. Mutations associated with decreased susceptibility to sulfas include Ser to Alanine (Ala) at position 436, Ala to Glycine (Gly) at position 437, Ala to Gly at position 581, and Ser to Phenylalanine (Phe) at position 436 coupled with Ala to Threonine (Thr)/Ser at position 613 (Plowe *et al*., 1998; Marks *et al*., 2005; McCollum *et al*., 2006). Both DHFR and DHPS mutations occurs in a progressive, step-wise fashion, with higher levels of in vitro resistance occurring in the presence of multiple mutations (Plowe *et al*., 1998; Marks *et al*., 2005)

In the absence of effective and practical preventive measures, the only current options for reducing the morbidity and mortality of malaria especially in Africa are chemoprophylaxis and chemotherapy. For this reason, the increasing prevalence of strains of *P. falciparum* resistant to antimalarial drugs poses a serious problem for the control of malaria.

In Nigeria, malaria accounts for high infant and childhood mortality and it imposes great burden on the country in terms of pains and trauma suffered by its victims as well as loss in outputs and cost of treatments. In addition to the use of orthodox medicine for treatment, personal communication has shown that malaria is often treated in Nigeria with local herbs and the services of spiritualists/traditional priests. Similarly, common prevention measures include use of drugs (prophylaxis), insecticides (coils and sprays), ordinary mosquito nets, Insecticide-Treated Nets (ITNs) and window/door nets (Ojo, 2005).

1.2 Justification/Rationale of the Study

Emerging drug-resistant *P. falciparum* strains are making malaria a resurging infectious disease. Moreover, in recent years the situation has worsened in many ways mainly due to the malaria parasite becoming resistant to almost all available antimalarial drugs.

Newer drug therapies, unfortunately, have not eluded drug resistant strains of the malaria parasite as there have also been case reports of resistance against new drugs such as Atovaquone-proguanil, (malarone®) cotrifazid, doxycycline, mefloquine (Lariam®).

Effective usage of existing antimalarial drugs requires continuous input of the drug resistance pattern in the field. *In-Vitro* drug sensitivity assays provide information on the quantitative drug response of *P. falciparum;* they are therefore important tools for monitoring the drug response of *P. falciparum* and they provide background information for development and evaluation of drug policies.

In addition, *in-vitro* drug sensitivity assays serve as epidemiological tools to assess baseline sensitivity which can be an indicator of future therapeutic failure. They can also guide on the partner drug in Artemisinin Combination Therapies (ACTs).

This study was therefore designed to assess the *in vitro* sensitivity pattern and the molecular characteristics of *P. falciparum* that are likely to influence the observed resistance to these new antimalarial drugs; and also to test the efficacy of some selected Nigerian herbs used as local antimalarial drugs. This will contribute to the development of novel antimalarial drugs thus circumventing the resistance potentials of this parasite.

1.3 Objectives of the Study

The objectives of this study are to:

1. establish the incidence of malaria (*Plasmodium* infection) in Ogun State, Southwestern Nigeria
2. assess the resistance patterns of *P. falciparum* to the current antimalarial drugs
3. determine the prevalence of molecular markers of resistance to different classes of antimalarial drugs
4. establish the factors that may contribute to the development of antimalarial drug resistance in Southwestern Nigeria.
5. determine the efficacy of some Nigerian herbs used as local antimalarial drugs/drinks *in vitro*.

1.4 Scientific Hypotheses

1. There is a wide spread of multidrug drug resistant *P. falciparum*
2. *In vitro* susceptibility testing can provide information on drug resistance pattern of *P. falciparum* to the new drugs that are currently in use.
3. Molecular studies using various markers can provide advance information on the emergence of drug resistance pattern in the field.
4. The misuse/abuse of antimalarial drugs results in fast development of resistance of *P. falciparum* to antimalarial drugs.
5. There is efficacy in the local herbs used as antimalarial drugs

CHAPTER TWO

LITERATURE REVIEW

2.1. Disease Incidence and Trends

Malaria remains an important public health concern in countries where transmission occurs regularly, as well as in areas where transmission has been largely controlled or eliminated. In particular, young children, pregnant women, and non-immune visitors to malarious areas are

at greatest risk of severe or fatal illness. Ninety percent of the global burden of malaria occurs in Africa, south of Sahara and in spite of the considerable efforts in the campaign against malaria, the number of cases and deaths associated with the disease remain almost unvaried (WHO, 2001). Reasons for this include the emergence of parasite resistance to drugs, resistance of their vectors to insecticides, demographic growth with ensuing deterioration of living and infrastructure standards in endemic areas, environmental degradation, armed conflicts leading to large movement of refugees, uncontrolled movement through endemic areas and natural disasters (Bourdy *et al.*, 2008)

2.1.1. Geographical Distribution and Populations at Risk

Malaria occurs in over 90 countries worldwide (Bloland, 2001). WHO estimated that 36% of the global population live in areas where there is risk of malaria transmission, 7% reside in areas where malaria has never been under meaningful control and 29% live in areas where malaria was once transmitted at low levels or not at all, but where significant transmission has been re-established (WHO, 1996a). The development and spread of drug-resistant strains of malaria parasites has been identified as a key factor in this resurgence (Bloland, 2001) and is one of the greatest challenges to malaria control today. Malaria transmission occurs primarily in tropical and subtropical regions in sub-Saharan Africa, Central and South America, the Caribbean island of Hispaniola, the Middle East, the Indian subcontinent, South-East Asia, and Oceania (Hay and Snow, 2006). In areas where malaria occurs, however, there is considerable variation in the intensity of transmission and risk of malaria infection. Highlands (greater than 1500 m) and arid areas with less than 1000 mm rainfall per annum typically have less malaria, although they are also prone to epidemic malaria when parasitaemic individuals provide a source of infection and climate conditions are favourable to mosquito development (WHO 1996). Although urban areas have typically been at lower risk, explosive, unplanned population growth has contributed to the growing problem of urban malaria transmission (Knudsen and Slooff, 1992).

Each year an estimated 300 to 650 million clinical cases of malaria occur, making it one of the most common infectious diseases worldwide (Hay *et al*., 2004). Malaria can be, in certain epidemiological circumstances, a devastating disease with high morbidity and mortality, demanding a rapid, comprehensive response. In other settings, it can be a more pernicious

public health threat. In many malarious areas of the world, especially sub-Saharan Africa, malaria is ranked among the most frequent causes of morbidity and mortality among children (Greenwood *et al*., 2005). It has been estimated that more than 90% of the 1.5 to 3.0 million deaths attributed to malaria each year occur in African children (Hay *et al*., 2004). Other estimates based on a more rigorous attempt to calculate the burden of disease in Africa support this level of mortality (Snow *et al.,* 1999). In addition to its burden in terms of morbidity and mortality, the economic effects of malaria infection can be tremendous. These include direct costs for treatment and prevention, as well as indirect costs such as lost productivity from morbidity and mortality; time spent seeking treatment, and diversion of household resources (Sachs and Malaney, 2002). The annual economic burden of malaria infection in 1995 was estimated at US$ 0.8 billion, for Africa alone (Bloland 2001). This heavy toll can hinder economic and community development activities throughout the region. More than ever before malaria is both a disease of poverty and a cause of poverty (Bourdy *et al.*, 2008).

Nigeria is known for high prevalence of malaria and it is a leading cause of morbidity and mortality in the country (Ademowo *et al*., 2006). Available records show that at least 50 per cent of the population of Nigeria suffers from at least one episode of malaria each year and that malaria accounts for over 45% of all out patient visits. It was reported that malaria prevalence (notified cases) in year 2000 was about 2.4 million (Sowunmi *et al*., 2004). The disease accounts for 25 per cent of infant mortality and 30 per cent of childhood mortality in Nigeria. Therefore, it imposes great burden on the country in terms of pains and trauma suffered by its victims as well as loss in outputs and cost of treatments (Ogungbamigbe *et al*., 2005).

2.2. Causative Agents

Malaria is caused by intracellular protozoan parasites of the genus *Plasmodium*. The parasite belongs to Kingdom Protista, Phylum Apicomplexa, Class Aconoidasida, Order Haemosporida, Family Plasmodiidae, Genus *Plasmodium* and Species *falciparum* The most serious forms of the disease are caused by *P. falciparum* and *P. malariae*, but other related species (*P. ovale*, *P. vivax*) can also infect humans. This group of human-pathogenic *Plasmodium* species is usually referred to as *malaria parasites* (Trampuz *et al*., 2003). *P.*

falciparum, *P. vivax*, *P. ovale*, and *P. malariae* differ in geographical distribution, microscopic appearance, clinical features, periodicity of infection, potential for severe disease, ability to cause relapses, and potential for development of resistance to antimalarial drugs. To date, drug resistance has only been documented in two of the four species, *P. falciparum* and *P. vivax* (Bloland, 2001).

2.3 Transmission and Biology of *P. falciparum*

Malaria can be transmitted by several species of female anopheline mosquitoes that differ in behavior (Greenwood *et al*., 2005). There are about 460 species of the *Anopheles* mosquito, but only 68 transmit malaria. *Anopheles gambiae*, found in Africa is one of the best malaria vectors since it is long-lived, prefers feeding on humans, and lives in areas near human habitation (Cowman, 2006).

Prior to transmission, *P. falciparum* resides within the salivary gland of the mosquito. The parasite is in its sporozoite stage at this point. As the mosquito takes its blood meal, it injects a small amount of saliva into the skin wound. The saliva contains antihemostatic and anti-inflammatory enzymes that disrupt the clotting process and inhibit the pain reaction (Bruce Chwatt, 1985**)**. Typically, each infected bite contains 5-200 sporozoites which proceed to infect the human (Gilles *et al.*, 1993). Once in the human bloodstream, the sporozoites circulate for a few minutes before infecting liver cells.

Liver Stage

After circulating in the bloodstream, the *P. falciparum* sporozoites enter hepatocytes to initiate the exoerythrocytic stage. At this point, the parasite loses its apical complex and surface coat, and transforms into a trophozoite. Within the parasitophorous vacuole of the hepatocyte, *P. falciparum* undergoes schizogonic development. In this stage, the nucleus divides multiple times with a concomitant increase in cell size, but without cell segmentation. This exoerythrocytic schizogony stage of *P. falciparum* has a minimum duration of roughly 5.5 days. After segmentation, the parasite cells are differentiated into merozoites (Miller *et al.*, 1994). After maturation, the merozoites are released from the hepatocytes and enter the erythrocytic portion of their life-cycle. The released merozoites do not reinfect hepatocytes.

Erythrocytic Stage

After release from the **hepatocytes**, the **merozoites** enter the bloodstream prior to infecting red blood cells. At this point, the **merozoites** are roughly 1.5μm in length and 1 μm in diameter, and use the apicomplexan invasion organelles (**apical complex**, pellicle and surface coat) to recognize and enter the host erythrocyte (**Bruce Chwatt 1985).**The parasite first binds to the erythrocyte in a random orientation. It then reorients such that the apical complex is in proximity to the erythrocyte membrane. A tight junction is formed between the parasite and erythrocyte. As it enters the red blood cell, the parasite forms a parasitophorous vesicle, to allow for its development inside the **erythrocyte** (Gilles *et al*., 1993).

After invading the erythrocyte, the parasite loses its specific invasion organelles (apical complex and surface coat) and differentiates into a round trophozoite located within a parasitophorous vacuole in the red blood cell cytoplasm. The young trophozoite (or "ring" stage, because of its morphology on stained blood films) grows substantially before undergoing schizogonic division (Arora and Arora, 2005). The growing parasite replicates its DNA multiple times without cellular segmentation to form a schizont. These schizonts then undergo cellular segmentation and differentiation to form roughly 16-18 merozoite cells in the erythrocyte (Gills *et al.*, 1993). The merozoites burst from the red blood cell, and proceed to infect other erythrocytes. The parasite then stays in the bloodstream for roughly 60 seconds before invading another erythrocyte. This infection cycle occurs in a highly synchronous fashion, with roughly all of the parasites throughout the blood in the same stage of development. This precise clocking mechanism has been shown to be dependent on the human host's own circadian rhythm. Specifically, human body temperature changes, as a result of the circadian rhythm, seem to play a role in the development of *P. falciparum* within the erythrocytic stage (Bruce Chwatt, 1985).

Within the red blood cell, the parasite metabolism depends greatly on the digestion of hemoglobin. Infected erythrocytes are often sequestered in various human tissues or organs, such as the heart, liver and brain. This is caused by parasite-derived cell surface proteins being present on the red blood cell membrane and it is these proteins that bind to receptors on human cells. Sequestration in the brain causes cerebral malaria, a very severe form of the disease, which increases the victim's likelihood of death. The parasite can also alter the

morphology of the red blood cell, causing knobs on the erythrocyte membrane (Miller *et al*., 1994).

Gametocyte Differentiation

During the erythrocytic stage, some merozoites develop into male and female gametocytes in a process called gametocytogenesis (Billker *et al*., 1998). These gametocytes take roughly 8-10 days to reach full maturity and remain within the erythrocytes until taken up by the mosquito host.

Mosquito Stage

The gametocytes of *P. falciparum* are taken up by the female *Anopheles* mosquito as it takes its blood meal from an infected human. Upon being taken up by the mosquito, they leave the erythrocyte shell and differentiate into gametes. The female gamete maturation process entails slight morphological changes, as it becomes enlarged and spherical. On the other hand, the male gamete maturation involves significant morphological development. The male gamete's DNA divides three times to form eight nuclei and concurrently, eight flagella are formed. Each flagella pairs with a nucleus to form a microgamete, which then separates from the parasite cell in a process referred to as exflagellation (Gilles *et al.,* 1993).Gametogenesis (formation of gametes) has been shown to be caused by: 1) a sudden drop in temperature upon leaving the human host, 2) a rise in pH within the mosquito, and 3) xanthurenic acid within the mosquito (Billker *et al.*, 1998).

Fertilization of the female gamete by the male gamete occurs rapidly after gametogenesis. The fertilization event produces a zygote. The zygote then develops into an ookinete. The zygote and ookinete are the only diploid stages of *P. falciparum*. The diploid ookinete is an invasive form of *P. falciparum* within the mosquito. It traverses the peritrophic membrane of the mosquito midgut and crosses the midgut epithelium. Once through the epithelium, the ookinete enters the basil lamina, and forms an oocyst. During the ookinete stage, genetic recombination can occur. This takes place if the ookinete was formed from male and female gametes derived from different populations. This can occur if the human host contained multiple populations of the parasite, or if the mosquito fed on multiple infected individuals within a short time-frame (Bruce Chwatt, 1985).

Over the period of 1-3 weeks, the oocyst grows to a size of tens to hundreds of micrometres. During this time, multiple nuclear divisions occur. After maturation, it divides to form multiple haploid sporozoites in a process referred to as sporogony. Immature sporozoites break through the oocyst wall into the haemolymph, then migrate to the salivary glands and complete their differentiation. Once mature, the sporozoites can proceed to infect a human host during a subsequent mosquito bite (Gilles *et al.*, 1993).

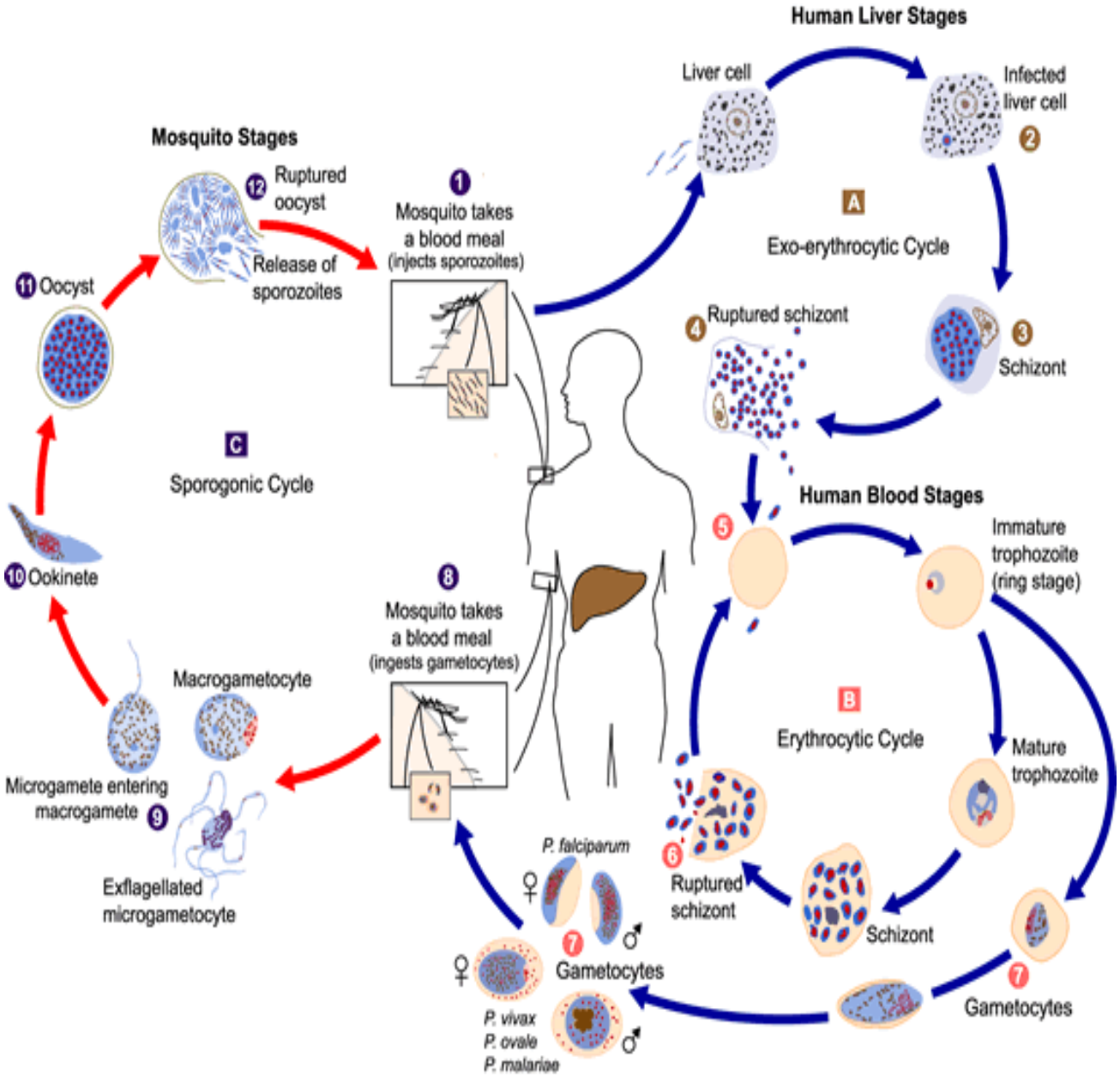

Fig 2.1 Life cycle of *Plasmodium* Species.

(Bruce Chwatt, 1985)

2.4. Symptoms

Malaria is a complex disease that varies widely in epidemiology and clinical manifestation in different parts of the world. This variability is due to factors such as the species of malaria parasites that occur in a given area, their susceptibility to commonly used or available antimalarial drugs, the distribution and efficiency of mosquito vectors, climate and other environmental conditions and the behavior and level of acquired immunity of the exposed human populations (Greenwood *et al.*, 1991; Mockenhaupt *et al.*, 2000). The parasites multiply within the red blood cells, causing symptoms that include symptoms of anemia (light headedness, shortness of breath, tachycardia etc.), as well as other general symptoms such as fever, chills, nausea, flu-like illness, arthralgia (joint pain), vomiting, anemia caused by hemolysis, hemoglobinuria, and convulsions and in severe cases, coma and death (WHO 1991).

The classical symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting four to six hours, occurring every two days in *P. vivax* and *P. ovale* infections, while it occurs every three days in *P. malariae* (Boivin, 2002). *P. falciparum* can have recurrent fever every 36-48 hours or a less pronounced and almost continuous fever (Trampuz *et al.*, 2003). For reasons that are poorly understood, but which may be related to high intracranial pressure, children with severe malaria frequently exhibit abnormal posturing, a sign indicating severe brain damage (Idro *et al*., 2007). Malaria has been found to cause cognitive impairments, especially in children. It causes widespread anemia during a period of rapid brain development and also direct brain damage. This neurologic damage results from cerebral malaria to which children are more vulnerable (Boivin, 2002).

Consequences of severe malaria include coma and death if untreated, young children and pregnant women are especially vulnerable. Splenomegaly (enlarged spleen), severe headache, cerebral ischemia, hepatomegaly (enlarged liver), hypoglycemia, and hemoglobinuria with renal failure may occur (Trampuz *et al.*, 2003). Renal failure may cause blackwater fever, where hemoglobin from lysed red blood cells leaks into the urine (Idro *et al.*, 2007). Severe malaria can progress extremely rapidly and cause death within hours or days. In the most severe cases of the disease fatality rates can exceed 20%, even with intensive care and

treatment (Kain *et al*., 1998). In endemic areas, treatment is often less satisfactory and the overall fatality rate for all cases of malaria can be as high as one in ten (Mockenhaupt *et al*., 2004). Over the longer term, developmental impairments have been documented in children who have suffered episodes of severe malaria (Trampuz *et al*., 2003). Chronic malaria is seen in both *P. vivax* and *P. ovale*, but not in *P. falciparum*. Here, the disease can relapse months or years after exposure, due to the presence of latent parasites (hypnozoites) in the liver (Kain *et al.*, 1998). Severe malaria is almost exclusively caused by *P. falciparum* infection and usually arises 6-14 days after infection.

2.5. Diagnosis

Direct microscopic examination of intracellular parasites on stained blood films is the gold standard for definitive diagnosis in nearly all settings. However, several other approaches exist or are in development, some of which are discussed here.

2.5.1. Microscopy

Simple light microscopic examination of Giemsa stained blood films is the most widely practiced and useful method for definitive malaria diagnosis. Advantages include differentiation between species, quantification of the parasite density and ability to distinguish clinically important asexual parasite stages from gametocytes which may persist without causing symptoms (WHO, 1991). These advantages can be critical for proper case-management and evaluating parasitological response to treatment. Specific disadvantages are that slide collection, staining, and reading can be time-consuming and microscopists need to be trained and supervised to ensure consistent reliability. While availability of microscopic diagnosis has been shown to reduce drug use in some trial settings (Chanda *et al.*, 2009). Any programme aimed at improving the availability of reliable microscopy should also retrain clinicians in the use and interpretation of microscopic diagnosis.

Another method is a modification of light microscopy called the Quantitative Buffy Coat Method (QBCTM, Becton-Dickinson). Originally developed to screen large numbers of specimens for complete blood cell counts, this method has been adapted for malaria diagnosis (Levine *et al*., 1989). The technique uses microhaematocrit tubes precoated with fluorescent acridine orange stain to highlight malaria parasites. With centrifugation, parasites are

concentrated at a predictable location. Advantages to QBC are that less training is required to operate the system than for reading Giemsa-stained blood films and the test is typically quicker to perform than normal light microscopy. Disadvantages are that electricity is always required, special equipment and supplies are needed, the per-test cost is higher than simple light microscopy, and species-specific diagnosis is not reliable. Field trials have shown that the QBC system may be marginally more sensitive than conventional microscopy under ideal conditions (Levine *et al*., 1989; Tharavanij, 1990).

2.5.2. Clinical (presumptive) Diagnosis

Although reliable diagnosis cannot be made on the basis of signs and symptoms alone because of the non-specific nature of clinical malaria, clinical diagnosis of malaria is common in many malarious areas (WHO, 1997). In much of the malaria-endemic world, resources and trained health personnel are so scarce that presumptive clinical diagnosis is the only realistic option (Smith *et al.*, 1994). Clinical diagnosis offers the advantages of ease, speed, and low cost. In areas where malaria is prevalent, clinical diagnosis usually results in all patients with fever and no apparent other cause being treated for malaria. This approach can identify most patients who truly need antimalarial treatment, but it is also likely to misclassify many who do not (Olivar *et al.*, 1991). Over-diagnosis contributes considerably to misuse of antimalarial drugs (Ogungbamigbe *et al*., 2008). Considerable overlap exists between the signs and symptoms of malaria and other frequent diseases, especially acute lower respiratory tract infection and can greatly increase the frequency of misdiagnosis and mistreatment (Redd *et al*., 1992).

Attempts to improve the specificity of clinical diagnosis for malaria by including signs and symptoms other than fever or history of fever have met with only minimal success (Smith *et al*., 1994). The Integrated Management of Childhood Illnesses (IMCI) programme defined an algorithm that has been developed in order to improve diagnosis and treatment of the most common childhood illnesses in areas relying upon relatively unskilled health care workers working without access to laboratories or special equipment. With this algorithm, every febrile child living in a “high-risk” area for malaria should be considered to have, and be treated for, malaria. “High risk” has been defined in IMCI Adaptation Guides as being any situation where as little as 5% of febrile children between the ages of 2 and 59 months are

parasitaemic (WHO, 1997), a definition that will likely lead to significant over-diagnosis of malaria in areas with low to moderate malaria transmission.

2.5.3. Antigen detection tests (rapid or "dipstick" diagnostic tests)

A third diagnostic approach involves the rapid detection of parasite antigens using rapid immunochromatographic techniques. Multiple experimental tests have been developed targeting a variety of parasite antigens (WHO, 1996; Bloland, 2001). A number of commercially available kits (e.g. ParaSight-F®, Becton-Dickinson; Malaquick®, ICT, Sydney, New South Wales, Australia) are based on the detection of the histidine-rich protein 2 (HRP-II) of *P. falciparum*. Compared with light microscopy and QBC, this test yielded rapid and highly sensitive diagnosis of *P. falciparum* infection (WHO, 1996; Craig and Sharp, 1997). Advantages to this technology are that no special equipment is required, minimal training is needed, the test and reagents are stable at ambient temperatures and no electricity is needed. The principal disadvantages are a currently high per-test cost and an inability to quantify the density of infection. Furthermore, for tests based on HRP-II, detectable antigen can persist for days after adequate treatment and cure; therefore, the test cannot adequately distinguish a resolving infection from treatment failure due to drug resistance, especially early after treatment (WHO, 1996).

Additionally, a test based on detection of a specific parasite enzyme (lactate dehydrogenase or pLDH) has been developed (OptiMAL®, Flow Inc. Portland, OR, USA) and reportedly only detects viable parasites, which if true, eliminates prolonged periods of false positivity post-treatment (Makler *et al.,* 1998; Palmer *et al.,* 1999). Newer generation antigen detection tests are able to distinguish between *falciparum* and non-*falciparum* infections, greatly expanding their usefulness in areas where non-*falciparum* malaria is transmitted frequently (Bloland, 2001).

2.5.4. Molecular tests

Detection of parasite genetic material through polymerase-chain reaction (PCR) techniques has become a more frequently used tool in the diagnosis of malaria, as well as the diagnosis and surveillance of drug resistance in malaria. Specific primers have been developed for each of the four species of human malaria. One important use of this new technology is in detecting

mixed infections or differentiating between infecting species when microscopic examination is inconclusive (Beck, 1999). In addition, improved PCR techniques could prove useful for conducting molecular epidemiological investigations of malaria clusters or epidemics (Purfield *et al.,* 2004). Primary disadvantages to these methods are overall high cost, high degree of training required, need for special equipment, absolute requirement for electricity, and potential for cross-contamination between samples (Berzins and Anders, 1999).

2.5.5 Serology

Techniques also exist for detecting anti-malaria antibodies in serum specimens. Specific serological markers have been identified for each of the four species of human malaria. A positive test generally indicates a past infection. Serology is not useful for diagnosing acute infections because detectable levels of anti-malaria antibodies do not appear until weeks into infection and persist long after parasitaemia has resolved. Moreover, the test is relatively expensive, and not widely available (Bloland, 2001).

2.6. Antimalarial Drugs

For the past 50 years, there have been two main classes of antimalarial agents in use, the antifolates and the cinchona alkaloids or the quinoline-containing drugs (Philips, 2001). The quinoline-containing drugs include the cinchona alkaloids, quinine and quinidine, and the aminoalcohol quinine analogues mefloquine (a 4-quinoline methanol) and halofantrine (a 9-phenathrene methanol), which are recent introductions (ter Kuile 1993; Philips 2001). There are also the 8-aminoquinoline primaquine, which is used for its gametocidal effect and its action on the liver stage of *P. Vivax*, and the 4-aminoquinolines, chloroquine and its relative amodiaquine (White, 1997). The antifolates include the diaminopyrimidines, such as pyrimethamine and trimethoprim; the biguanides, represented by proguanil (cycloguanil) and chlorproguanil; and the sulfa drugs, including the sulfonamides and the sulfones.

2.6.1. Quinine and related compounds

Quinine is the oldest and most famous anti-malarial (Dorvault, 1982). It has a long history stretching from Peru, to the discovery of the Cinchona tree and the potential uses of its bark, to the current day and a collection of derivatives that are still frequently used in the prevention and treatment of malaria (Segurado *et al*., 1997). Quinine is an alkaloid that acts as a blood

schizonticidal and weak gametocide against *Plasmodium species*. As an alkaloid, it is accumulated in the food vacuoles of *Plasmodium*, especially *P. falciparum*. It acts by inhibiting the hemozoin biocrystallization, thus facilitating an aggregation of cytotoxic heme. Quinine is less effective and more toxic as a blood schizonticidal agent than Chloroquine; however it is still very effective and widely used in the treatment of acute cases of severe *P. falciparum* (Foley and Tilley, 1998). It is especially useful in areas where there is known to be a high level of resistance to Chloroquine, Mefloquine and sulfa drug combinations with pyrimethamine (Foley and Tilley, 1998). Quinine is also used in post-exposure treatment of individuals returning from an area where malaria is endemic (Foley and Tilley, 1997). Quinine, along with its dextroisomer, Quinidine, has been the drug of last resort for the treatment of malaria, especially severe disease.

Quinimax and Quinidine are the two most commonly used alkaloids related to Quinine, in the treatment or prevention of Malaria. Quinimax is a combination of four alkaloids (namely Quinine, Quinidine, Cinchoine and Cinchonidine) (Mills and Bone, 2000). This combination has been shown in several studies to be more effective than Quinine, supposedly due to a synergistic action between the four Cinchona derivatives. Quinidine is a direct derivative of Quinine. It is a distereoisomer, thus having similar anti-malarial properties to the parent compound. Quinidine is recommended only for the treatment or severe cases of malaria (Foley and Tilley, 1998).

Chloroquine

Chloroquine is a 4-aminoquinolone derivative of quinine, first synthesized in 1934 and has since been the most widely used antimalarial drug until recently (WHO, 2001). Historically, it has been the drug of choice for the treatment of non-severe or uncomplicated malaria and for chemoprophylaxis. It is believed to reach high concentrations in the vacuoles of the parasite, which, due to its alkaline nature, raises the internal pH. It controls the conversion of toxic heme to hemozoin by inhibiting the biocrystallization of hemozoin (Lobel and Campbell, 1986) thus poisoning the parasite through excess levels of toxicity. Other potential mechanisms through which it may act include interfering with the biosynthesis of parasitic nucleic acids, the formation of a chloroquine-haem or chloroquine-DNA complex. It was the original prototype from which most other methods of treatment are derived (Foley and Tilley,

1997). It is also the least expensive, best tested and safest of all available drugs. The emergence of drug resistant parasitic strains is rapidly decreasing its effectiveness (Rieckmann *et al*., 1978; Wellems and Plowe, 2001); however it is still the first-line drug of choice in most sub-Saharan African countries. It is now suggested that it is used in combination with other antimalarial drugs to extend its effective usage.

The most significant level of activity found is against all forms of the schizonts (with the obvious exception of chloroquine-resistant *P. falciparum* and *P. vivax* strains) and the gametocytes of *P. vivax*, *P. malariae*, *P. ovale* as well as the immature gametocytes of *P. falciparum*. Chloroquine also has a significant anti-pyretic and anti-inflammatory effect when used to treat *P. vivax* infections, thus it may still remain useful even when resistance is more widespread (Alene and Bennett, 1996). A slightly different drug called nivaquine or chloroquine phosphate was also invented (Bloland 2001).

Amodiaquine

Amodiaquine is a 4-aminoquinolone anti-malarial drug similar in structure and mechanism of action to Chloroquine (Winstanley *et al.*, 1987). It is thought to be more effective in clearing parasites in uncomplicated malarial than Chloroquine, thus leading to a faster rate of recovery (Olliaro and Taylor. 2002). However, some fatal adverse effects of the drug were noted during the 1980's, thus reducing its usage in chemoprophylaxis. The WHO's advice on the subject maintained that the drug should be used when the potential risk of not treating an infection outweighs the risk of developing side effects (Basco, 1991). It has also been suggested that it is particularly effective and less toxic than other combination treatments in HIV positive patients (Parise *et al*., 1998). Adverse reactions are generally similar in severity and type to that seen in Chloroquine treatment. In addition, bradycardia, itching, nausea, vomiting and some abdominal pain have been recorded. Some blood and hepatic disorders have also been seen in a small number of patients (Olliaro *et al.*, 1996).

Mefloquine

Mefloquine was developed during the Vietnam War and is chemically related to quinine. It was developed to protect American troops against multi-drug resistant *P. falciparum*. It is a very potent blood schizonticide with a long half-life (Mockenhaupt, 1995). It is thought to act

by forming toxic heme complexes that damage parasitic food vacuoles. Mefloquine (*Lariam*®) is effective in prophylaxis and for acute therapy (Palmer *et al.,* 1993). It is now strictly used for resistant strains (and is usually combined with Artesunate) (van Vugt *et al.*, 1998). It has been linked with an increased number of stillbirths (Palmer *et al.,* 1993) and is not recommended for use during the first trimester, although considered safe during the second and third trimesters. Mefloquine frequently produces side effects, including nausea, vomiting, diarrhea, abdominal pain and dizziness. Several associations with neurological events have been made, namely affective and anxiety disorders, hallucinations, sleep disturbances, psychosis, toxic encephalopathy, convulsions and delirium. Moreover cardiovascular effects have been recorded with bradycardia and sinus arrhythmia (Ridley, 1997)

Atovaquone

Atovaquone is a hydroxynapthoquinone that is currently being used most widely for the treatment of opportunistic infections in immunosuppressed patients. It is effective against chloroquine-resistant *P. falciparum*, but because when used alone, resistance develops rapidly, atovaquone is usually given in combination with proguanil (Looareesuwan, 1996). A fixed dose antimalarial combination of 250mg atovaquone and 100mg proguanil (*Malarone*^{TM}) was introduced to market worldwide and was additionally being distributed through a donation programme (Foege, 1997). Two drugs originally synthesized in China were recommended for field trials. Pyronaridine was reported to be 100% effective in one trial in Cameroon (Ringwald *et al.,* 1996); however, it was only between 63% and 88% effective in Thailand (Looareesuwan, 1996). Lumefantrinel, a fluoromethanol compound, was also produced as a fixed combination. Atovaquone produces no side-effects such as the cardiovascular effect with mefloquine which can trigger heart rhythm problems.

Primaquine

Primaquine is a highly active 8-aminoquinolone that was used in treating all types of malaria infection (Olliaro and Trigg, 1995). It was most effective against gametocytes but also acts on hypnozoites, blood schizonticytes and the dormant plasmodia in *P. vivax* and *P. ovale*. It is the only known drug to cure both relapsing malaria infections and acute cases (Looareesuwan, 1996). The mechanism of action is thought to mediate some effect through creating oxygen

free radicals that interfere with the plasmodial electron transport chain during respiration (Bloland *et al*., 1997). There are few significant side effects such as anorexia, nausea, vomiting, cramps, chest weakness, anaemia, some suppression of myeloid activity and abdominal pains; In cases of over-dosage granulocytopenia may occur (Bruce-Chwatt,, 1985).

Halofantrine

Halofantrine was developed by the Walter Reed Army Institute of Research in the 1960s (Mills and Bone, 2000). It is a phenanthrene methanol, chemically related to Quinine and acts as a blood schizonticide effective against all *Plasmodium* parasites (ter Kuile *et al.*, 1993). Its mechanism of action is similar to other anti-malarials. Cytotoxic complexes are formed with ferritoporphyrin XI that cause plasmodial membrane damage (Bloland, 2001). Despite being effective against drug resistant parasites, Halofantrine was not commonly used in the treatment (prophylactic or therapeutic) of malaria due to its high cost, very variable bioavailability and most importantly it has been shown to have potentially high levels of cardiotoxicity (Nosten *et al*., 1993). A popular drug based on halofantrine is Halfan.

2.6.2 Antifolate drugs

This class of drugs includes effective casual antimalarial prophylactic and therapeutic agents, some of which act synergistically when used in combination. They are of various combinations of dihydrofolate-reductase inhibitors (proguanil, chlorproguanil, pyrimethamine and trimethoprim) and sulfa drugs (dapsone, sulfalene, sulfamethoxazole, sulfadoxine, and others). Although these drugs have antimalarial activity when used alone, parasitological resistance can develop rapidly (Kupferschmidt *et al.*, 1988). When combined, they produce a synergistic effect on the parasite and can be effective even in the presence of resistance to the individual components (Kublin *et al.*, 2002). Typical combinations include sulfadoxine/pyrimethamine (SP or Fansidar1), sulfalene-pyrimethamine (metakelfin) and sulfamethoxazole-trimethoprim (co-trimoxazole). A newer antifolate combination drug which is a combination of chlorproguanil and dapsone, also known as Lapdap, has a much more potent synergistic effect on malaria than existing drugs such as SP. Benefits of this combination include a greater cure rate, even in areas currently experiencing some level of SP resistance, a lower likelihood of resistance developing because of a more advantageous pharmacokinetic and pharmacodynamic profile and probable low cost (Kublin *et al.*, 2002).

Sulphadoxine Pyrimethamine

Sulfadoxine-pyrimethamine (SP) has been widely used as first-line therapy for uncomplicated *P. falciparum* malaria throughout sub-Saharan Africa, because of its affordability, its ease of administration and until recently, its effectiveness. It is currently the only option for intermittent treatment of malaria during pregnancy (McCollum *et al*., 2006). SP, which has potent efficacy against chloroquine-resistant and pyrimethamine-resistant *P. falciparum*, became available in 1971 and became the standard second-line therapy against chloroquine-resistant falciparum malaria. Pyrimethamine acts by inhibiting dihydrofolate reductase in the parasite, thus preventing the biosynthesis of purines and pyrimidines and therefore halting the processes of DNA synthesis, cell division and reproduction. It acts primarily on the schizonts during the hepatic and erythrocytic phases (Kuznetsov *et al.*, 1984). The action of sulphadoxine is focused on inhibiting the use of para-aminobenzoic acid during the synthesis of dihydropteroic acid. When combined the two key stages in DNA synthesis in the plasmodia are prevented.

Proguanil

Proguanil (Chloroguanadine) is a biguanide; a synthetic derivative of pyrimidine. It was developed in 1945 by a British Antimalarial research group (Mills and Bone, 2000). It has many mechanisms of action but primarily is mediated through conversion to the active metabolite cycloguanil pamoate. This inhibits the malarial dihydrofolate reductase enzyme. Its most prominent effect is on the primary tissue stages of *P. falciparum*, *P. vivax* and *P. ovale*. It has no known effect against hypnozoites therefore is not used in the prevention of relapse. It has a weak blood schizonticidal activity when combined with Atovaquone (a hydroxynaphthoquinone). Proguanil is used as a prophylactic treatment in combination with another drug, most frequently Chloroquine (Kublin *et al.*, 2002). Proguanil has been used in combination with dapsone for prophylaxis and treatment (Shanks *et al*., 1992; Mutabingwa *et al*., 2005) and recently, proguanil/dapsone has been combined with artesunate (Nzila *et al*., 2002). There are very few side effects to Proguanil, with slight hair loss and mouth ulcers being occasionally reported following prophylactic use.

2.6.3 Antibiotics

Tetracycline and derivatives such as doxycycline are very potent antimalarials and are used for both treatment and prophylaxis (Kremsner *et al.,* 1994). In areas where response to quinine has deteriorated, tetracyclines are often used in combination with quinine to improve cure rates. Clindamycin has been used but offers only limited advantage when compared to other available antimalarial drugs as parasitological response is slow and recrudescence rates are high (Kremsner *et al.*, 1994).

Doxycycline

Doxycycline is a Tetracycline compound derived from Oxytetracycline. The tetracyclines were one of the earliest groups of antibiotics to be developed and are still used widely in many types of infection. It is a bacteriostatic agent that acts to inhibit the process of protein synthesis by binding to the 30S ribosomal subunit thus preventing the 50s and 30s units from bonding (Kremsner *et al.,* 1989). Doxycycline is used primarily for chemoprophylaxis in areas where quinine resistance exists. It can be used in resistant cases of uncomplicated *P. falciparum* but has a very slow action in acute malaria. The most commonly experienced side effects are permanent enamel hypoplasia, transient depression of bone growth, gastrointestinal disturbances and some increased levels of photosensitivity. Due to its effect on bone and tooth growth it is not used in children under 8, pregnant or lactating women and those with a known hepatic dysfunction (Kremsner *et al.,* 1994). Tetracycline is only used in combination for the treatment of acute cases of *P. falciparum* infections due to its slow onset. Unlike Doxycycline it is not used in chemoprophylaxis. Oesophageal ulceration, gastrointestinal upset and interferences with the process of ossification and depression of bone growth are known to occur. The majority of side effects associated with Doxycycline are also experienced.

Clindamycin

Clindamycin is a derivative of Lincomycin, with a slow action against blood schizonticides. It is only used in combination with Quinine in the treatment of acute cases of resistant *P. falciparum* infections and not as a prophylactic (Kremsner *et a*l., 1989; 1994)

2.6.4. Artemisinin compounds

Artemisinin is a Chinese herb (Qinghaosu) that has been used in the treatment of fevers for over 1,000 years (WHO, 1996), thus predating the use of Quinine in the western world. It is derived from the plant *Artemisia annua*, with the first documentation as a successful therapeutic agent in the treatment of malaria in 340 AD (Mueller *et al.* 2000; 2004). The active compound was isolated first in 1971 and named Artemsinin (Mills and Bone, 2000). It is a sesquiterpene lactone with a chemically rare peroxide bridge linkage, which is thought to be responsible for the majority of its anti-malarial action (Hien and White, 1993). It has proven to be effective against all forms of multi-drug resistant *P. falciparum*, thus every care is taken to ensure compliance and adherence together with other behaviours associated with the development of resistance. It is also only given in combination with other anti-malarials.

Artemesinin has a very rapid action and the vast majority of acute patients treated show significant improvement within 1-3 days of receiving treatment. It has demonstrated the fastest clearance of all anti-malarials currently used and acts primarily on the trophozoite phase, thus preventing progression of the disease. It is converted to active metabolite dihydroartemesinin that then inhibits the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) encoded by *P. falciparum* (Eckstein-Ludwig *et al.*, 2003). It was one of many candidates then tested by Chinese scientists from a list of nearly 200 traditional Chinese medicines for treating malaria. It was the only one that was effective, but it was found that it cleared malaria parasites from their bodies faster than any other drug in history. *Artemisia annua* is a common herb and has been found in many parts of the world, including along the Potomac River, in Washington, D.C (Hien and White, 1993).

A number of sesquiterpine lactone compounds have been synthesized from the plant *Artemisia annua* (artesunate, artemether, arteether) (Mills and Bone, 2000). These compounds are used for treatment of severe malaria and have shown very rapid parasite clearance times and faster fever resolution than occurs with quinine. Artemisinin drugs first introduced in South-East Asia have proven to be well tolerated and the most potent of antimalarials (White, 1999). They exhibit the following properties: rapid significant reduction of parasite biomass, rapid resolution of clinical symptoms, effective against multidrug resistant *P. falciparum* and reduction of gametocyte carriage, which may reduce transmission. However,

artemisinin drugs have a very short half-life and thus a multiple dose regimen of seven days is required to achieve an acceptable cure rate (WHO, 2003).

In some areas of South-East Asia, combinations of artemisinins and mefloquine offer the only reliable treatment for uncomplicated malaria, due to the development and prevalence of multidrug resistant *falciparum* malaria (White, 1999). Combination therapy (an artemisinin compound given in combination with another antimalarial), typically a long half-life drug like mefloquine) has reportedly been responsible for inhibiting intensification of drug resistance and for decreased malaria transmission levels in South-East Asia (Price *et al.*, 1996; White, 1999). Artesunate (AS) plus amodiaquine (AQ) is one artemisinin-based combination (ACT) recommended by the WHO for treating *P. falciparum* malaria in Africa (Sodiomon *et al*., 2009).

Artemether is a methyl ether derivative of dihydroartemesinin. It is similar to artemesinin in mode of action but demonstrates a reduced ability as a hypnozoiticidal compound, instead acting more significantly to decrease gametocyte carriage. Similar restrictions are in place, as with artemesinin, to prevent the development of resistance, therefore it is only used in combination therapy for severe acute cases of drug-resistant *P. falciparum*. It was discovered in 1982 (Dorvault, 1982). Artesunate, discovered in 1983 (Mills and Bone, 2000) is a hemisuccinate derivative of the active metabolite dihydroartemisin. Currently it is the most frequently used of all the artemesinin-type drugs. Its only effect is mediated through a reduction in the gametocyte transmission. It is used in combination therapy and is effective in cases of uncomplicated *P. falciparum*.

Dihydroartemisinin is the active metabolite to which Artemisinin is reduced. It is the most effective Artemisinin compound and the least stable (Krettli, 2001). It has a strong blood schizonticidal action and reduces gametocyte transmission. It is used for therapeutic treatment of cases of resistant and uncomplicated *P. falciparum*. As with artesunate, no side effects to treatment have thus far been recorded.

Arteether is an ethyl ether derivative of dihydroartemisinin. It is used in combination therapy for cases of uncomplicated resistant P. falciparum (Krettli, 2001). No side effects have been recorded.

2.7 COMBINATION THERAPY WITH ANTIMALARIALS

The problem of the development of malaria resistance must be weighed against the essential goal of anti-malarial care; that is to reduce morbidity and mortality. Thus a balance must be reached that attempts to achieve both goals whilst not compromising either too much by doing so. The most successful attempts so far have been in the administration of combination therapy. The key driver for combination antimalarial therapy is the need to slow development of acquired drug resistance to a New Chemical Entity (NCE) and so maintain high levels of efficacy for a longer period of time. This is best achieved by combining molecules which individually have high levels of efficacy (WHO, 2001)

Combination therapy can be defined as, 'the simultaneous use of two or more blood schizonticidal drugs with independent modes of action and different biochemical targets in the parasite' (Price *et al.*, 1999). Much evidence has supported the use of combination therapies. The use of two antimalarial drugs simultaneously, especially when the antimalarials have different mechanisms of action, has the potential for inhibiting the development of resistance to either of the components. The efficacy of a combination of a 4-aminoquinoline drug (chloroquine or amodiaquine) with Sulfadoxine/Pyrimethamine (SP) has been reviewed (McIntosh and Greenwood, 1998). It was found that the addition of either chloroquine or amodiaquine to SP marginally improved parasitological clearance (compared with SP alone). The studies reviewed were mostly done in areas and at times when both SP and Chloroquine/Amodiaquine retained a fair amount of efficacy, and it is not clear from these studies how well such a combination would act in areas where one of the components was significantly compromised. However several problems prevent the wide use in the areas where its use is most advisable. These include: problems identifying the most suitable drug for different epidemiological situations, the expense of combined therapy (it is over 10 times more expensive than traditional mono-therapy), how soon the programmes should be introduced and problems linked with policy implementation and issues of compliance (WHO,

2001). The combinations of drugs currently prescribed can be divided into two categories: Non-artemesinin and quinine-based combinations and artemesinin based combinations.

2.7.1 Non-Artemesinin based combinations

Sulfadoxine-pyrimethamine plus Amodiaquine: This is a combination that has been shown to produce a faster rate of clinical recovery than SP and Chloroquine (McIntosh and Greenwood 1998). However there are serious adverse reactions associated which have limited its distribution. It is thought to have a longer therapeutic lifetime than other combinations and may be a more cost-effective option to introduce in areas where resistance is likely to develop.

Sulfadoxine-Pyrimethamine plus Mefloquine: This is produced as a single dose pill (***Fansimef*®)** and has obvious advantages over some of the more complex regimes. This combination of drugs has very different pharmokinetic properties with no synergistic action. This characteristic is potentially thought to delay the development of resistance; however it is counteracted by the very long half life of Mefloquine which could exert a high selection pressure in areas where intensive malaria transmission occurs. It is also an expensive combination.

Tetracycline or Doxycycline plus Quinine: Despite the increasing levels of resistance to Quinine this combination have proven to be particularly efficacious (Kremsner *et al*., 1994). The longer half-life of the Tetracycline component ensures a high cure rate. Problems with this regime include the relatively complicated drug regimen, where Quinine must be taken every 8 hours for 7 days. Additionally, there are severe side effects to both drugs (Cinchonism in Quinine) and Tetracyclines are contraindicated in children and pregnant women. For these reasons this combination is not recommended as first-line therapy but can be used for non-responders who remain able to take oral medication.

2.7.2 Artemesinin-based combinations

Artemesinin has a very different mode of action from conventional anti-malarials. This makes it particularly useful in the treatment of resistant infections (Mueller *et al*., 2000). However, in order to prevent the development of resistance to this drug it is only recommended in combination with another non-artemesinin based therapy. It produces a very rapid reduction

in the parasite biomass with an associated reduction in clinical symptoms and is known to cause a reduction in the transmission of gametocytes thus decreasing the potential for the spread of resistant alleles. Artemisinin combination therapy (ACT) has been widely adopted as first-line treatment for uncomplicated *falciparum* malaria (Ashley *et al*., 2007; Nosten and White, 2007). Although these drug combinations appear to be safe and well-tolerated, experience with their use in Africa is still limited (Talisuna *et al.,* 2006; Staedke *et al.,* 2008). Artesunate and chloroquine combination has been thoroughly tested in randomized controlled trials and has demonstrated that it is well tolerated with few side effects (Nosten and White, 2007). However, in one study there was less than 85% cure in areas where Chloroquine resistance was known. It is not approved for use in combination therapy and is unadvised in areas of high *P. falciparum* resistance. Artesunate and Amodiaquine combination has also been tested and proved to be more efficacious and similarly well tolerated than the Chloroquine combination. The cure rate was greater than 90%, potentially providing a viable alternative where levels of Chloroquine resistance are high (Sodiomon *et al.,* 2009). The main disadvantage is a suggested link with neutropenia (Mutabingwa *et al.,* 2005).

Artesunate and mefloquine have been used as an efficacious first-line treatment regimen in areas of Thailand for many years (Adjuik *et al.,* 2004). Mefloquine is known to cause vomiting in children and it induces some neuropsychiatric and cardiotoxic effects, interestingly these adverse reactions seem to be reduced when the drug is combined with Artesunate, it is suggested that this is due to a delayed onset of action of Mefloquine. This is not considered a viable option to be introduced in Africa due to the long half-life of Mefloquine, which potentially could exert a high selection pressure on parasites (Bloland, 2001).

Artemether and Lumefantrine (*Coartem®, Riamet®, and Lonart®*) is a combination that has been extensively tested in 16 clinical trials, proving effective in children less than 5years and has been shown to be better tolerated than Artesunate plus Mefloquine combinations (Mutabingwa *et al.,* 2005). There are no serious side effects documented but the drug is not recommended in pregnant or lactating women due to limited safety testing in these groups. This is the most viable option for widespread use and is available in fixed-dose formulae thus increasing compliance and adherence (Lefevre *et al.,* 2001).

Artesunate and Sulfadoxine-Pyrimethamine is a well tolerated combination but the overall level of efficacy still depends on the level of resistance to Sulfadoxine and Pyrimethamine thus limiting its usage (WHO, 2001). Piperaquine-dihydroartemisinin-trimethoprim (*Artecom*®) alone and in combination with Primaquine has been studied in resistant areas of China and Vietnam (Yeka *et al.,* 2008). The drug has been shown to be highly efficacious (greater than 90%) even to strains resistant to Primaquine. More information is required on safety and tolerability in pregnant women and children and toxicology data. Pyronaridine and Artesunate has been tested and was shown to have a clinical response rate of 100% in one trial in Hainan (an area with high levels of *P. falciparum* resistance to Pyronaridine) (Nosten and White, 2007). Chlorproguanil-Dapsone and Artesunate (Lapdap plus) is the most tested drug currently under development and could be introduced in African countries imminently. It is not recommended as a monotherapy due to concerns of resistance developing, thus threatening the future use of related compounds (Nosten and White, 2007).

2.7.3. Traditional Antimalarial Herbs

The use of plants for therapeutic purposes dates back to the human history (Ogbonna *et al*., 2008). Medicinal plants, since time immemorial, have been used in virtually all cultures as a source of medicine (Hoareau and Dasilva, 1999) and for a long time, natural products were the only sources of medication (Bourdy *et al.,* 2008). Several medicinal plants have been used locally to treat malaria infection. Some of such plants are *Enantia chloranta, Nauclea natifolia, Salacia Nitida* (Ogbonna *et al*., 2008), *Acalypha fruticosa*, *Azadirachta indica*, *Cissus rotundifolia*, *Echium rauwalfii*, *Dendrosicyos socotrana* and *Boswellia elongate* (Merlin, 2004; Clarkson *et al*., 2004; Alshwash *et al.*, 2007), *Cymbopogon giganteus and Morinda lucida* etc .Medicinal plants such as *Momordica charantia* (Ejirin wewe), *M balsamina* (Ejirin), *Ageratum conyzoides* (Imi Eshu), *Ocimum gratissimum Cardiospermum grandiofiorum* (Ako Ejirin), *Diospyros monbuttensis* (Egun Eja) etc have been used to treat one ailment or the other in Africa, especially Nigeria (Awe and Makinde, 1997; Azas *et al*., 2002 Otimenyin *et al*., 2008).

The urgency generated by drug-resistant strains of malaria parasites has accelerated antimalarial drug research over the last two decades. While synthetic pharmaceutical agents

continue to dominate research, attention has increasingly been directed to natural products (Etkin, 2003). The success of artemisinin, isolated from *Artemisia annua* and its derivatives for the treatment of resistant malaria has focused attention on the plants as a source of antimalarial drugs (Tan *et al*., 1998). Moreover, plants have been the basic source of sophisticated traditional medicine systems for thousands of years and were instrumental to early pharmaceutical drug discovery and industry (Elujoba *et al*., 2005). The world's poorest are the worst affected, and many treat themselves with traditional herbal medicines. These are often more available and affordable, and sometimes are perceived as more effective than conventional antimalarial drugs (Merlin, 2004).

Ethnobotanical information about antimalarial plants used in traditional herbal medicine, is essential for further evaluation of the efficacy of plant antimalarial remedies and efforts are now being directed towards discovery and development of new chemically diverse antimalarial agents (Clarkson *et al*., 2004). Several rural dwellers depend on traditional herbal medicine for treatment of many infectious diseases including malaria (Ali *et al.*, 2004). The reputed efficacies of these plants have been recognized and passed on from one generation to the other.

About 75% of the population in Africa does not have direct access to chemical treatment, such as chloroquine, but they have access to traditional medicine for treating fevers. Treatment with these remedies has suffered a number of deficiencies; diagnosis is often a problem, identification of plant extracts may be insecure and the chemical content of extracts may vary considerably (Azas *et al*., 2002). Natural products isolated from plants used in traditional medicine, which have potent antiplasmodial action *in vitro*, represent potential sources of new antimalarial drugs (Wright *et al*,. 1994; Gasquet *et al*., 1993). It had been advocated that direct crude drug formulation of the herbs following toxicological absolution may not only produce dosage forms faster but will also lead to cheaper and more affordable drugs for the communities that need them (Elujoba, 1998). Also, there is a belief that these medicines are safe because they are natural and have been used traditionally over a period of time (Sofowora, 1993; Willcox *et al*., 2003).

Plant materials remain an important resource to combat serious diseases in the world (Tshibangu *et al*., 2002) and pharmacognostic investigations of plants are carried out to find novel drugs or templates for development of new therapeutic agents (König, 1992). Moreover herbs can be highly effective for treating malaria if government can educate those involved in the practice regarding the normal dose to be taken before getting well. Therefore, government should provide subvention for the Ministry of Health incorporating National Agency for Food and Drug Administration and Control (NAFDAC) to go into more Malaria research in local herb just to develop new and more effective drug for prevention and control, particularly in view of the rapid spread of drug resistance. Nevertheless, much work need to be done to educate the community and the producers of indigenous herbal products to strictly adhere to environmental hygiene

2.8 Antimalarial Drug Resistance

Resistance to antimalarial drugs has been described for two of the four species of malaria parasite that naturally infect humans, *P. falciparum* and *P. vivax* (Nguyen-Dinh *et al.*, 1981; Singh, 2000). *P. falciparum* has developed resistance to nearly all antimalarials in current use, although the geographical distribution of resistance to any single antimalarial drug varies greatly (Wongsrichanalai *et al*., 2002; Talisuna *et al*., 2004). Chloroquine-resistant *falciparum* malaria has been described everywhere that *P. falciparum* malaria is transmitted (Verdrager, 1986; Wernsdorfer, 1991; Wellems and Plowe, 2001; Sidhu *et al.*, 2002; White 2004;) except for malarious areas of Central America (north-west of the Panama Canal), the island of Hispaniola, and limited areas of the Middle East and Central Asia (Bloland, 2001). Verdrager (1995) has described Localized permanent epidemics as the genesis of chloroquine resistance in *P. falciparum*.

Sulfadoxine/Pyrimethamine (SP) resistance occurs frequently in South-East Asia and South America and is more prevalent in Africa as the drug is increasingly being used as a replacement for chloroquine (Price *et al*., 1999; Purfield *et al*., 2004). Mefloquine resistance is frequent in some areas of South-East Asia and has been reported in the Amazon region of South America and sporadically in Africa (Mockenhaupt, 1995). Cross-resistance between halofantrine and mefloquine is suggested by reduced response to halofantrine when used to treat mefloquine failures (ter Kuile *et al*., 1993). Noedl and others (2008) in a recent study

have shown that artemisinin is losing its potency in Cambodia and increased efforts are required to prevent artemisinin-resistant malaria from spreading across the globe.

2.8.1 Definition of antimalarial drug resistance

Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject" (WHO, 1972). This definition was later modified to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Wernsdorfer and Wernsdorfer, 1995). Most researchers interpret this as referring only to persistence of parasites after treatment doses of an antimalarial rather than prophylaxis failure, although the latter is a useful tool for early warning of the presence of drug resistance (Lobel and Campbell, 1986).

This definition of resistance requires demonstration of malaria parasitaemia in a patient who has received an observed treatment dose of an antimalarial drug and simultaneous demonstration of adequate blood drug and metabolite concentrations using established laboratory methods (such as high performance liquid chromatography) or *in vitro* tests (Basco and Ringwald, 2000). In practice, this is rarely done with *in vivo* studies. *In vivo* studies of drugs for which true resistance is well known (such as chloroquine) infrequently include confirmation of drug absorption and metabolism. Demonstration of persistence of parasites in a patient receiving directly observed therapy is usually considered sufficient. Some drugs, such as mefloquine, are known to produce widely varying blood levels after appropriate dosing and apparent resistance can often be explained by inadequate blood levels (Slutsker, 1990; Basco, 1991).

2.8.2 Malaria treatment failure

Drug resistant parasites are often used to explain malaria treatment failure. However, they are two different clinical scenarios. These are failure to clear parasitaemia and recover from an acute clinical episode when a suitable treatment has been given and anti-malarial resistance in its true form. A distinction must be made between a failure to clear malarial parasitaemia or resolve clinical disease following a treatment with an antimalarial drug and true antimalarial

drug resistance. While drug resistance can cause treatment failure, not all treatment failures are due to drug resistance (Plowe, 2003). Many factors can contribute to treatment failure including incorrect dosing, non-compliance with duration of dosing regimen, poor drug quality, drug interactions, poor or erratic absorption, and misdiagnosis. Probably all of these factors, while causing treatment failure (or apparent treatment failure) in the individual, may also contribute to the development and intensification of true drug resistance through increasing the likelihood of exposure of parasites to suboptimal drug levels (Ariey and Robert, 2003).

2.8.3 Mechanisms of antimalarial resistance

In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs, only a single point mutation is required to confer resistance, while for other drugs, multiple mutations appear to be required (Lobel and Campbell, 1986). Provided the mutations are not deleterious to the survival or reproduction of the parasite, drug pressure will remove susceptible parasites while resistant parasites survive (Watkins and Mosobo, 1993). Single malaria cases have been found to be made up of heterogeneous populations of parasites that can have widely varying drug response characteristics, from highly resistant to completely sensitive (Thaithong, 1983). Similarly, within a geographical area, malaria infections demonstrate a range of drug susceptibility. Over time, resistance becomes established in the population and can be very stable; persisting long after specific drug pressure is removed. The biochemical mechanism of resistance has been well described for chloroquine, the antifolate combination drugs, and atovaquone (Looareesuwan, 1996; Alene and Bennett, 1996; Bloland, 2001; Alifrangis *et al.*, 2003).

2.8.3.1 Chloroquine resistance

The first type of resistance to be acknowledged was to Chloroquine in Thailand in 1957 (Mills and Bone, 2000). As the malaria parasite digests haemoglobin, large amounts of a toxic by-product are formed. The parasite polymerizes this by-product in its food vacuole, producing non-toxic haemozoin (malaria pigment). It is believed that resistance of *P. falciparum* to chloroquine is related to an increased capacity for the parasite to expel chloroquine at a rate that does not allow chloroquine to reach levels required for inhibition of haem polymerization

(Foley and Tilley, 1997). This chloroquine efflux occurs at a rate of 40 to 50 times faster among resistant parasites than sensitive ones (Martin *et al*., 1987).

Further evidence supporting this mechanism is provided by the fact that chloroquine resistance can be reversed by drugs which interfere with this efflux system (Martin *et al.,* 1987). It is unclear whether parasite resistance to other quinoline antimalarials (amodiaquine, mefloquine, halofantrine, and quinine) occurs via similar mechanisms but resistances are thought to have occurred by similar mechanisms (Foley and Tilley, 1997).

2.8.3.2 Antifolate combination drugs

Antifolate combination drugs, such as sulfadoxine plus pyrimethamine, act through sequential and synergistic blockade of two key enzymes involved with folate synthesis. Pyrimethamine and related compounds inhibit the step mediated by dihydrofolate reductase (DHFR) while sulfones and sulfonamides inhibit the step mediated by dihydropteroate synthetase (DHPS) (Bruce-Chwatt, 1985; Kublin *et al.*, 2002). Specific gene mutations encoding for resistance mutations have been associated with varying degrees of resistance to antifolate combination drugs (Plowe *et al.,* 1998; Marks *et al*., 2005). Talisuna *et al* in Uganda [Talisuna *et al.*,, 2004] have shown higher levels of resistance to chloroquine and SP in zones of higher transmission intensity. On the other hand, history shows that chloroquine resistance emerged first in low transmission zones and that antifolate resistance has increased more rapidly in low transmission areas (White 2004).

2.9 Spread of resistance

There is no single factor that confers the greatest degree of influence on the spread of drug resistance, but a number of plausible causes associated with an increase have been acknowledged. These include aspects of economics, human behaviors, pharmacokinetics, and the biology of vectors and parasites. The most influential causes are examined below:

2.9.1 Biological influences on resistance

The biological influences are based on the parasite's ability to survive the presence of an anti-malarial thus enabling the persistence of resistance and the potential for further transmission

despite treatment. In normal circumstances parasites that persist after treatment are destroyed by the host's immune system, therefore factors that act to reduce the elimination of parasites could facilitate the development of resistance (Bloland, 2001). This explains the poorer response associated with immunocompromised individuals, pregnant women and young children.

Based on data on the response of sensitive parasites to antimalarial drugs *in vitro* and the pharmacokinetic profiles of common antimalarial drugs, there is thought to always be a residuum of parasites that are able to survive treatment (Wernsdorfer, 1991). Under normal circumstances, these parasites are removed by the immune system. Factors that decrease the effectiveness of the immune system in clearing parasite residuum after treatment also appear to increase survivorship of parasites and facilitate development and intensification of resistance. This mechanism has been suggested as a significant contributor to resistance in South-East Asia, where parasites are repeatedly cycled through populations of non-immune individuals (Verdrager, 1986, 1995). The non-specific immune response of non-immune individuals is less effective at clearing parasite residuum than the specific immune response of semi-immune individuals (White, 1997). The same mechanism may also explain poorer treatment response among young children and pregnant women (White, 1997). Among refugee children in the former Zaire, those who were malnourished (low weight for height) had significantly poorer parasitological response to both chloroquine and SP treatment (Wolday *et al.,* 1995). Similarly, evidence from prevention of malaria during pregnancy suggests that parasitological response to treatment among individuals infected with the human immunodeficiency virus (HIV) may also be poor. HIV-seropositive women require more frequent treatment with SP during pregnancy in order to have the same risk of placental malaria as is seen among HIV-seronegative women (Parise *et al.,* 1998*)*. Some characteristics of recrudescent or drug resistant infections appear to provide a survival advantage or to facilitate the spread of resistance conferring genes in a population (White., 1999). In one study, patients experiencing chloroquine treatment failure had recrudescent infections that tended to be less severe or even asymptomatic (Handunnetti *et al.,* 1996). Schizont maturation may also be more efficient among resistant parasites (Lobel and Campbell, 1986; Warsame *et al.*, 1991).

There has been evidence to suggest that certain parasite-vector combinations can alternatively enhance or inhibit the transmission of resistant parasites, causing 'pocket-like' areas of resistance. In South-East Asia, two important vectors, *Anopheles stephensi* and *A. dirus*, appear to be more susceptible to drug-resistant malaria parasites than to drug sensitive malaria parasites (Wilkinson *et al*., 1976; Sucharit *et al*., 1977). In Sri Lanka, researchers found that patients with chloroquine-resistant malaria infections were more likely to have gametocytaemia than those with sensitive infections and that the gametocytes from resistant infections were more infective to mosquitoes (Handunnetti *et al*., 1996). The reverse is also true; some malaria vectors may be somewhat refractory to drug-resistant malaria, which may partially explain the pockets of chloroquine sensitivity that remain in the world in spite of very similar human populations and drug pressure as found in Haiti (Bloland *et al.*, 1997).

Many antimalarial drugs in current usage are closely related chemically and development of resistance to one can facilitate development of resistance to others. Chloroquine and Amodiaquine are both 4-aminoquinolines and cross-resistance between these two drugs is well known (Hall, 1975; Basco, 1991). Development of resistance to mefloquine may also lead to resistance to halofantrine and quinine (Foley and Tilley, 1998). Antifolate combination drugs have similar action and widespread use of SP for the treatment of malaria may lead to increased parasitological resistance to other antifolate combination drugs (Watkins and Mosobo, 1993). Development of high levels of SP resistance through continued accumulation of DHFR mutations may compromise the useful life span of newer antifolate combination drugs such as chlorproguanil/dapsone (LapDap) even before they are brought into use. This increased risk of resistance due to SP use may even affect non malarial pathogens as the use of SP for treatment of malaria had been found to result in increased resistance to trimethoprim/sulfamethoxazole among respiratory pathogens (Hastings, 2003).

There is an interesting theory that development of resistance to a number of antimalarial drugs among some *falciparum* parasites produces a level of genetic plasticity that allows the parasite to rapidly adapt to a new drug, even when the new drug is not chemically related to drugs previously experienced (Rathod *et al.*, 1997). The underlying mechanism of this plasticity is currently unknown, but this capacity may help explain the rapidity with which strains of *falciparum* develop resistance to new antimalarial drugs. The choice of using a long half-life

drug (SP, Mefloquine) in reference to one with a short half-life (Chloroquine, LapDap, Quinine) has the benefit of simpler, single dose regimens which can greatly improve compliance or make directly observed therapy feasible (Lefevre *et al.*, 2001). Unfortunately, that same property may increase the likelihood of resistance developing, due to prolonged elimination periods (Newton *et al*., 2006). Parasites from new infections or recrudescent parasites from infections that did not fully clear will be exposed to drug blood levels that are high enough to exert selective pressure but are insufficient to provide prophylactic or suppressive protection (Wernsdorfer, 1991). When blood levels drop below the minimum inhibitory concentration (the level of drug that fully inhibits parasite growth), but remain above the concentration of drug that produces 5% inhibition of parasite growth (EC5), selection of resistant parasites occurs. This selection was illustrated in one study in Kenya that monitored drug sensitivity of parasites reappearing after SP treatment (Watkins and Mosobo, 1993). Parasites reappearing during a period when blood levels were below the point required to clear pyrimethamine-resistant parasites, but still above that level required to clear pyrimethamine-sensitive parasites, were more likely to be pyrimethmine-resistant than those reappearing after levels had dropped below the level required to clear pyrimethamine-sensitive parasites (Watkins and Mosobo, 1993). This period of selective pressure lasts for approximately one month for mefloquine, whereas it is only 48 hours for quinine. In areas of high malaria transmission, the probability of exposure of parasites to drug during this period of selective pressure is high (Trape and Rogier, 1996). In Africa, for instance, people can be exposed to as many as 300 infective bites per year; in rare cases, even as much as 1000 infective bites per year and during peak transmission, as many as five infective bites per night (Trape and Rogier, 1996).

Mismatched pharmacokinetics can also play a role in facilitating the development of resistance. For instance the elimination half-life of pyrimethamine is between 80 and 100 hours and is between 100 and 200 hours for sulfadoxine, leaving an extended period when sulfadoxine is “unprotected” by synergy with pyrimethamine (Watkins and Mosobo, 1993). This sort of mismatched pharmacokinetics is even more apparent in the mefloquine plus sulfadoxine-pyrimethamine combination used in Thailand where mefloquine has an elimination half-life of approximately 336 to 432 hours (Palmer *et al.*, 1993; McIntosh and Greenwood, 1998). It is apparent that there are more genetically distinct clones per person in

areas of more intense transmission than in areas of lower transmission (Babiker and Walliker, 1997). However, the interpretation of this and its implications for development of resistance has variously been described as resistance being more likely in low-transmission environments (Paul and Day, 1998), high-transmission environments (Mackinnon, 1997; Mackinnon and Hastings, 1998; Babiker and Walliker, 1997), or either low- or high- but not intermediate-transmission environments (Hastings 1997; Hastings and Mckinnon, 1998).

The relationship between transmission intensity and parasite genetic structure is obviously complex and subject to other confounding/contributing factors (Hastings and Mckinnon, 1998). What is clear is that the rate at which resistance develops in a given area is sensitive to a number of factors beyond mere intensity of transmission. Such factors are initial prevalence of mutations, intensity of drug pressure, population movement between areas, the nature of acquired immunity to the parasite or its strains, etc. However, reducing the intensity of transmission will likely facilitate prolonging the useful life span of drugs (Hastings and Mckinnon, 1998).

2.9.2 Programmatic influences on resistance

Programmatic influences on development of antimalarial drug resistance include overall drug pressure, inadequate drug intake (poor compliance or inappropriate dosing regimens), pharmacokinetic and pharmacodynamic properties of the drug or drug combination, and drug interactions (Wernsdorfer and Wernsdorfer, 1995). Additionally, reliance on presumptive treatment can facilitate the development of antimalarial drug resistance. Overall drug pressure especially that exerted by programmes utilizing mass drug administration (e.g Eko free malaria programme, Federal government Malaria intervention programme using Coartem) probably has the greatest impact on development of resistance (Payne, 1988; Wernsdorfer, 1999). Studies have suggested that resistance rates are higher in urban and periurban areas than rural communities, where access to and use of drug is greater (Ettling *et al*., 1995).

Confusion over proper dosing regimen has been described. In Thailand the malaria control programme recommended 2 tablets (adult dose) of SP for treating malaria based on studies suggesting that this was effective. Within a few years, this was no longer effective and the programme increased the regimen to 3 tablets (Price *et al.*, 1996). Although unproven, this

may have contributed to the rapid loss of SP efficacy there. Similar confusion over the proper SP dosing regimen exists in Africa (Salako, 1998). To simplify treatment, many programmes dose children based on age rather than weight and depending on the regimen being recommended, this has been shown to produce systematic under dosing among children of certain weight and age groups (Plowe, 2003).

The use of presumptive treatment for malaria has the potential for facilitating resistance by greatly increasing the number of people who are treated unnecessarily but will still be exerting selective pressure on the circulating parasite population (Wernsdorfer *et al*, 1994). In some areas and at some times of the year, the number of patients being treated unnecessarily for malaria can be very large (Persidis, 2000). Concurrent treatment with other drugs can increase the likelihood of treatment failure and may contribute to development of drug resistance. For instance, folate administration for treatment of anaemia and possibly when used as a routine supplement during pregnancy can increase treatment failure rates (Ballereau, 1997). Similarly, concurrent illness and malnourishment may have an influence on drug resistance (Payne, 1988).

Drug quality has also been implicated in ineffective treatment and possibly drug resistance (Shakoor *et al*., 1997). Either through poor manufacturing practices, intentional counterfeiting, or deterioration due to inadequate handling and storage, drugs may not contain sufficient quantities of the active ingredients. In an analysis of chloroquine and antibiotics available in Nigeria and Thailand, between 37% and 40% of samples assayed had substandard content of active ingredients, mostly from poor manufacturing practices (Shakoor *et al*., 1997). Another study in Africa found chloroquine stored under realistic tropical conditions lost at least 10% of its activity in a little over a year (Ballereau, 1997).

2.10 Detection of resistance

In general, four basic methods have been routinely used to study or measure antimalarial drug resistance: *in vivo*, *in vitro*, animal model studies, and molecular characterization (Basco, 2007). Additionally, less rigorous methods have been used, such as case reports, case series,

or passive surveillance. Much discussion has occurred regarding the relative merits of one test over another, with the implication always being that one type of test should be used preferentially. Careful consideration of the types of information each yields indicates, however, that these are complementary, rather than competing, sources of information about resistance. Recognition of drug resistance (or, treatment failure) in individual patients is made difficult in many settings by operational issues such as availability and quality of microscopy. Especially in Africa, where presumptive diagnosis and treatment for malaria is most common, detection of treatment failures also tends to be presumptive (persistence or reappearance of clinical symptoms in a patient recently receiving malaria treatment). Because of the non-specific nature of clinical signs and symptoms of malaria and the many other causes of febrile disease, this can lead to a false sense that a particular drug is not working when it is, or, potentially, that an ineffective drug is working when it is not. In cases where microscopy is used, presence of parasitaemia in a supposedly fully treated patient may indicate treatment failure, but is not necessarily evidence of drug resistance.

2.10.1 *In vivo* tests

An *in vivo* test consists of the treatment of a group of symptomatic and parasitaemic individuals with known doses of drug and the subsequent monitoring of the parasitological and/or clinical response over time (Basco, 2007). One of the key characteristics of *in vivo* tests is the interplay between host and parasite. Diminished therapeutic efficacy of a drug can be masked by immune clearance of parasites among patients with a high degree of acquired immunity (White, 1997). Of the available tests, *in vivo* tests most closely reflect actual clinical or epidemiological situations i.e. the therapeutic response of currently circulating parasites infecting the actual population in which the drug will be used. Because of the influence of external factors (host immunity, variations of drug absorption and metabolism, and potential misclassification of reinfections as recrudescences), the results of *in vivo* tests do not necessarily reflect the true level of pure antimalarial drug resistance. However, this test offers the best information on the efficacy of antimalarial treatment under close to actual operational conditions. This should be expected to occur among clinic patients if provider and patient compliance is high. The original methods for *in vivo* tests required prolonged periods of follow-up (minimum of 28 days) and seclusion of patients in screened rooms to prevent the possibility of re-infection. These methods have since been modified extensively and the most

widely used methods now involve shorter periods of follow-up (7 to 14 days) without seclusion, under the assumption that reappearance of parasites within 14 days of treatment is more likely due to recrudescence rather than re-infection (WHO, 1996b). Additional modifications reflect the increased emphasis on clinical response in addition to parasitological response. Traditionally, response to treatment was categorized purely on parasitological grounds as sensitive, resistance I (RI), RII, and RIII (WHO, 1996). Later modifications have combined, to varying extent, parasitological and clinical indicators (WHO, 1996).

Since anaemia can be a major component of malaria illness, *in vivo* methodologies allow investigation of haematological recovery after malaria therapy (Boland, 2001). Failure of complete parasitological clearance, even in situations where recurrence of fever is rare, can be associated with lack of optimal haematological recovery among anaemic patients. Standardization is however a limitation in this methodology. Major differences in sample size, enrolment criteria, exclusion criteria, length and intensity of follow-up, loss-to-follow-up rates, and interpretation and reporting of results are apparent in published papers on *in vivo* trials (Ringwald and Basco, 1999). These differences make it difficult, if not impossible, to compare results from one study to another with any level of confidence (Bloland, 2001). The methodology currently being used and promoted, especially in sub-Saharan Africa, is a system that emphasizes clinical response over parasitological response (WHO, 1996). Close adherence to this protocol does provide comparable data; however, these data are not readily comparable to data collected using other *in vivo* methods. Although not called for in the protocol, categorization of the parasitological response using the standard WHO definitions (WHO 1996) would allow some ability to compare to historical levels and provide useful parasitological results that would aid in interpreting the clinical results.

2.10.2 *In vitro* tests

From the point of view of a researcher interested in pure drug resistance, *in vitro* tests avoid many of the confounding factors which influence *in vivo* tests by removing parasites from the host and placing them into a controlled experimental environment. In the most frequently used procedure, the micro-technique, parasites obtained from a finger-prick blood sample are exposed in microtitre plates to precisely known quantities of drug and observed for inhibition of maturation into schizonts (Rieckmann *et al*., 1978; Jenson, 1988; Basco, 2007). This test more accurately reflects “pure” antimalarial drug resistance. Multiple tests can be performed

on isolates, several drugs can be assessed simultaneously, and experimental drugs can be tested. In addition, *falciparum* erythrocytic parasites can be evaluated in-vitro. However, the test has certain significant disadvantages. The correlation of *in vitro* response with clinical response in patients is neither clear nor consistent, and the correlation appears to depend on the level of acquired immunity within the population being tested. Prodrugs, such as proguanil, which require host conversion into active metabolites, cannot be tested. Neither can drugs that require some level of synergism with the host's immune system (Golenda and Rosenberg, 1997). These tests are technologically more demanding and relatively expensive, which makes them potentially more difficult to adapt successfully to routine work in the field.

In vitro assays for the sensitivity of human malaria parasites to antimalarial drugs provide information complementary to that derived from the epidemiology of drug-resistant malaria (Basco and Ringwald, 2007). Its principles were initially based on a suboptimal short-term culture method reported in 1912 which was applied for the WHO macrotest system in the 1960s (Rieckmann *et al*., 1978) and used until the 1980s. The principles of *in vitro* culture underwent a major modification in 1976, when a new method for continuous culture of *P. falciparum* was reported by Trager & Jensen (1976). The essential components of the complete blood-medium mixture are Roswell Park Memorial Institute (RPMI) 1640 medium buffered with *N*-(2-hydroxyethyl) piperazine-*N´*-(2-ethanesulfonic acid) (HEPES) and sodium bicarbonate, human serum (or serum substitutes, including animal sera and lipid enriched bovine albumin) and *P. falciparum*-infected human erythrocytes. This technical improvement led to the elaboration of several *in vitro* assay systems in the late 1970s and in the 1980s, including the WHO microtest system (morphological assay), the 48-h test (morphological assay) and the radioisotope microtest (Basco, 2007). Over the past few years, two novel *in vitro* drug sensitivity assays have been evaluated: an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies directed against either plasmodial lactate dehydrogenase (LDH) or histidine-rich protein II (HRP II) and a fluorometric assay with DNA-binding fluorescent dyes (WHO, 1997). These two assays are non-morphological and non-radioactive and are also based on Trager & Jensen's (1976) culture method.

2.10.3 Animal model studies

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

2.10.4 Molecular techniques

These tests offer promising advantages to the methods described above. Molecular tests use polymerase chain reaction (PCR) to indicate the presence of mutations encoding biological resistance to antimalarial drugs (Plowe *et al.,* 1998). Theoretically, the frequency of occurrence of specific gene mutations within a sample of parasites obtained from patients from a given area could provide an indication of the frequency of drug resistance in that area analogous to information derived from *in vitro* methods (Basco, 2007). Advantages include the need for only small amounts of genetic material as opposed to live parasites, independence from host and environmental factors and the ability to conduct large numbers of tests in a relatively short period of time (Beck, 1999). Disadvantages include the obvious need for sophisticated equipment and training and the fact that gene mutations that confer antimalarial drug resistance are currently known or debated for only a limited number of drugs primarily for dihydrofolate reductase inhibitors (pyrimethamine), dihydropteroate synthase inhibitors (sulfadoxine), and chloroquine (Plowe *et al.,* 1998). Confirmation of the association between given mutations and actual drug resistance is difficult, especially when resistance involves more than one gene locus and multiple mutations (Sidhu *et al* 2002).

2.10.5 Case reports and passive detection of treatment failure

Additional methods for identifying or monitoring antimalarial drug resistance include the use of case reports or case series of spontaneously reported treatment failure. In general, these methods require far less investment in time, money and personnel and can be done on an ongoing basis by individual health care centers (Bloland, 2001). They suffer however, from presenting a potentially biased view of drug resistance primarily because denominators are typically unknown and rates of resistance cannot be calculated. Nonetheless, case reports can be useful and may indicate a problem that should be confirmed using one of the other methods. In the United States for instance, case reports, especially when occurring in clusters, of prophylaxis failure have been used to help formulate recommendations for chemoprophylaxis of non-immune travellers to endemic areas (White, 1999).

Another method that has been used is passive detection of treatment failure. In this system, patients are treated following usual treatment guidelines and told to come back to the clinic or hospital if symptoms persist or return. Those cases which do return are considered to represent the population of treatment failures. Because this system does not ensure compliance with treatment regimens through directly observed therapy and does not attempt to locate and determine the outcome of patients who do not return on their own, data are seriously biased. In a study conducted in Ethiopia and Eritrea using this method, only 1706 (4.3%) out of 39824 patients returned to the clinic (Alene and Bennett, 1996). The assumption was that those patients who did not return did not have resistant parasites, yielding a very low prevalence of resistance (1.8% to 4.8%, depending on region). These results contrast dramatically with results from standard 7-days *in vivo* trials conducted at two sites in Eritrea in 1994 (Bloland, 2001) and one site in Ethiopia in 1993–1994 which found between 58% and 86% RII/RIII level resistance (Tulu, 1996).

2.11 The future: prevention of drug resistance

The future of antimalarial drug resistance and efforts to combat it is defined by a number of assumptions. First, antimalarial drugs will continue to be needed long into the future (Breman, 2001). No strategy in existence or in development, short of an unforeseen scientific breakthrough or complete eradication, is likely to be 100% effective in preventing malaria infection. Secondly, as long as drugs are used, the chance of resistance developing to those

drugs is present (Plowe, 2003). *P. falciparum* has developed resistance to nearly all available antimalarial drugs and it is highly likely that the parasite will eventually develop resistance to any drug that is used widely. Thirdly, development of new drugs appears to be taking longer than development of parasitological resistance. The development of resistance to antimalarial drugs in South-East Asia has been far quicker than the estimated 12 to 17 years it takes to develop and market a new antimalarial compound (Ridley, 1997). Fourthly, affordability is an essential consideration for any strategy to control drug-resistant malaria, especially in Africa (Foster and Phillips 1998; Goodman *et al.*, 1999).

The future, especially in Africa, will also be defined by how well the central tenets of malaria control can be reconciled with the central tenets of control of drug resistance. One of the cornerstones of the current approach to malaria control is the provision of prompt, effective malaria treatment. In much of Africa, easy access to public sector health care is limited and when it is accessible, health care staff are often inadequately trained, insufficiently supplied and supported, ineffectively supervised and poorly motivated (Goodman *et al.*, 2000). One response to this situation has been the intentional liberalization of access to drugs; instead of relying so heavily on the formal public sector to distribute antimalarial drugs, some people are suggesting that the best way to reduce the time between onset of illness and first treatment with an antimalarial drug is by making these drugs widely available on the open market, from unofficial sources of health care and at the household level (Salako, 1998). This approach is gaining support internationally. This approach is also in direct conflict with the primary methods for inhibiting development of drug resistance, limited access to and judicious use of chemotherapeutic agents. Clearly, some middle ground will need to be identified that will improve access to antimalarial drugs for those who need to be treated while at the same time reducing the inappropriate use of those same drugs. Prevention strategies can be divided into those aimed specifically at preventing malaria infection and those aimed at reducing the likelihood of development of drug resistance. Reduction of overall malaria infection rates or transmission rates have an indirect impact on development of drug resistance by reducing the number of infections needing to be treated (and therefore, overall drug pressure) and by reducing the likelihood that resistant parasites are successfully transmitted to new hosts. The transmission reduction methods include the use of insecticide-treated bednets, indoor residual insecticide spraying, environmental control (mosquito breeding site or “source” reduction),

other personal protection measures (e.g. use of repellent soap or screening windows) and chemoprophylaxis in defined populations (Ariey and Robert, 2003). An effective and deliverable vaccine would also be greatly beneficial (Good, 2001).

Interventions aimed at preventing drug resistance, generally focus on reducing overall drug pressure through more selective use of drugs; improving the way drugs are used and also by improving prescribing by clinicians, follow-up practices and patient compliance; or using drugs or drug combinations which are inherently less likely to foster resistance or have properties that do not facilitate development or spread of resistant parasites (Plowe, 2003).

In Nigeria, the methods that are widely used to study antimalarial drug resistance are *in-vivo* methods, animal model studies and molecular methods (Oduola *et al*., 1993; Ogbonna *et al*., 2008; Ogunfowokan *et al*., 2009). Using *in-vitro* sensitivity test more accurately reflects "pure" antimalarial drug resistance, multiple tests can be performed on isolates, several drugs can be assessed simultaneously and experimental drugs can be tested (Basco, 2007). In addition, *falciparum* erythrocytic parasites can be evaluated *in-vitro*. The method is also a good epidemiological tool to assess baseline sensitivity and to monitor the drug response of *P. falciparum* (Druilhe *et al.*, 2007). Moreover, it provides background information for development and evaluation of drug policies, can be an indicator of future therapeutic failure and can also guide on the partner drug in ACT (Basco, 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area:

This study was carried out in Ogun State, located in the tropical zone of Southwestern Nigeria. With its state capital in Abeokuta, Ogun State has a land area of 16,409.26 square kilometers and a population of 2,759,109 (Unpublished report, Ministry of Health, 2003). Ogun State is bounded on the West by Benin Republic, on the South by Lagos State and the Atlantic Ocean, on the East by Ondo State and on the North by Oyo State. The State is made up of six ethnic groups viz, Egba, Ijebu, Remo, Egbado, Awori and Egun. The language of the majority of the people of Ogun State is Yoruba but this is however broken into scores of dialects. Ogun State is in the transitional zone between the tropical rain forest and derived savannah zone in the southwest, Nigeria. The area experiences two seasons, the dry season (November to March) and the wet season (April to October). The annual temperature range is from 22.8ºC to 34.9ºC and the mean annual rainfall is about 107.3mm. Malaria is present throughout the year with a marked increase during the rainy season (Ojo, 2005).

3.2 Study subjects

Children between 1-15 years, pregnant women and other adults were included in this study. This is because majority of malaria cases occur in children under the age of 12 years, pregnant women are also especially vulnerable. The blood samples were collected and analyzed between April 2008 and June 2009.

3.3 Sampling Procedure

Ogun State, which was the study site, was divided into 4 zones for sample collection. The 4 zones are

- Sango – Ota (Yewa)
- Abeokuta (Egba)
- Ijebu Ode (Ijebu) and
- Sagamu (Remo)

Fig 2.1 Map of Ogun State, Southwestern Nigeria

(http://ogunstate.gov.ng/eGovernment/index.php)

Using the population size of each zone and the rate of prevalence of malaria in the state, the sample size for each of the four zones was calculated with a 95% CI and precision level of 5% as follows: $n = Z^2 P(1-P)^2 / d^2$

Where n=sample size

Z=1.96 at 95%

P=Prevalence rate

d=Sampling error that can be tolerated (0.05)

State general hospitals located in the different zones were used as sample collection centers. Both in and out patients who presented with uncomplicated malaria in the hospitals were recruited for the research work. Four thousand and sixty six patients were recruited into this study. The total number of subjects recruited in Sango-ota, Abeokuta, Ijebu-ode and Sagamu were 1120, 1116, 995 and 835 respectively. The mean age was 19 years (>1 –70) with 93% less than 25 years.

3.4 Ethical Consideration

Scientific and Ethical clearance was obtained from the Nigerian Institute of Medical Research - Institutional Review Board (NIMR-IRB) and Covenant University Ethics Committee for this work. The Ogun State Ministry of health (Hospitals Management Board) was also informed and clearance obtained for this study. Written informed consent was obtained from patients prior to recruitment into this study. Consent for the children was provided by the parents/guardians while some of the participants provided the assents.

3.5. Sample collection

Blood samples were collected for malaria screening from both fingerprick and venepuncture . This is to check the presence of healthy asexual parasites in the peripheral smear of patients. Safety procedures were adopted in the collection of finger-prick blood samples by swabbing the area to be sampled with 70% alcohol and allowing it to dry before collection. The bleeding was done in the hospitals by clinicians and medical laboratory scientists. About 2-5 ml of blood was then drawn (venepuncture) with a sterile disposable syringe and transferred to a heparinised centrifuge tube. The blood samples were transported to the laboratory at 4^0C. Drops of peripheral blood were placed on 1.5 x 7.0-cm strips of Whatman (Brentford, United

Kingdom) 3MM filter paper so that the blood covered half the length of the strip. The strips were air-dried and kept in plastic bags until use.

3.6 Cryopreservation

The infected blood was centrifuged at 3000 revolutions per minute (rpm) for 10 min in a refrigerated centrifuge. The supernatant/plasma was removed and cells were suspended in equal volume of cryopreservative. The suspension was distributed into cryotubes and quickly frozen in cryofreezer at -80^{0}C and then transferred into liquid nitrogen (-196^{0}C). The cryopreservative was prepared by adding 28ml glycerol to 72ml of 4.2% Sorbitol in normal saline. The solution was sterilized by filtration through a Millipore filter of 0.22µm porosity.

3.7 Processing of Sample

3.7.1 Microscopic examination

Thick and thin films stained with Giemsa were prepared for the microscopic examination of the malaria parasite. The thin films were fixed with methanol and all films were stained with 3% Giemsa stain of pH 7.0 for 30 min as recommended by WHO (WHO, 2000). Blood films were examined microscopically using 100X (oil immersion) objectives as described by Cheesbrough (2000). The thick films were used to determine the parasite densities while thin films were used to identify the parasite species and infective stages. Parasite density per microlitre of blood (parasitemia) was estimated from the thick film, taking the number of leucocytes per microliter of blood as 8,000 and was expressed as follows:

$$\text{Parasite density/µL} = \frac{\text{Parasite count x 8,000}}{\text{No of WBC counted}}$$

3.8. Antimalarial sensitivity testing

3.8.1 Revival of Cryopreserved parasites

The vial was taken out of the liquid nitrogen tank and thawed quickly in a 37^{0}C water bath. The content was transferred to a centrifuge tube and centrifuged at 3000rpm for 10min. The supernatant was then removed and an equal volume of 3.5% NaCl was added. The suspension

was centrifuged again and the supernatant removed. The pellet was washed twice with complete medium supplemented with 15%serum.

3.8.2 In vitro microtest (Mark III Test)

Drug samples were prepared in picomole (pM) quantities according to WHO *in vitro* micro test procedure (WHO, 1990; Druilhe *et al*., 2001). Blood collected on heparin was washed twice in RPMI 1640 medium (Gibco BRL, Paisley, United Kingdom) and once with complete culture medium (Appendix I). Chloroquine sulphate, amodiaquine, mefloquine, SP and artemisinin were dosed into 96-well microplates. Stock solutions of chloroquine sulphate (600 ng/ml) and the other antimalarial drugs (ng/ml) were prepared in sterile distilled water (chloroquine, amodiaquine, mefloquine and quinine) or ethanol (sulphadoxine-pyrimethamine and artemisinin) and used in twofold dilutions with the culture medium in 96-well culture microplates (Nunc, Denmark) to obtain nine final dilutions (600 to 2.34 ng/ml for chloroquine) and (appropriate dilution factor was determined for the other drugs). Parasitized RBCs was added to each well and to three control wells (without drug) to a final volume of 250µl/well at a 2% hematocrit. Each isolate was tested in duplicate and the plates were incubated at 37°C for 24 – 30 hours. At the end of the assay, the plates were read by preparing thick smear from each well and determining the IC_{50} for each drug using the HN-NonLinn software package.

3.9 Antimalarial Activity Testing of Crude Organic Extracts of Medicinal Plants: *Momordica charantia (Ejirin), Diospyros monbuttensis (Eegun eja)* and *Morinda lucida (Oruwo)*

3.9.1 Preparation of Plant Extract

10g of dried extract was dissolved in 50ml alcohol (95%) for 7 days at room temperature. The alcohol was allowed to evaporate at room temperature. 10ml Dimethyl sulphoxide (DMSO) was added to 10mg of each extract to make 1mg/ml.

3.9.2 *In vitro* Test

The assay was performed in duplicate in a 96-well microtiter plate, according to WHO method [*in vitro* micro test (Mark III)] that is based on assessing the inhibition of schizont

maturation. RPMI 1640 (Gibco BRL, Paisley, United Kingdom) was the culture medium used for cultivation of *P. falciparum* (Flores *et al*., 1997). Dilution was prepared from the crude plant extract and the final concentrations prepared by dilution were (125, 62.5, 31.25, 15.6, 7.8, 3.9 and 1.95µg/ml). Negative controls treated by solvent and positive controls (Chloroquine phosphate) were added to each set of experiments. 50µl from the blood mixture media was added to each well in plate and incubated in CO_2 condition at 37.5°C for 24–30 h. After incubation, contents of the wells were harvested and stained for 30 min in a 2% Giemsa solution pH 7.2, after that the developed schizonts were counted against the total asexual parasite count of 200. The count process was done in duplicate, and the data were analyzed by using HN-NonLin software to estimate IC_{50}

3.10 Molecular studies

Polymerase Chain Reaction/Restriction fragment Length Polymorphism (PCR/RFLP) was used to determine the resistant genes and study the genetic diversity/genetic variation of antimalarial resistant *Plasmodium falciparum.*

3.10.1 DNA extraction

DNA was extracted from patient blood spotted on the filter paper ("pre-culture") and cultured parasites ("post-culture") using the QiaAmp DNA Blood Mini kit Blood and Body Fluid Spin Protocol (Qiagen, Valencia, CA). The protocol for the extraction was carried out according to manufacturer's instruction.

3.10.2 PCR for detection of *Pfcrt* gene

The oligonucleotides primers were designed from published sequences. For amplification of the 1.6-kb fragment of *pfcrt*, the lower primer was 5'-CCGTTAATAATAAATACAGGC-3'.The upper primer was 5'-CTTTTAAAAATGGAAGGGTGT-3', (Dorsey *et al.*, 2001). The primary PCR components, in a final volume of 20µL, was 2.5mM $MgCl_2$, 640µM deoxynucleotide triphosphate (dNTPs), buffer 10x, 10pM of each primer, 1U of Taq polymerase (Ampli Taq Gold; Applied Biosystems, Foster City, CA, USA) and 2µL of DNA samples. 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.

3.10.3 Nested PCR and RFLP for *Pfcrt* mutation-specific detection

Product from primary PCR (2µl of 10x dilution) was used in a follow-up, nested, allele-specific PCR amplifications to detect the codons for *pfcrt* 76K or 76T. These diagnostic PCR amplifications used a common inner primer pair 5'-GGCTCACGTTTAGGTGGA-3' and 5'-TGAATTTCCCTTTTTATTTCCAAA-3' (detects the 76T codon) or 5'-GTTCTTTTAGC AAAAATCT-3' (detects the 76K codon). The PCR stages for these diagnostic amplifications were at 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. Purified genomic DNA from *P. falciparum* clones HB3 (chloroquine sensitive) and Dd2 (chloroquine resistant) were used as positive controls, and water, extracted uninfected blood smears, and uninfected blood spots on filter paper were used as negative controls. The PCR products from the amplification reactions were evaluated by electrophoresis on 2% agarose gels containing ethidium bromide.

10µl of the nested PCR product reaction mixture were treated directly with 3U of the restriction enzyme *Apo* I for 6 to 16 hours at 50°C as recommended by the manufacturer (New England Biolabs, Beverly, MA). The enzyme *Apo* I recognise and cut the 76K codon, releasing fragment from product. It does not cut the product containing the 76T codon found in chloroquine-resistant *P. falciparum*.

3.10.4 PCR and RFLP for detection of *Pfmdr1* gene

Gene segments spanning codon 86 of the *Pfmdr*1 gene were amplified in 20µl of standard PCR mixture containing 5µl of extracted DNA and primers MDR1 5'-ATGGGTAAA GAGCAGAAAGA-3' and MDR2 5'-AACGCAAGTAATACATAAAGTCA-3'. 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 then performed under the same PCR conditions using 1µl of the product solution and primers MDR3 5'-TGGTAACCTCAG-TATCAAAGAA-3' and MDR4 5'-ATAAACCTAAAAAGGAACTGG-3'.

Presence of the mutant 86Y codon was detected by digestion of 8µl of the second amplification product solution with 1.5U of *Afl* III according to the manufacturer's instruction (New England Biolabs). The products of restriction digestion were separated by electrophoresis on a 2% agarose gel and detected by staining with ethidium bromide.

3.10.5 PCR assays for the detection of *Pfdhfr* and *Pfdhps* genes

Pfdhfr, and *Pfdhps* PCRs were performed as described by May and Meyer (2003a) and Marks *et a,l* (2004). For the *Pfdhps* PCR, two primers (primer *Pfdhps*-F 5-ATGATTCTTTT TCAGATG-3_ and primer *Pfdhps*-R 5-CCAATTGTGTGATTTGTCCAC-3 were designed to amplify 747 bp of the region exhibiting mutations relevant to Sulphadoxine resistance. PCR was performed with a volume of 20µl (each primer at 0.2M, dNTPs at 200M, and 1U of Hotstar-*Taq* with the appropriate buffer [Qiagen, Valencia, Calif.] and $MgCl_2$ at a final concentration of 2.0 mM) and approximately 80ng of template genomic human DNA, with parasite DNA concentrations corresponding to the parasite burden in the individual. After an initial denaturation (15 min at 95°C), 31 cycles of 30s at 94°C, 40s at 53°C, and 1min at 72°C were run. Elongation of the amplicons was completed by a final cycle of 10min at 72°C. Subsequently, a nested PCR was performed to increase the yields of the specific amplicons using primers primer *pfdhps*-F1 (5-GTTGAACCTAAACGTGCTG-3) and *pfdhps*-R1 (5-ATTACAACATTTTGATCATTC-3). 3µl of the primary PCR product was used in a reaction volume of 25µl containing 0.2M of each primer, dNTPs at 200M, reaction buffer with $MgCl_2$ at a final concentration of 2.0 mM, and 1U of Hotstar-*Taq*). In the nested PCR, a high initial annealing temperature (AT), which ensures a high level of specificity of initial primer binding, is followed by a gradual decrease in the AT toward the pre-calculated optimal AT. The parameters consisted of an initial denaturation step (15 min at 95°C) and 43 cycles of 30s at 94°C, the AT for 40 s, and 72°C for 1 min, in which the ATs were 65°C (5 cycles), 60°C (5 cycles), 56°C (7 cycles), 54°C (13 cycles), and 53°C (13 cycles). Fragment elongation was performed by use of a cycle of 10 min at 72°C. The amplicons were monitored for quality and the expected size on 1% ethidium bromide-stained agarose gels. Statistical analysis (_χ^2 tests) was performed by the use of STATA software (version 8.2; Corp., College Station, Tex.).

3.10.6 PCR and RPLP assay for (SERCA) *PfATPase*6

P. falciparum positive samples were amplified by PCR using *PfATPase6*-specific primary and nested primer pairs. DNA extract for each sample was subjected to nested PCR amplification with primers flanking nucleotide codon 2307of the *PfATPase6* gene. Both the primary and secondary reactions comprised 2µl template, 0.25µM primer, 1.5mM $MgCl_2$, 200µM dNTP's, 1x PCR buffer and 1U Taq DNA polymerase, in 25µl reactions. Amplification cycles for both primary and secondary reactions consisted of an initial denaturation at 94^0C for 2 minutes, followed by 25 cycles of denaturation at 94^0C for 45 seconds, annealing at 46^0C for 45 seconds and extension at 65^0C for 1 minute, final extension was run at 65^0C for 2 minutes. For primary amplification, the primers are 2307FW 5'-TGA GCA TGG CAC AAG TIT 3'; 2307RV- 5'TCA ATA ATA CCT AAT CCA CCT AAA TA-3'.For nested PCR the primers are FW-EN 5'-TGA GCA TGG TAG AAG TTT T-3' and RV-EN 5'- TCA TCT GTA TTC TTA ATA ATA TTT AAA TCT GTA CTA-3' (Zhang *et al*., 2008).

Internal primers for the nested PCR amplification (2307FW-EN and 2307RV-EN, were engineered to create Csp6 I restriction sites. One site, at *PfATPase6* nucleotide codon 1916, serves as the internal control for the restriction digestion assay, which is always cut by the enzyme. Csp6 I digestion assay comprised 4µl of secondary PCR amplicon (432bp), 1x buffer and 1.5U of Csp6 I restriction enzyme, in 30µl reactions. Digestion assays were incubated for 12 hours at 37 ^{0}C. PCR amplicon and restriction digests were analyzed by electrophoresis on a 2% ethidium bromide-stained agarose gel and visualized under UV transillumination. Restriction digests were loaded in 15 µl volumes per lane while undigested product was run in 5µ loading volumes. Band sizes were measured using Syngene gel imaging analysis software.

3.10.7 Molecular Genotyping of isolates using MSP1&2 and Glurp

Primers and PCR protocols were followed as previously described by Snounou *et al.* (1999) for family specific allele analysis of *msp-1* (block 2) and *msp-2* (block 3). For PCR amplification, the primers for each of the families in *msp-1 and msp-2* are as shown in Table 3.1. and described by Snounou *et al*., 2007. PCR condition consisted of an initial denaturation step (5 min at 95°C) and 35 cycles of 30s at 95°C, 72°C for 30 s, and 72°C for 5 min and a final elongation at 72°C for 5min.

Polymerase Chain Reaction (PCR) amplification was performed on thermal cycler (Perkin Elmer 9700/2400, UK) in a final volume of 20µl. The PCR products were visualized by UV transillumination at 302nm on gel documentation system (Syngenta, USA) after electrophoresis on 2% agarose gel (Promega/Boehringer) using 0.5 × TBE buffer at 80–100 volts. Allele sizes were calculated using Genetool programme. To understand the identity of Nigerian isolates with respect to isolates of other regions, sequence data available in public domains were downloaded for allelic families of *msp-1&2* and details are given below; Thailand(K1-M77730, MAD20-M77722, R033-AAA29684, 3D7- U91676), Vietnam(K1-AF509651, MAD20-AF509653&94, FC27-AF104696, 3D7-AF104693), Tanzania(K1-AF061134, FC27-AY532386), Brazil(K1-AF509714, MAD20-AY714585, FC27-DQ115973, 3D7-AF177389), China(MAD20-AF251345), Sudan(MAD20-AF034635), Iran(MAD20-AY138509, R033-AY138507, FC27-DQ338451), Indonesia(K1-AF191061, R033-AAF18431), Western Africa (R033-PFAMSA1), Kenya(R033-AAM21583), Ghana(FC27-AF329577), PNG(FC27-AF329579), Gambia(FC27-U91668, 3D7- U91665) and Nigeria(3D7-AF148224) (Joshi *et al*., 2007).

Table 3.1 PCR Primers for MSP1, MSP2 and Glutamate rich protein

Primer Name	Sequence 5'-3'
Glurp GOF	TGAATTGAAGATGTTCACACTGGAAC
Glurp GOR	GTGGAATTGCTTTTTCTTCAACACTAA
Glurp GNF	TGTTCACACTGAACAATTAGATTTAGATCA
Msp 1 F	CTAGAAGCTTTAGAAGATGCAGTATTG
Msp 1 R	CTTAAATAGTATTCTAATTCAAGTGGATCA
Msp 1F M	AAATGAAGGAACAAGTGGAACAGCTGTTAC
Msp 1R M	ATCTGAAGGATTTGTACGTCTTGAATT ACC
Msp 1F K	AAATGAAGAAGAAATTACTACAAAAGGTGC
Msp 1R K	GCTTGCATCAGCTGGAGGGCTTGCACCAGA
Msp 1F R	TAAAGGATGGAGCAAATACTCAAGTTGTTG
Msp 1R R	CATCTGAAGGATTTGCAGCACCTGGAGATC
Msp 2 F	ATGAAGGTAATTAAAACATTG TCTATTATA
Msp 2 R	CTTTGTTACCATCGGTACATTCTT
Msp 2F Fc	AATACTAAGAGTGTAGGTGCARATGCTCCA
Msp 2R Fc	TTTTATTTGGTGCATTGCCAGAACTTGAAC
Msp 2F D	AGAAGTATGGCAGAAAGTAAKCCTYCTACT
Msp 2R D	GATTGTAATTCGGGGGATTCAGTTTGTTCG

Statistical Analysis.

Statistical analysis was performed using Stat View 5.0.1 (SAS Institute Inc., Cary, NC). Statistical significance was based on a α level of 0.05. The data/statistical analysis for significance were done using Microsoft excel, SPSS and HN-NonLin analysis methods. One-way analysis of variances was used to compare the geometric mean parasites density in patients from different age groups. The Student's *t*-test was used to compare mean number of alleles of *P. falciparum* MSP-1, MSP-2, and GLURP. *P* values < 0.05 was considered significant for all statistical analysis. Data from all the questionnaires were coded, entered and analysed using Epi Info 6.04 and SPSS software.

3.11 Questionnaire Administration

Structured Questionnaires were administered to patients, parents of infants and older children. The questionnaire seeks to identify the locality, age and sex of the respondents. In addition, the patients' attitude to use of antimalarial drugs and Insecticide treated mosquito nets or other means of controlling man-mosquito contact as practiced in the locality and malaria management practices by the study population were identified. A cross sectional study was conducted in the four zones of Ogun State, Southwestern Nigeria.

CHAPTER FOUR

RESULTS

4.1. Incidence of Malaria in Ogun State, Southwestern Nigeria.

4.1.1Patients Characteristics

A total of 4066 subjects comprising of 1839 males and 2227 females presenting with malaria in four different senatorial districts of Ogun state were recruited into the study. The total number of subjects recruited in Sango-ota, Abekuta, Ijebu-ode and Sagamu were 1120, 1116, 995 and 835 respectively. The mean age was 19 years (>1 –70) with 93% less than 25 years.

4.1.2 Incidence of Malaria

The overall incidence of *falciparum* malaria as determined by microscopy in the study area was 62.7% (2250/4066). The highest incidence was observed in Sango (75.7%) while the lowest (48.4%) was observed in Abeokuta (Table 4.2). Table 4.1 shows the incidence of *P. falciparum* infection in Ogun State, Nigeria according to age and sex. Age group 1-5yrs had the highest incidence of infection (70.8) followed by age group <1year (63.9%) while age group 6-15years had the lowest incidence (52.8). The difference according to age was statistically significant ($p<0.0001$). The highest *falciparum* malaria mean parasitaemia of 1080p/µl was recorded among the age group 1-5years while age group >40years recorded the lowest mean parasitaemia of 800p/µl. Females had a higher incidence of malaria infection of 51.8% compared to males 48.2% in this study. The difference in the incidence of infection by sex was significant ($p<0.0001$).

4.2. *In Vitro* Drug sensitivity Tests

In-Vitro antimalarial drugs and local herbal extracts sensitivity tests were carried out and the IC_{50} and IC_{99} for each drug was determined. Sample of HN-NonLinn software statistical package used for the data analysis is shown in Figure 4.4. Table 4.3 shows the mean IC_{50} and IC_{99} values for chloroquine, amodiaquine, mefloquine, quinine, sulphadoxine/pyrimethamine and artesunate. The IC_{50} value is defined as the concentration of an antimalarial drug that inhibits 50 % of schizont maturation as compared with the development in drug-free control wells. IC_{99} gives a result that closely approximates a reliable Minimum Inhibitory

Concentration (MIC). The MIC is generally defined as the lowest drug concentration that inhibits the development of rings to schizonts. Moreover, the *in vitro* threshold values for antimalarials have been defined statistically as >2 SD above the mean (Pradines *et al*., 1998). IC_{50} implies that 50% of the parasite could not mature to schizont stage at that drug concentration while IC_{99} implies that the antimalarial drugs inhibited 99% of the parasites from maturing to schizont stage at that concentration.

All the isolates tested were sensitive to quinine, mefloquine and artesunate while *in vitro* resistance was observed to chloroquine, amodiaquine and SP (Table 4.4). Highest percentage of resistance to chloroquine (69.8%) was recorded among isolates from Yewa zone while highest percentage resistance to amodiaquine (30%) was observed in Ijebu zone. Highest resistance against SP (10%) was recorded in Yewa and Egba zones. A significant positive correlation was observed between the responses to artemisinin and mefloquine (P=0.001), artemisinin and quinine (P=0.05), quinine and mefloquine (P= 0.01), (Table 4.4). A significant negative correlation was observed between the responses to chloroquine and mefloquine (P=0.05).

4.3 Prevalence of drug resistant molecular markers

Prevalence of established antimalarial drug resistant markers was assayed for in 100 isolates collected from the study area. The molecular markers assayed for in the study were *Pfcrt, Pfmdr1, PfDhfr*, *PfDhps* and *PfATpase6.*Table 4.5 shows the overall and zonewise prevalence of the molecular markers to various antimalarial drugs in *P. falciparum* isolates from Ogun state, Nigeria. Sixty percent of the isolates had *Pfmdr1* (Y86) resistance gene while 48% had *Pfcrt* (T76) chloroquine resistance gene. None of the isolate had the PfATpase artemisinin resistant gene. Most of the isolates that showed *in-vitro* resistance to the drugs also harbored the genes coding for resistance to the drugs. Plates 4.1 – 4.5 show gel electrophoresis results for the detection of *Pfcrt, pfmdr1,dhfr, dhps andATPase6* genes/mutation respectively.

4.4 *In vitro* antimalarial activity of herbal extracts

The results of *in vitro* antimalarial activity of the three herbal extracts tested for antimalarial activity are as shown in Table 4.6. Of the selected three herbal extracts, the highest activity

was obtained with extract of *Diospyros monbuttensis* (IC_{50} = 32µg/ml) while the lowest was obtained from *Morinda lucida* (IC_{50} =250 µg/ml).

4.5 Genetic Diversity of *P. falciparum*

As an estimate of multiclonality of infection, the minimal number of *P. falciparum* strains in each individual was determined by assessing the number of alleles of the genes encoding MSP-1 and MSP-2 families. The MSP-1 and MSP-2 alleles were defined by gene family-specific PCR assays and analysis of the resulting length polymorphisms of the PCR products. One hundred *P. falciparum* isolates analyzed during the study demonstrated the highly diverse nature of the field isolates in respect of *msp-1* (block 2) and *msp-2* (central repeat region, block3). All the three reported families of *MSP-1*(K1, MAD20 and RO33) families and two MSP-*2* (FC27 and 3D7) families were observed among the isolates (Plates 4.6 - 4.10).

In MSP-1, Proportion of isolates with K1 family was 68% with 4 alleles in the range of 100 to 300 base pairs (bp). Proportion of isolates with MAD20 family was 40% and a total of 3 alleles were observed within 100 to 300 bp. RO33 proportion was 20% and the family was observed to be monomorphic with an allele size of 200 bp. Observed proportions, numbers and size range of alleles among the isolates are given in Table 4.7.

In MSP-*2*, the reported families FC27 and 3D7 were observed among the isolates (Table 4.7). Proportion of FC27 family was 76% and that of 3D7 was 56%. Proportional prevalence of FC27 and 3D7 families was significantly different ($\chi 2$ = 16.5, P=0.002). Three alleles of FC27 in the range of 300–500 bp and 3 alleles of 3D7 in the range of 450–600 bp were observed in the study. Proportion of multiclonal isolates (multiple infection) is given in Table 4.7 and multiplicity of infection, MOI was estimated by dividing the total number of fragments detected in the individual system by the number of samples positive in the particular system (either MSP-1 or MSP-2) and 80% of the isolates harbour the genes that code for glutamate rich protein with size ranging between 700 and 900.

The zonewise genetic diversity of *P. falciparum* from Ogun State is as shown on Table 4.8. A high polymorphism was recorded in all the zones for *MSP*-1 (block 2) and *MSP*-2 (central repeat region, block 3). Highest genetic polymorphism was recorded among the field isolates from Yewa zone.

4.6 Knowledge and practice on the use of antimalarial drugs

Questionnaires were administered to respondents resident in the study sites who were either malaria patients or parents/guardians of malaria infected children. The age of respondents ranged between 18 and 75 (Table 4.9). A total of 946 questionnaires were administered to assess the malaria related knowledge on issues regarding the use of antimalarial drugs and malaria management practices. Most of the respondents were students (52.2%). The responses of the participants are summarized in table 4.10. About 32.1% of participants have malaria attack at least once in 3 months while only 26.3% normally complete their antimalarial drugs. The malaria management practices showed that 24.6% attend hospitals, 12.0% use local healers while 25.0% buy antimalarial drugs without prescription by a physician. It was also found that some use more than one method in their management of malaria. Those who combined antimalarial drugs with traditional medicine from local healers were found to be 17.4%, while 1.2% reported doing nothing about malaria. Table 4.10 also shows the methods used in preventing mosquito bites by respondents. Only 18% of the sample population used Insecticide treated mosquito nets. Majority of the people (42.3%) used only window and door nets; 24% of the population used insecticides while 13% do not prevent mosquito bite at all.

Table 4.1. Incidence of *P. falcifarum* infection in Ogun State.

AGE	*No of Samples*			*No of Positive Cases*			*Mean*	
(Yrs)	**Male**	**Female**	**Total**	**Male**	**Female**	**Total**	***Parasitaemia***	**p.-value**
				(%)	**(%)**	**(%)**	***p/µl***	
< 1	**52**	**56**	**108**	**33**	**36**	**69**	**900**	**<0.0001**
				(47.8)	**(52.2)**	**(63.9)**		**(χ^2=46.863**
1-5	**300**	**324**	**624**	**193**	**249**	**442**	**1080**	
				(43.7)	**(56.3)**	**(70.8)**		
6-15	**330**	**412**	**742**	**195**	**197**	**392**	**890**	
				(47.4)	**(50.3)**	**(52.8)**		
16-25	**901**	**965**	**1866**	**639**	**500**	**1139**	**850**	
				(60.8)	**(44.0)**	**(61.0)**		
26-40	**208**	**221**	**429**	**110**	**155**	**265**	**990**	
				(41.5)	**(58.5)**	**(61.8)**		
> 40	**100**	**305**	**405**	**60**	**183**	**243**	**800**	
				(24.7)	**(75.3)**	**(60.0)**		
Total	**1839**	**2227**	**4066**	**1230**	**1320**	**2550**	**918.3**	
(%)	**(45.2)**	**(54.8)**	**(100)**	**(48.2)**	**(51.8)**	**(62.7)**		
p- value				**<0.0001 (χ^2=24.632)**				

Table 4.2: Zonewise Incidence of Malaria in Ogun State

Zone	No of Samples collected			No of Positive Samples			Mean parasitaemia
	Male	Female	Total	Male	Female	Total	
Ijebu Ode (Ijebu)	455	540	995	315	348	663 (66.6)	920
Sango Ota (Yewa)	536	584	1120	412	436	848 (75.7)	914
Abeokuta (Egba)	502	614	1116	251	289	540 (48.4)	860
Sagamu (Remo)	346	489	835	252	247	499 (59.8)	980
Total (%)	1839 (45.2)	2227 (54.8)	4066 (100)	1230 (48.2)	1320 (51.8)	2550 (62.7)	918.3

Table 4.3: *In vitro* susceptibility of *P. falciparum* isolates to Antimalarial Drugs

Drug	IC_{50} Mean (nM/L)	IC_{99} Mean (nM/L)	Resistance Threshold (nM/L)
Chloroquine (CQ)	24.4	164.2	> 160
Amodiaquine (AQ)	6.3	32.4	> 80
Artesunate (AS)	3.2	7.8	> 10.5
Mefloquine (MQ)	42.1	60.8	> 64.0
Sulphadoxine – Pyrimethamine	55.0	200	>300
	0.7	2.5	>4.0
Quinine	60.3	298.6	>300

Table 4.4: Zonewise resistance pattern of *P. falciparum* to antimalarial drugs

Origin	No. Isolates	CQ res	AQ res	AS res	MQ res	SP res (%)	Q res

	Cultured	(%)	(%)	(%)	(%)		(%)
Ijebu-Ode (Ijebu)	**20**	**9 (45)**	**6 (30)**	**0 (0)**	**0 (0)**	**1 (5)**	**0 (0)**
Sango Ota (Yewa)	**43**	**30 (69.8)**	**4 (9.3)**	**0 (0)**	**0 (0)**	**2 (10)**	**0 (0)**
Abeokuta (Egba)	**25**	**5 (20)**	**1 (4)**	**0 (0)**	**0 (0)**	**2 (10)**	**0 (0)**
Sagamu (Remo)	**12**	**7 (58.3)**	**2 (16.7)**	**0 (0)**	**0 (0)**	**0 (0)**	**0 (0)**
Total	**100**	**51 (51)**	**13 (13)**	**0 (0)**	**0 (0)**	**5 (5)**	**0 (0)**

CQ-Chloroquine; AQ-Amodiaquine; AS-Artesunate; MQ-Mefloquine; SP-Sulphadoxine/Pyrimethamine; Q-Quinine; res-resistance

Table 4.5: Zonewise Prevalence of molecular markers of resistance to antimalarial drugs in *P. falciparum* from Ogun State, Southwestern Nigeria.

Zone	No. of	Prevalence of resistance genes

	Isolates	*pfcrt*K76T (%)	*Pfmdr*1Y86N (%)	*Pfdhfr*S108N (%)	*pfdhps*K540E (%)	*pfATPase*S769N (%)
Ijebu	**20**	**9 (45)**	**10 (50)**	**3 (15)**	**0 (0)**	**0 (0)**
Yewa	**43**	**28 (65.1)**	**30(69.8)**	**4 (9.3)**	**1 (2.3)**	**0 (0)**
Egba	**25**	**5 (20.0)**	**12 (48)**	**4 (16)**	**1 (4)**	**0 (0)**
Remo	**12**	**6 (50.0)**	**8 (66.7)**	**1 (8.3)**	**0 (0)**	**0 (0)**
Total	**100**	**48 (48)**	**60 (60)**	**12 (12.0)**	**2 (2)**	**0 (0)**

Table 4.6 *In vitro* susceptibility of *P. falciparum* isolates to Local Antimalarial Herbs

Herbal Drug

Momordica charantia (Ejirin)	**125.0**
Morinda lucida (Oruwo)	**250.0**
Diospyros monbuttensis (Eegun eja)	**32.0**

Table 4.7: Genetic diversity of *Plasmodium falciparum* isolates from Ogun State, Southwestern Nigeria

Families	***No. positive by PCR (%)***	***No. of distinct alleles***	***Sizes of alleles (bp)***	***MOI***

MSP-1				
K1		4	100-300	
68(68)		3	100-300	**1.1**
MAD20	**40(40)**	1	200	
RO33				
20(20)				
		3	450-600	**1.2**
MSP-2		3	300-500	
3D7				
76(76)				
FC27				
56(56)				
GLURP		**5**	**700-900**	**1.3**
80 (80)				

* bp _ basepairs; MSP _ merozoite surface protein; GLURP _ glutamate-rich protein.

MOI Multiplicity of Infection

Table 4.8: Zonewise Genetic Diversity of *P. falciparum* from Ogun State, Southwestern Nigeria

Zone	No of Isolates	MSP 1			MSP 2		*Glurp* (%)
		K1 (%)	MAD20 (%)	RO33 (%)	3D7 (%)	FC27 (%)	
Ijebu	20	14 (20.6)	9 (22.5)	6 (30)	16 (21.1)	13 (23.2)	16 (20)
Yewa	43	31 (45.6)	21 (52.5)	8 (40)	38 (50)	18 (32.1)	33 (41.3)
Egba	25	15 (22.1)	7 (17.5)	4 (20)	12 (15.8)	18 (32.1)	21 (26.3)
Remo	12	8 (11.8)	3 (7.5)	2 (10)	10 (13.2)	7 (12.5)	10 (12.5)
Total	100	68 (100)	40 (100)	20 (100)	76 (100)	56 (100)	80 (100)

Legend: MSP 1- Merozoite surface protein 1; MSP 2- Merozoite surface protein 2 *Glurp*- Glutamate rich protein.

Prediposing Factors to Antimalarial Drug resistance

Table 4.9: Age Range of respondents

Age range (Years)	Frequency	(%)
18-25	527	55.7
26-35	231	24.4
36-45	126	13.3
>45	62	6.6
Total	946	100

Table 4.10: Knowledge on prevention and control of malaria among respondents

Variable	N = 946	%
Episode of Malaria Infection		
Once in a month	312	33
Once in three months	304	32.1
Twice in a year	144	15.2
Once in a year	141	14.9
Others	45	4.8
Reasons for stopping drug usage		
When I feel okay/cured	340	35.9
Price of drugs	236	24.9
When I complete dosage	249	26.3
Anytime I like	121	12.8

Antimalarial used as prophylaxis		
Quinolines	385	40.7
Sulphonamides	316	33.5
Artesunate	74	7.9
Artemisinin Combination Therapies	30	3.2
Local herbs	142	15.1
Others	2	0.3
Mosquito bite Preventive methods used		
Insecticide treated Bed nets	170	18
Insecticides	227	24
Mosquito repellant cream	26	2.7
Window nets	400	42.3
None	123	13

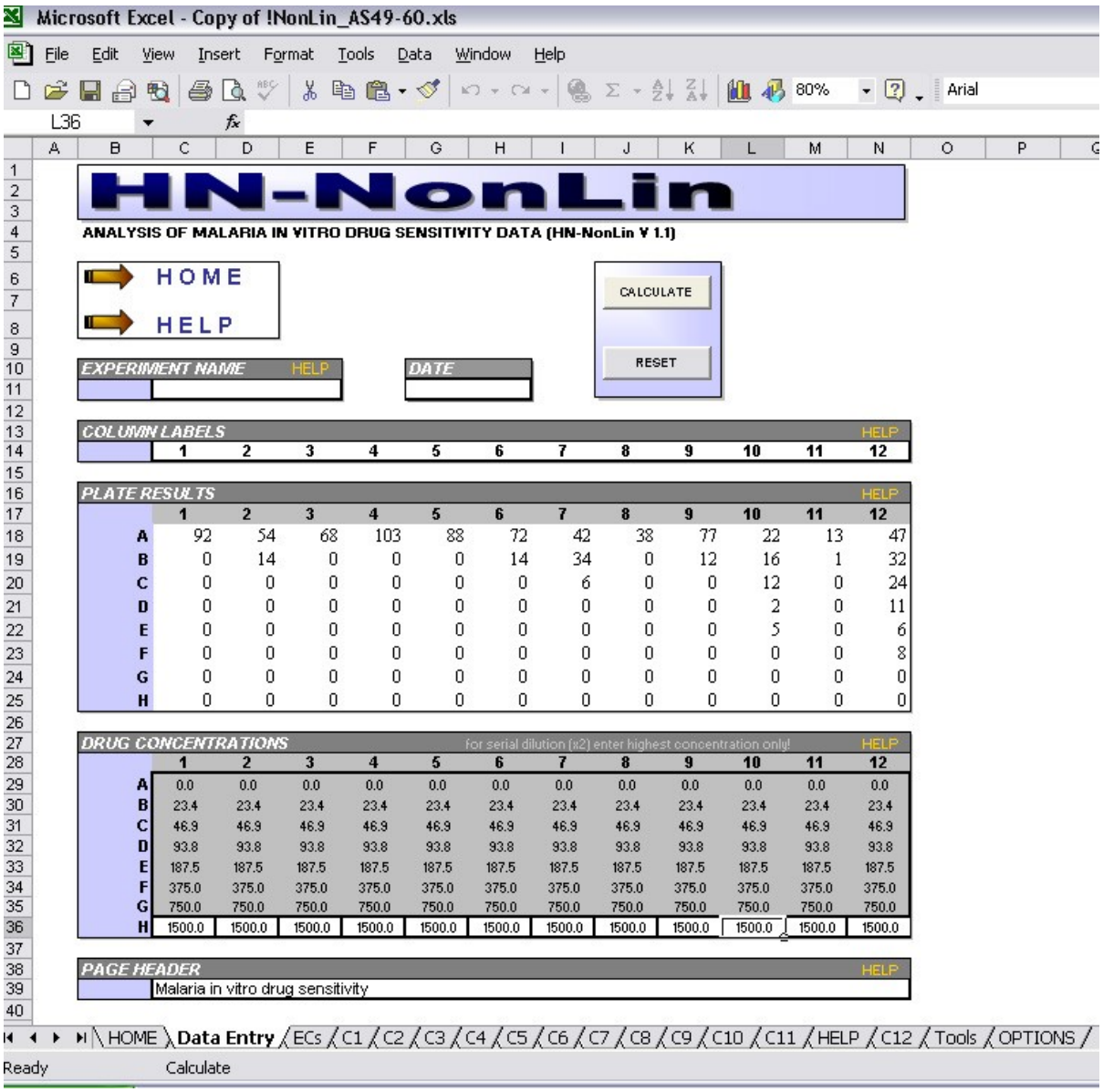

Figure 4.1. Sample of HN-NonLinn Software Statistical Package for the analysis of *in-vitro* drug sensitivity data for *Plasmodium* species

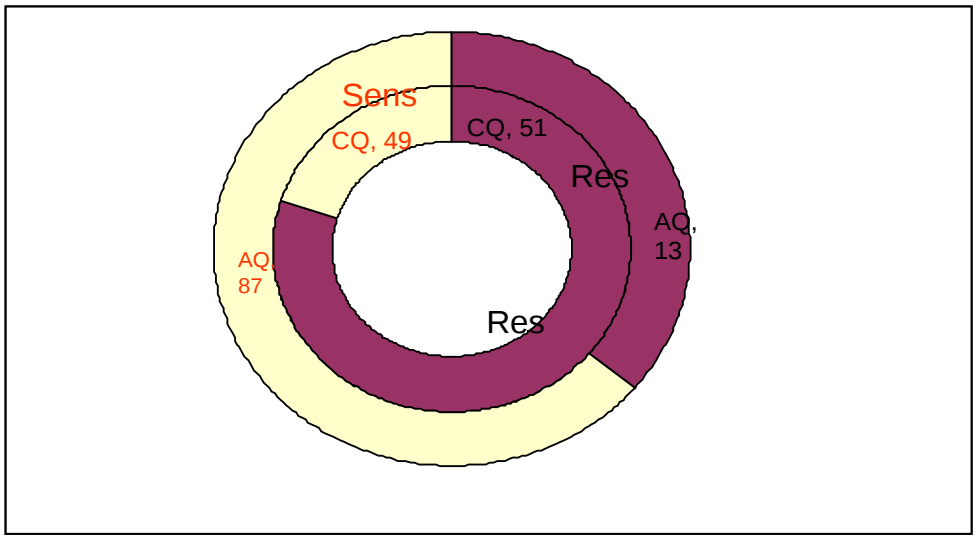

Fig 4.2 Cross Resistance between Chloroquine and Amodiaquine, n=64. The figure shows a cross resistance between chloroquine and amodiaquine. All the *P. falciparum* isolates that showed resistance to amodiaquine were resistant to chloroquine.

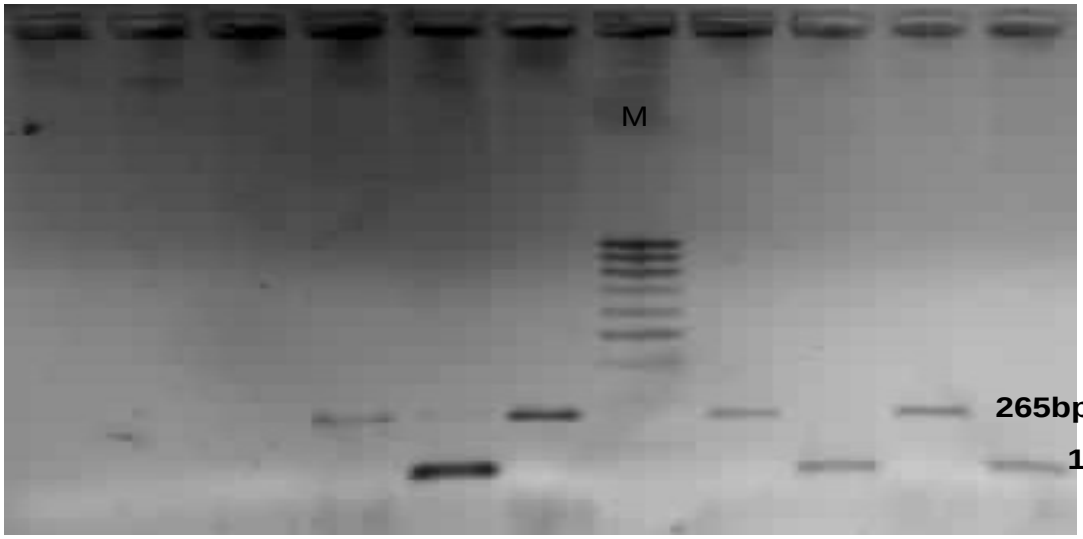

Plate 4.1: DNA bands of wild type and mutated *P. falciparum* chloroquine resistance genes

Plate 4.1: Gel Electrophoresis showing the DNA bands of wild type and mutated *Pfcrt* (K76T) gene after digestion with APO I enzyme. The undigested DNA fragments (265 bp) are the resistance genes while the digested DNA fragments with 132 bp are the sensitive strains as shown on plates.

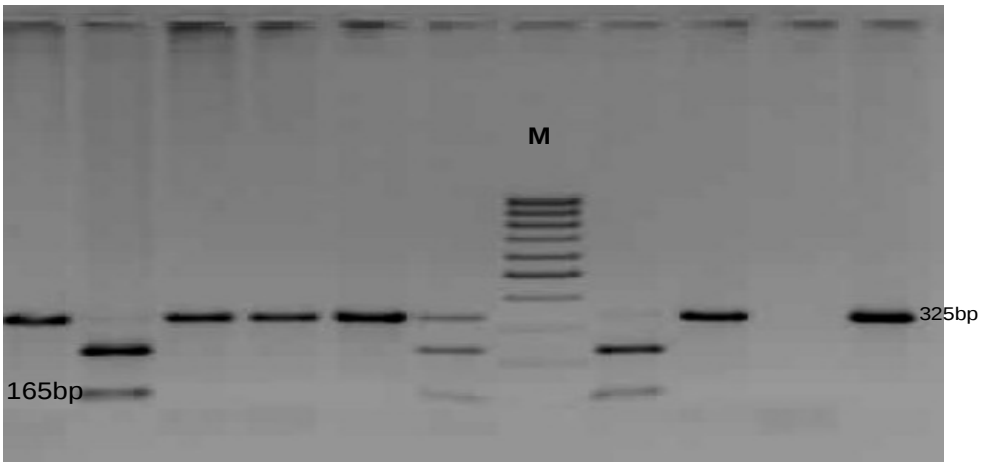

Plate 4.2: *P. falciparum* Multidrug Resistance Genes showing the wild type and mutated genes

Plate 4.2 shows the *Pfmdr1* (Y86N) genes on gel electrophoresis after digestion with *Afl III* enzyme. The isolates with DNA fragments of size 325bp are the wild type i.e. sensitive strains while the mutated strains were cut by the restriction enzyme to 165bp. M respresent the molecular size marker

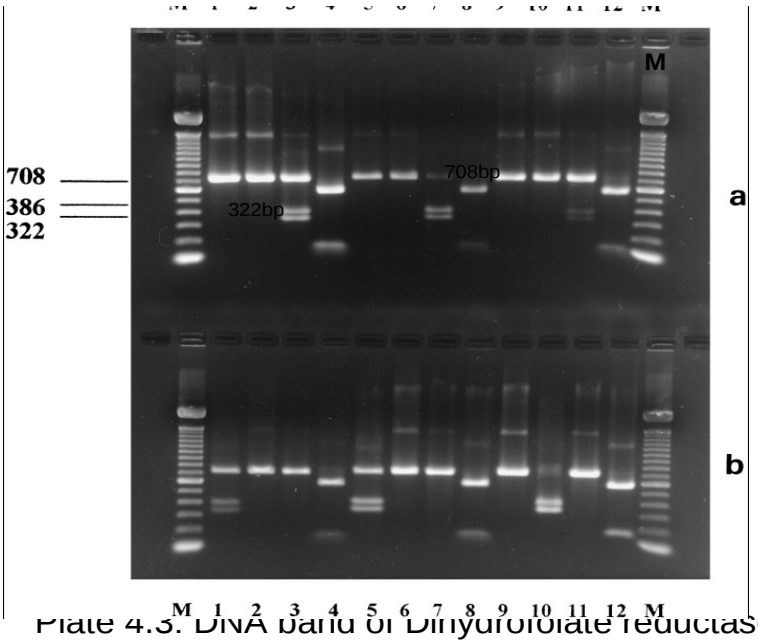

Plate 4.3: DNA band of Dihydrofolate reductase gene (DHFR 108)

Plate 4.3 shows the wild type and mutated genes of DHFR 108 for Sulphadoxine resistance. The wild type gene has a band size of 708bp while the mutated gene has a band size of 322bp.

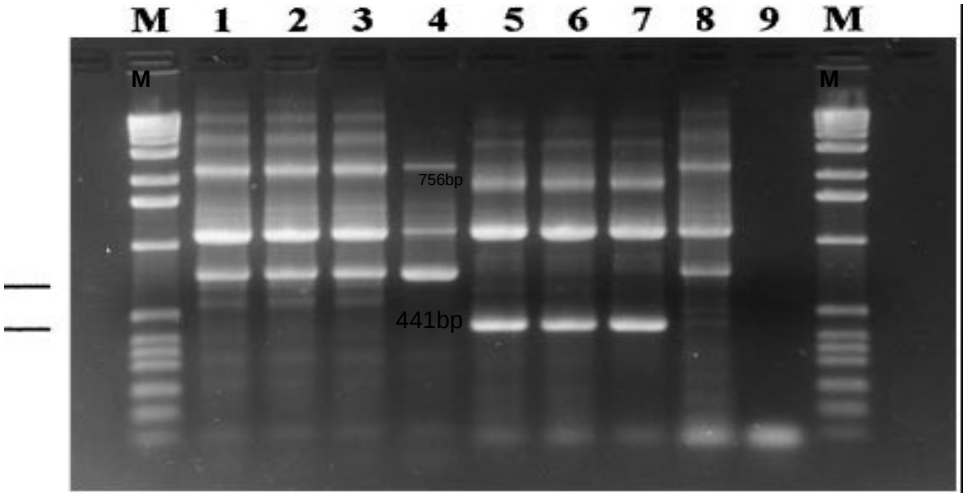

Plate 4.4: DNA band of Di hydropteroate synthase gene (DHPS 540)

Plate 4.4 shows the DNA band of wild type and mutated genes of Dihydropteroate synthase (DHPS 540). The band size of wild type is 756bp while that of the mutated gene is 441bp

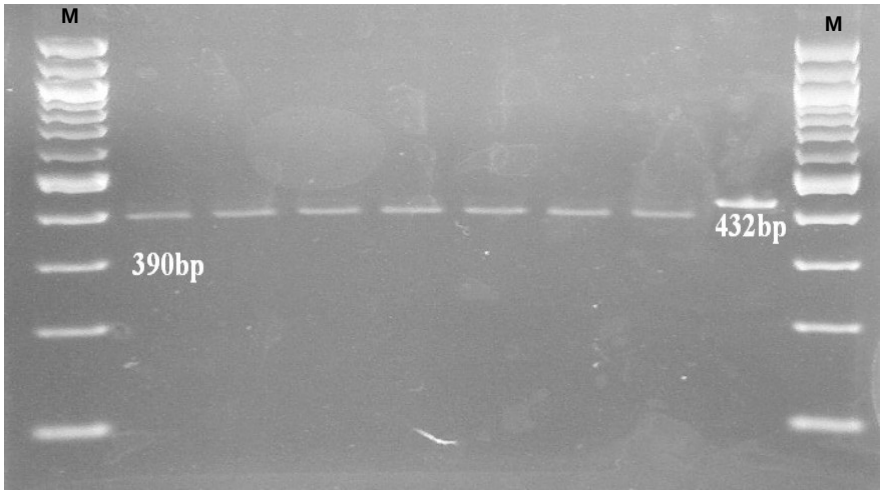

Plate 4.5: DNA band of wild type Pf ATPase6

The band size of the genes coding for pfATPase6 of between 430bp and 432bp is as shown on plate 4.5. There was no digestion which means that none of the isolates harbour resistance genes coding for artemisinin resistance.

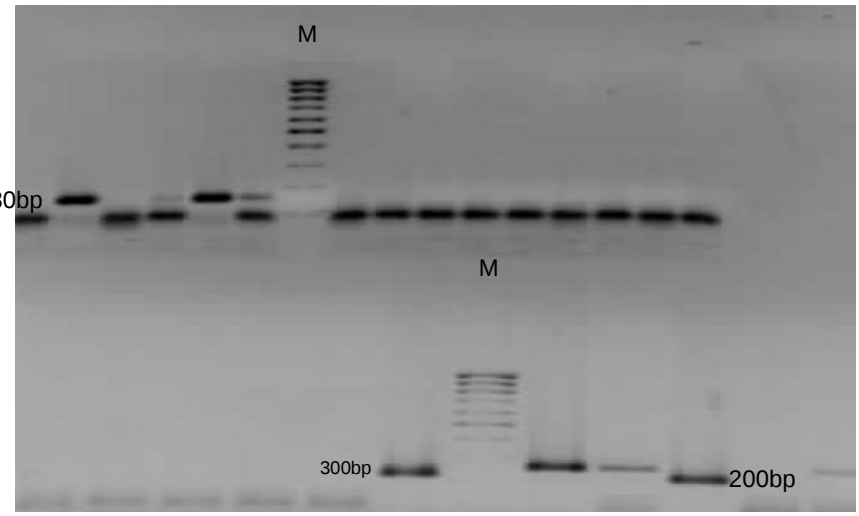

Plate 4.6: DNA bands of *P. falciparum* MSP1 MAD20 on Gel

Legend: M-marker; bp-base pair; 180bp, 200bp 300bp - different alleles for MSP1 MAD family

The DNA bands of genes coding for *P. falciparum* Merozoite Surface Proteins 1 (MSP1) MAD and K and RO33 are as shown on Plates 4.6, 4.7 and 4.8 respectively. The band size for MAD is between 180 and 300 basepairs (bp) while that of K is 100-300bp. For RO33 the band size in the range of 200 bp. M is the molecular size marker

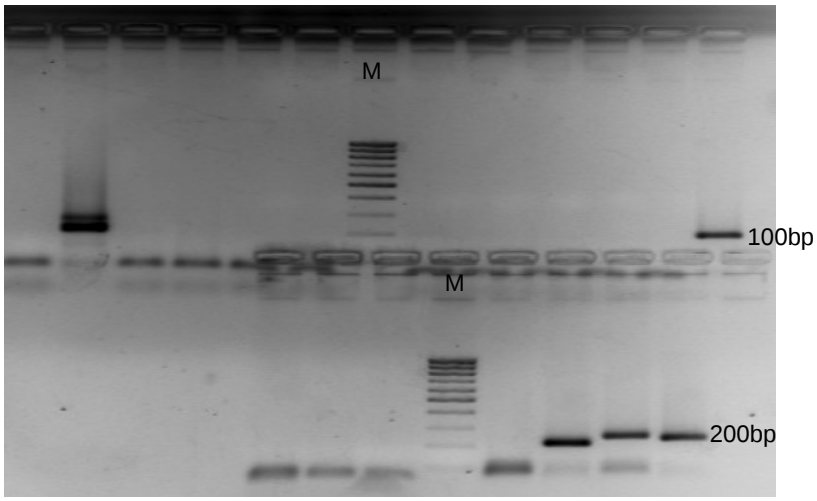

Plate 4.7: DNA bands of *P. falciparum* MSP1 K1 on Gel

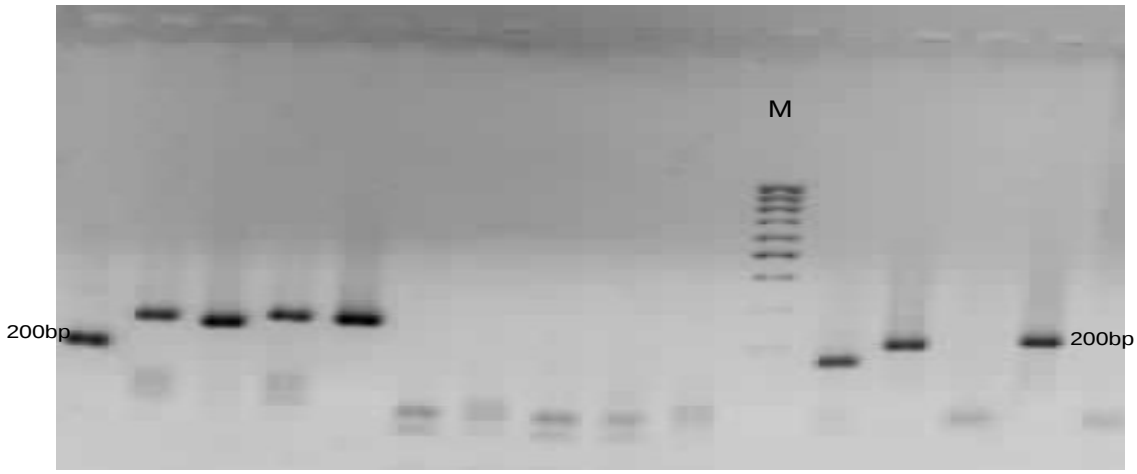

Plate 4.8: DNA bands of *P. falciparum* MSP1 RO33 on Gel

Legend: M-Molecular marker; bp-basepairs; 200bp-allelic size

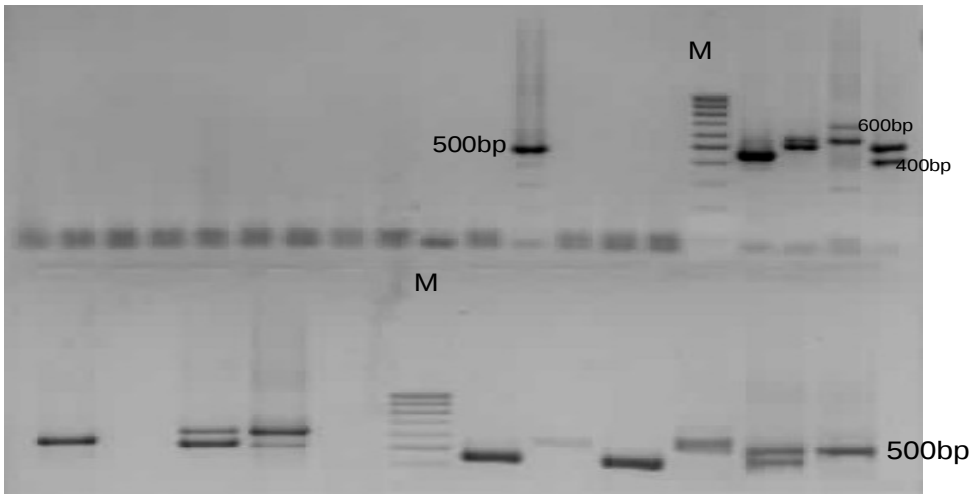

Plate 4.9: DNA bands of *P. falciparum* MSP2 3D7 on gel

Legend: M-molecular marker; bp-basepairs; 400-600-allelic sizes

The DNA bands of genes coding for *P. falciparum* Merozoite Surface Proteins 2 (MSP-2) 3D7 and FC27 are as shown on Plates 4.9 and 4.10 respectively. The band size for 3D7 is between 400 and 600bp while that of FC27 is 400-500bp. M is the molecular size marker.

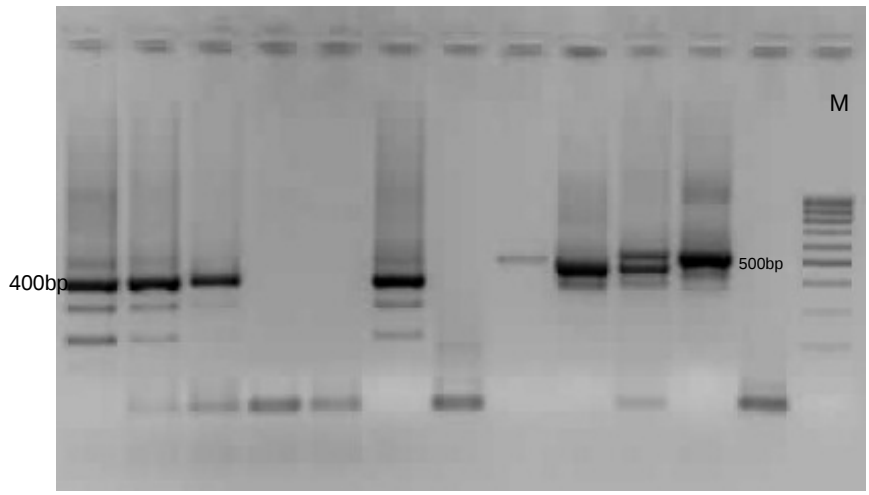

Plate 4.10: DNA bands of *P. falciparum* Merozoite Surface Protein 2 FC27 on gel

Legend: M-Marker; 400bp, 500bp- allelic sizes

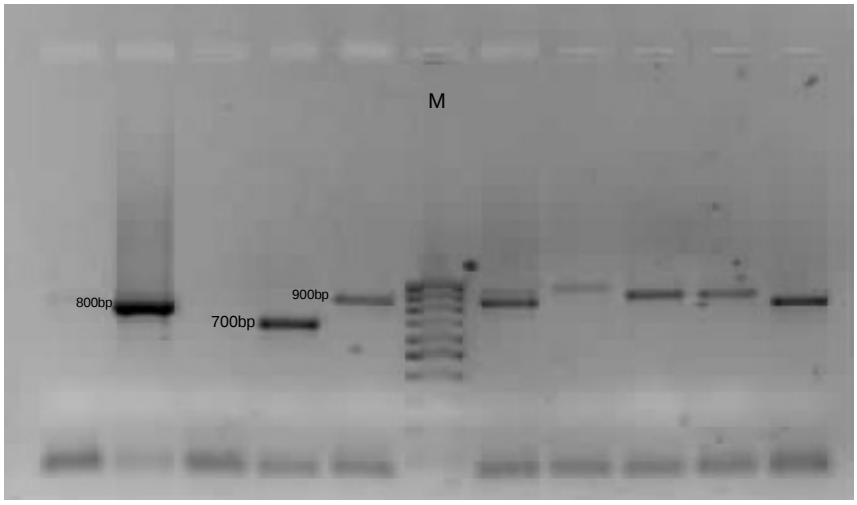

Plate 4.11: DNA band of ***P. falciparum*** Glutarmate rich protein

Legend: M-molecular marker; bp-basepairs; 700-900bp-allelic sizes

The DNA bands of genes coding for *P. falciparum* Glutamate Proteins are as shown on Plate 4.11. The band size is between 700 and 900 basepairs (bp).is 400-500bp. M is the molecular size marker

CHAPTER FIVE

DISCUSSION

Plasmodium falciparum which causes the most serious type of malaria especially in sub Saharan Africa was studied in this research work among the people of Ogun State Nigeria. Malaria is the most prevalent tropical disease in the world today and in sub-Saharan Africa, it is ranked among the most frequent causes of morbidity and mortality especially among children and is often the leading identifiable cause (Greenwood *et al*., 2005). Four thousand and sixty six (4066) subjects were included in the study on the incidence of *P. falciparum* infection in Ogun state, South Western Nigeria. Two thousand, five hundred and fifty (62.7%) were positive for *falciparum* malaria with an average parasitaemia of 918. This result is in agreement with earlier observations that Nigeria is known for high prevalence of malaria and it is a leading cause of morbidity and mortality in the country (Sowunmi *et al*., 2004; Ademowo *et al*., 2006). The high incidence rate of *falciparum* malaria in the selected study site is similar to the results of earlier studies in other parts of the country and other neighboring countries. Adefioye *et al*. (2007) and Marielle *et al*. (2003) recorded a prevalence rate of 72% and 70% among pregnant women in Oyo state, Nigeria and Gabon respectively. A prevalence rate of 76% was also reported by Aribodor *et al*. (2003) in Anambra State, South East Nigeria.

The result of this study was differs from that of Uko *et al.* (1998) who recorded a low prevalence rate of (6.8%). This may be due to the fact that the study was carried out during dry season alone when infection rate was low. Chanda *et al*., (2009) reported a very low incidence of 0.7% among children less than five years attending a local health facility in Zambia. In this study, 63.9% of children below one year and 70.8% of the children 1-5 years were positive for *falciparum* malaria and highest parasitaemia was also observed in this age group. This was followed by age range 16-25years with a prevalence of 52.8%. This findings was in agreement with those of Aribodor *et al*. (2003) who also observed a high prevalence rate among age group 10-19 years followed by age group 20-29 years old. In highly endemic malarious area where semi-immune adults usually have substantially acquired resistance to local strains of plasmodia, the prevalence of clinical malaria is higher and its severity greater in pregnant women, children and young adults. The high prevalence rate in the study area

could result to cerebral malaria in children, maternal anaemia and low birth weight and death as reported by other workers (Mockenhaupt *et al*., 2000; WHO, 2003). This is probably because they are more exposed to malaria parasite due to bad environmental conditions and their life styles

In-vitro method was used in this study to determine the quantitative drug response of *P. falciparum* to various antimalarial drugs that are in use in Nigeria. Resistance to antimalarial drugs can be assessed *in vitro* by the degree of parasite growth inhibition exerted by particular antimalarial drugs (Basco and Ringwald, 2007; Oduola *et al*., 1993). In any therapeutic strategy, establishment of the baseline sensitivity of local *P. falciparum* isolates to commonly prescribed drugs that are still effective in some endemic areas and new drugs that are not yet widely available in a country but might be employed in the near future (e.g. artemisinin derivatives) is important. The *in vitro* assay is particularly useful for fulfilling this function as it provides an objective, quantitative measure.

In-vitro resistance against chloroquine, amodiaquine and sulphadoxine/pyrimethamine was observed among Nigerian isolates of *P. falciparum* tested. In the cross sectional study, an IC_{50} of 24.4nM/L and IC_{99} of 164.2nM/L was recorded for chloroquine respectively. These results were similar to earlier findings of Wongsrichanalai *et al*. (2000) where a high IC_{50} was also recorded. Resistance threshold of isolates against chloroquine and amodiaquine were above 160nM/L and 80nM/L respectively which denoted resistance against the drugs. Moreover, resistance threshold of isolates against Sulphadoxine and pyrimethamine were 300nM/L and 4.0nM/L respectively. All the isolates tested against mefloquine, quinine and artesunate in this study were sensitive to the drugs. The IC_{50} of 42.1nM/L observed in mefloquine in this study is slightly higher than that recorded in earlier findings of Reber-Liske (1983) where IC_{50} of 39.7nM/L was observed. The sensitivity ranges observed in the current study was also within the range for sensitivity of isolates to the drug (WHO, 2001). *In-vivo* resistance against chloroquine, amodiaquine, mefloquine and sulphadoxine /pyrimethamine has been reported by researchers in Nigeria (Happi *et al*., 2004; Ogunfowokan *et al*., 2009). The *in vivo* response of *P.*

falciparum to antimalarial drugs is modulated by a number of factors. These include the pharmacokinetic properties of antimalarial drugs, innate and acquired immunity in the patient, as well as the complexity of infections in high transmission areas (Happi *et al*., 2004). Several of these factors may contribute to the range of variations in the clinical expression of chloroquine resistance and *in vitro* resistance patterns.

All the isolates tested *in vitro* against artemisinin were sensitive to the drug. However treatment failures have been observed by other workers from Southwestern Nigeria during *in vivo* studies (Happi *et al*., 2004). Bioavailability of the drugs in the system amongst other factors has been attributed to treatment failures (Ibrahim *et al*., 2007). Although dihydroartemisinin is the active ingredient in the artemisinin based drugs. Artesunate® which contains artemisinin was used for the *in vitro* drug testing in this study. This is because it has been reported that dihydroartemisinin is unstable on drug plates and that artemisinin is the most appropriate drug for *in vitro* drug assays due to its stability in pre-dosed plates (Tanariya *et al*., 2000).

High resistance against chloroquine and amodiaquine were observed among the isolates collected from Yewa and Ijebu zones while SP resistance was not observed in isolates from Remo zone. There was cross resistance between chloroquine and amodiaquine as some of the isolates that showed resistance to chloroquine also showed resistance to amodiaquine in all the zones. Cross-resistance between chloroquine and amodiaquine has been reported both *in vitro* and *in vivo*. Pradines *et al*. (1999) have observed cross resistance of chloroquine and amodiaquine in earlier studies. It has also been observed that parasites may quickly develop resistance to amodiaquine in areas where extensive chloroquine resistance has been documented (Winkler *et al*., 1994).

Antimalarial drug resistance is now generally acknowledged to be one of the greatest threats to our ability to “Roll Back Malaria (Molta, 1995; Federal Ministry of Health 2004). The situation is worsening, with the geographic spread of resistance widening to previously unaffected areas and a remorseless increase both in the prevalence and degree of drug resistance. Resistance to chloroquine and sulfadoxine-pyrimethamine (SP) is widespread in Asia and South America and is spreading in Africa. This is evidenced in the results of this study as there was *in vitro* resistance against chloroquine. The burden of disease caused by

malaria and its consequences has been documented in terms of childhood mortality, anemia, maternal and infant morbidity and mortality, neurologic disability, and economic and social costs. The burden caused specifically by antimalarial drug resistance is more difficult to quantify. Resistance against chloroquine was observed in all the four zones where this present study was carried out.

There is an increasing acceptance that the ideal approach to antimalarial treatment is the use of combination of two or more drugs, rather than a single antimalarial drug, preferably with artemisinin derivative as one of the drugs (WHO, 2001; Ashley *et al*., 2007). Amodiaquine in combination with artesunate has been introduced as first-line treatment of malaria to replace chloroquine in Nigeria and other malaria-endemic countries of Africa (Sodiomon *et al*., 2009). Although the role of artesunate in this combination is to prevent the development of amodiaquine resistance, parasites may quickly develop resistance to amodiaquine in areas where extensive chloroquine resistance has been documented. In addition, little is known about the mechanism or epidemiology of amodiaquine resistance. Resistant parasites may then likely recrudesce under the selective force of the second drug in the combination and be transmitted to mosquitoes (WHO, 2003).Therefore, we cannot rule out the possibility of increasing selection of amodiaquine-resistant parasites with the increasing use of amodiaquine in combination with artesunate in Nigeria.

Reduced *in-vitro* susceptibility is not synonymous with diminished therapeutic effectiveness, but it is the probable first step of an alarming cascade and definitely pleads for increased vigilance and a coordinated and rapid deployment of drug combinations. A previous study in Nigeria assessed the quality of drugs from retail outlets and pharmacies and attributed problems to a lack of quality control in manufacture and degradation during storage (Onwujekwe *et al*., 2009). A major problem with the treatment of malaria is the high level of treatment failures resulting in the large part from the high prevalence of counterfeit drugs bought by the patients (Hall *et al*., 2006; Newton *et al*., 2006; Onwujekwe *et al*., 2009). Anti- malarials are among the most widely consumed drugs in tropical countries that have been particularly targeted by counterfeiters and of the anti-malarial drugs used in the world today

Developing countries, where malaria is one of the most prevalent diseases, still rely on traditional medicine as a source for the treatment of this disease. While synthetic pharmaceutical agents continue to dominate research, increasing attention has been directed to natural products (Alshwash *et al*., 2007). The success of artemisinin, isolated from *Artemisia annua*, and its derivatives for the treatment of resistant malaria has focused attention on plants as a source of antimalarial drugs (Tan *et al*., 1998). In this study, three crude organic extracts obtained from medicinal plants used in Nigerian folk medicine for the treatment of fever and malaria were tested *in vitro* against *P. falciparum*. The most active extract was obtained from *Diospyros monbuttensis* that showed appreciable inhibition to the parasites at all the concentrations used and an IC_{50} of 32µg/L in the study. *Diospyros monbuttensis*, which is locally used for the treatment of fevers, headaches and stomach disorders, (Awe and Makinde, 1997; Azas *et al*., 2002) has not been widely studied. This study represents the first conducted for antimalarial activity of crude extracts of *Diospyros monbuttensis*. The results confirm that those plants which are used in traditional medicine against malaria may possess *in vitro* and significant antimalarial potential and justify their use in traditional medicine. This observation suggests that the active constituents in the extract may be cytotoxic for *P. falciparum* trophozoites, thereby inhibiting their development to the schizont stage.

However, *in vivo* studies on these medicinal plants are necessary and should seek to determine toxicity of the active constituents, their side effects, serum-attainable levels, pharmacokinetic properties and diffusion in different body sites. Additional pharmacokinetic investigations are therefore advisable to identify host-related factors, such as poor absorption, accelerated gastrointestinal passage of the test drug, or metabolic peculiarities of some patients, which might lead to a faster-than-normal inactivation or elimination of the test drug (Azas *et al*., 2002). The use of herbs by some local people proved 100% sensitive to *P. falciparum* (Merlin, 2004; Ogbonna, *et al*., 2008).

An IC_{50} observed for *Momordica charantia* in this study was 125µg/L. These observations suggest that the active constituents in the extract might also be cytotoxic for *P. falciparum* trophozoites, thereby inhibiting their development to the schizont stage. The antimalarial activity of *Momordica charantia* has been previously reported (Alshwash *et al*., 2007). They found that the aqueous extract of. *M. charantia* leaves showed IC_{50} values less than 100 µg/ml which is in agreement with the observations in this study; the methanolic extract showed

moderate activity with IC_{50} =125 µg/ml. *Morinda lucida* also exhibited antimalarial activities in this study. The IC_{50} of 250µg/L observed in this study is comparable with other studies. Also for *M. lucida,* dose-dependent inhibitory outcomes were marked. Awe and Makinde, (1997) reported the dose-dependent and seasonal variation in the activity of *M. lucida* using both *in vitro* and *in vivo* techniques. *M. lucida* was reported to contain anthraquinones which showed *in vitro* activity against *P. falciparum* and also possesses antifungal properties. *Morinda lucida* is used locally in the treatment of yellow fever and jaundice (Guido *et al*., 1995).

One of the factors to be considered in the prophylaxis, treatment, and control of *Plasmodium falciparum* malaria is the resistance of parasite strains that may arise against virtually every drug available. Identification of *Pfcrt* as the central determinant of chloroquine-resistant *P. falciparum* malaria provides a molecular marker that can be used for surveillance of resistance and to evaluate drug treatment and prophylaxis policies. The present results further support this role of the *Pfcrt* gene. In the current study, 100 *P. falciparum* clinical isolates were collected during 2007-2008 and 49 (49%) of these samples were found to be chloroquine-sensitive and 51 (51%) chloroquine-resistant using the WHO *in vitro* microtest method. Amplification products from all the chloroquine-sensitive samples carried the codon for *Pfcrt* 76K. Out of the 51 samples that were chloroquine-resistant by *in vitro* testing, 48 were found to carry the 76T codon, 5 were found to contain mixed 76T/76K codons, and 3 were discordantly found to contain the 76K codon. The results of work showed that there is a positive correlation between *in-vitro* resistance pattern of isolates and prevalence of molecular markers of resistance in the four zones. This finding corroborates with earlier findings where molecular markers of resistance were found in samples that gave *in vivo* resistance/drug failure (Djimde *et al*., 2001; Chen *et al*., 2001; Happi *et al*., 2004).

The high prevalence of mutations at codons 76T, that code for chloroquine resistance in Nigerian *P. falciparum* isolates suggests that the population of *P. falciparum* that circulates in South-Western Nigeria has been selected by the long use of CQ. The overall picture emerging from this study is that resistance to this drug is abundant in south- Western Nigeria, and this finding strongly supports withdrawal of CQ as the first-line drug for treatment of *falciparum* malaria in Nigeria. This observation also supports the view that the *Pfcrt* polymorphism at

position 76 is in fact a significant factor of CQ resistance, as shown in previous studies from Cameroon (Basco and Ringwald, 2001.), Mali (Djimde *et al*., 2001), Mozambique (Mayor *et al*., 2001), Nigeria (Adagu and Warhurst, 2001), Sudan (Babiker *et al*., 2001), Uganda (Kyosiimire-Lugemwa et al., 2002), Madagascar (Ariey *et al*., 2002), Laos (Labbe *et al*., 2001), Papua New Guinea (Maguire *et al*., 2001), and Thailand (Chen *et al*., 2001; Jürgen and Christian, 2003). CQ resistance has been attributed to a single mutation at codon 76 in the *Pfcrt* gene (Djimde *et al*., 2001; Basco and Ringwald, 2001).

It has been observed that the *Pfcrt* K76 and the *Pfmdr1* Y86 alleles are closely associated in chloroquine resistant strains (Adagu and Warhurst, 1999). A similar observation was made in this study as all isolates carrying *Pfcrt* mutated allele were also positive for *Pfmdr1* mutated allele. However, studies comparing the associations of the *Pfmdr* 1 variant and the *Pfcrt* K76 variant have shown that the impact of the *pfcrt* gene was stronger than that of the *pfmdr* gene (Djimde *et al*., 2001; Dorsey *et al*., 2001; Ojurongbe *et al*., 2007). It has been suggested that the degree of chloroquine resistance is further modulated by factors linked to genes other than *Pfcrt* or *Pfmdr* (Chen *et al*., 2001).

Consistent with other studies, one can assume that the prevalence of *Pfcrt* T76 is a function of the actual chloroquine level, age of the patient, and also influenced by acquired immunity and natural resistance factors of the host (Wellems and Plowe 2001). Furthermore, one can assume that chloroquine intake contributes essentially to the selection of the *Pfcrt* T76 allele. Based on these observations, chloroquine appears to have an extended influence on the distribution of the *Pfcrt* polymorphism in an isolate. Thus, sub-therapeutic blood levels of chloroquine promote the emergence of drug resistance by direct selection.

Molecular methods that detect genetic markers of drug resistance are potentially powerful tools for tracking drug-resistant malaria. In this study, the combination of *Pfcrt* and *Pfmdr1* mutations in isolates associated with *in vitro* amodiaquine resistance was observed. Mutant *PfcrtT76* and *Pfmdr1Y86* alleles were observed in 48% and 60% of the samples, respectively. A previous study in Sudan (Babiker *et al*., 2001) found that the mutant *PfcrtT76* allele is associated with amodiaquine treatment failure. The high prevalence of the mutant *PfcrtT76* allele (48%) and *Pfmdr*1 allele (60%) observed in Ogun State, Nigeria confirms recent reports of the high prevalence rate of this alleles in parasites obtained from similar studies (Happi *et*

al., 2006) and is also consistent with rates ranging from 60% to 100% reported in other malaria-endemic regions (Maguire *et al*., 2001; Basco and Ringwald, 2001; Djimde *et al* 2001). The non-significant selection of the mutant *PfcrtT76* by amodiaquine may be due to the high prevalence of this allele in the *P. falciparum* population from South Western Nigeria. Selection of *Pfmdr1Y86* by amodiaquine has also been reported previously in the Gambia (Happi *et al*., 2003). Although the importance of point mutations in *Pfcrt* in producing chloroquine resistance is beyond dispute (Basco and Ringwald, 2001; Maguire *et al*., 2001; Chen *et al*., 2001) recent transfection studies of *Pfcrt* have shown that isolates expressing the mutant *PfcrtT76* allele retain sensitivity to amodiaquine while showing a reduced susceptibility to monodesethyl amodiaquine, the active metabolite of amodiaquine (Happi *et al*., 2003). The selection of the mutant *PfcrtT76* and *Pfmdr1Y86* alleles indicates the primary involvement of these two genes in the mediation of amodiaquine resistance. Thus, similar to chloroquine resistance, amodiaquine resistance in *P. falciparum* may depend primarily on mutation(s) in *Pfcrt* and additional mutations in *Pfmdr1* or other *Plasmodium* genes may also have significant roles in increasing resistance to the drug.

The combination of *pfcrtT76* and *pfmdr1Y86* mutations was associated with amodiaquine treatment failure. These two alleles have been shown to be in linkage disequilibrium in chloroquine-resistant isolates of *P. falciparum* from The Gambia and Nigeria (Happi *et al*., 2003). The similarity in the chemical structures of chloroquine and amodiaquine and their possible likely common mode of action suggests that the molecular basis of resistance to these two drugs may be similar.

Resistance against Sulphadoxine and Pyrimethamine was observed in this study. Among the isolates analyzed for resistance markers, *Pfdhfr*S108N and *Pfdhps*K540E genes coding for Pyrimethamine and Sulphadoxine respectively were detected. It has been earlier observed that Resistance to pyrimethamine is primarily conferred by a non synonymous point mutation at codon 108 and is consecutively enhanced by mutations at codons 51 and 59 of the *P. falciparum pfdhfr* gene located on chromosome 4 (Bruce-Chwatt, 1985; Kublin *et al.*, 2002). The enzyme is part of the folate pathway and, thus, of DNA replication. In this study genes that code for sulphadoxine and pyrimethamine resistance were detected among the isolates screened for markers of resistance against these drugs. *Pfdhfr*S108N and *Pfdhps*K540E alleles were detected in 12 and 2 isolates respectively.

On the basis of evolutionary theories, biological disadvantages are expected for parasites carrying resistance-mediating mutations in the absence of drug pressure. The fitness deficit conferred by the *Pfdhfr*S108N mutation in the absence of pyrimethamine use is considered quite low. Enduring resistance in the absence of strong drug pressure implies that the expected decline in the prevalence of resistant parasites is balanced by mechanisms that confer biological advantages with regard to survival fitness, replication and transmission probability, invasion, reproduction, and vector properties that favor transmission. As Sub-Saharan African countries are confronted with the rapid emergence of resistance against virtually every drug that is used for the treatment of *P. falciparum*, malaria drug pressure is considered to essentially promote the emergence of SP resistance, which is now widespread in East Africa, but also well recognized in West Africa.

Selection for the Ser to Asn substitution at codon 108 of the *Pfdhfr* gene has been shown to be linked to parasite survival after treatment with pyrimethamine -containing regimens (Plowe *et al.*, 1998; Marks *et al.*, 2005). Accordingly, the high frequencies of resistant parasite populations have been attributed to increased pyrimethamine consumption (Marks *et al.*, 2005). An Asp to Ile substitution at codon *Pfdhfr*51 (*Pfdhfr*N51I) and/or a Cys to Arg exchange at codon *pfdhfr*59 (*pfdhfr*C59R) appears to enhance pyrimethamine resistance if one or both of these occur concurrently with *Pfdhfr*S108N. *Pfdhfr*S108N-N51I-C59R is the combination of mutations most strongly associated with pyrimethamine resistance. Point mutations at codons 437 and 540 of the *Pfdhps* gene located on chromosome 8 of *P. falciparum* are considered responsible for sulphadoxine resistance. *Pfdhps* encodes a key enzyme in the folate pathway, as does *Pfdhfr*. The Ala to Gly substitution at position 437 (*Pfdhps*A437G) is, in general, the first mutation to occur. In Africa this is followed by the Lys to Glu substitution at position 540 (*Pfdhps*K540E), which confers higher levels of resistance. It was recently shown that the presence of the three *Pfdhfr* mutations combined with the two *Pfdhps* mutations (quintuple mutation) is strongly associated with SP resistance (Marks *et al.*, 2005).

Geographical clustering was reported for chloroquine-resistant *Pfcrt* (Wootton *et al.*, 2002) and anti folate resistant *Pfdhfr* and *Pfdhps* haplotypes (Cortese *et al.*, 2003), indicating distinct ancestral selection events in different areas. One important aspect in the possible setting

leading to drug resistant parasites in Western Nigeria is the genetic background of the local parasite population. Multidrug-resistant *P falciparum* is common, with a high rate of *Pfcrt*, *Pfmdr1*, *Pfdhfr* and *Pfdhps* resistance haplotypes. Thus, selection pressures were exerted on a heavily mutated genetic background. The several *PfATPase6* haplotypes harbouring the S769N mutation indicate that selections of such mutants are therefore not impossible in the nearest future. As chloroquine and SP are replaced by more effective artemisinin-based combination therapies (ACTs), strategies for monitoring (and, if possible, deterring) drug-resistant malaria must be updated and optimized. *In vitro* methods for measuring resistance will be critical for confirming and characterizing resistance to ACTs. Molecular markers are useful for tracking the emergence and dissemination of resistance and guiding treatment policy where resistance is low or moderate.

One hundred samples that were positive for *P. falciparum* by microscopy were subjected to PCR genotyping for estimating prevalence of the *PfATPase* codon S769N mutation. All the 100 (100%) isolates carried the artemisinin sensitive wild type allele, S769. This observation is similar to the ones observed in several African countries where artemisinin and its derivatives are used as first line of treatment of uncomplicated malaria (Basco and Ringwald, 2001; Djimde *et al*., 2001; Mayor *et al.*, 2001; Adagu and Warhurst, 2001; Babiker *et al*., 2001; Kyosiimire-Lugemwa et al., 2002). However Artemisinin resistant genes have been detected by some workers especially in South Asia (Labbe *et al*., 2001; Maguire *et al*., 2001; Chen *et al*., 2001; Jürgen and Christian, 2003). Artemisinin derivatives are an essential component of treatment against multidrug-resistant *falciparum* malaria. The genes that code for artemisinin resistance was not detected in any of the isolates screened for antimalarial resistance genes. Widespread multidrug-resistant *falciparum* malaria led WHO to recommend combination drug therapy as first-line treatment, with formulations containing an artemisinin compound as policy standard. Artemisinin and its derivatives are the most potent and rapidly acting antimalarials. However, artemisinin resistance has been reported in murine models of malaria (Ferrer-Rodiriguez *et al.*, 2004). Diligent surveillance is needed to monitor continued susceptibility to artemisinin derivatives in endemic areas.

Numerous studies have demonstrated that children under five years and pregnant women are at a higher risk of suffering from clinical malaria (Sachs and Malaney, 2002; Greenwood *et*

al., 2005; Adefioye *et. al.*, 2007; Sotimehin *et al*., 2008), and drug prevention has been advocated for years, using either weekly or monthly prophylaxis or, more recently in several countries, intermittent presumptive treatment, IPT (Cot and Deloron, 2003). At a time, many countries in the Sub Saharan region recommended a weekly chloroquine prophylaxis for each pregnant woman from the beginning of pregnancy to delivery. One possible drawback of such increased drug consumption is an increased drug pressure that may lead to the selection of drug-resistant parasites. There is some evidence in favor of this hypothesis. For instance, in countries where Artemisinin resistance has been discovered, all resistant isolates came from areas with uncontrolled use of artemisinin derivatives (Ronan *et al*., 2005)

There is little existing knowledge about actual quality of drugs provided by different providers in Nigeria and in many sub-Saharan African countries. Such information is important for improving malaria treatment that will help in the development and implementation of actions designed to improve the quality of treatment. A study conducted in South-East Nigeria found that there was a high prevalence of poor quality drugs (Onwujekwe *et al*., 2009). Such findings provide areas for public intervention to improve the quality of malaria treatment services and to forestall resistance.

The Merozoite Surface Protein1 (MSP-*1*) and Merozoite Surface Protein2 (MSP-*2*) are highly polymorphic markers and the large allelic polymorphism has been reported in the block 2 of the *msp-1* gene and the central repetitive domain (block3) of the *msp-2* gene. Families differing in nucleotide sequences and in number of repetitive sequences (length variation) were used for genotyping purposes. This study evaluates the extent of genetic diversity in the field isolates of *P. falciparum* obtained from Ogun State in South Western Nigeria. The population structure of the isolates analyzed with the polymorphic markers *MSP-1*, *MSP-2*, and Glutamate Rich Protein (GLURP) in this study showed extensive diversity in parasite populations in the four zones in Ogun State, South Western Nigeria. The MSP-1, MSP-2, and GLURP markers showed 8, 6, and 5 allelic families, respectively. This diversity of the *P. falciparum* population in South Western Nigeria is reflected in the complexity of parasite populations in the samples. A catalog of genetically distinct parasite populations co-infecting those infected with malaria, based on PCR amplification of these markers, showed that multiplicity of infection was common. Multiclonality of infections has been shown to be a

common feature in most malaria-endemic areas (Happi *et al*., 2004; Ntoumi *et al*., 1995; Magesa *et al*., 2001; Nzila *et al*., 2006). Epidemiologic data from some study sites in Africa suggest that the multiplicity of *P. falciparum* infection may be directly related to the intensity of transmission (Arnot, 1998; Babiker *et al*., 1997). This multiplicity of infections may also have important implications for the epidemiology of drug-resistant *P. falciparum* malaria and the outcome of treatment in patients. The initial presence of several parasite populations with different drug response profiles would result in elimination of drug sensitive populations and selection of resistant parasites. The findings in this study are in agreement with those of previous studies involving asymptomatic carriers and symptomatic patients in holoendemic areas, both in the complexity of population structure and multiplicity of parasites in human hosts (Ntoumi *et al*., 1995; Snounou *et al*., 1999). All the MSP 1, MSP2 and Glurp families were represented in each of the four zones in Ogun state where samples were collected.

In considering possible strategies for the reduction of the burden of antimalarial drug resistance, it is useful to differentiate between the current burden of drug resistance and the potential burden in the future resulting from the continued emergence and spread of drug resistance. The factors that are likely to contribute to the development of antimalarial resistance were studied through administration of questionnaire to people who live in the four zones of Ogun State, South Western Nigeria. 33% of respondents do have malaria at least once in a month and 32.1 would have malaria at least once in three months respectively. This means about 80.3% will have malaria at least once in a year. It was also found in this study that the rate of exposure to antimalarial drugs including the artemisinins is very high. 40.7% of the sample population use quinolines as prophylaxis while more than 33% use sulphonamides and about 3.2% use artemisinins as prophylaxis. As long as drugs are used, the chance of resistance developing to those drugs is present (Plowe, 2003). It has been observed that the development of resistance to antimalarial drugs in South-East Asia has been far quicker than the estimated 12 to 17 years it takes to develop and market a new antimalarial compound (Ridley, 1997).

Affordability is an essential consideration for any strategy to control drug-resistant malaria, especially in Africa (Foster and Phillips 1998; Goodman *et al.*, 1999). In the current study, about 25% of the sample population stops the use of drugs because of price or when they

finish the one they could afford. The future, especially in Africa, will also be defined by how well the central tenets of malaria control can be reconciled with the central tenets of control of drug resistance. One of the cornerstones of the current approach to malaria control is the provision of prompt, effective malaria treatment. In much of Africa, easy access to public sector health care is limited and when it is accessible, health care staff are often inadequately trained, insufficiently supplied and supported, ineffectively supervised and poorly motivated (Goodman *et al.,* 2000). Central to achieving a reduction in both current and future burdens is an improvement in drug usage by patients and providers so that good quality drugs are available and taken at the correct dose and for a sufficient length of time to affect a radical cure and reduce the likelihood that partially resistant parasites will survive. Improving drug use is most effective where the parasite is still sensitive to the drug. Where resistance has rendered the drug ineffective, the current burden of resistance can only be reduced by replacing the failing drug regimen with one that is effective. The difficulty lies in deciding which drug regimen to switch to, since the choice of drug or drug combination will determine the subsequent development of drug resistance. Reducing the future burden of resistance requires that effective antimalarial drugs continue to be available in the future and requires the continuous search for and development of potential new antimalarial drugs.

However, the complete drug development process can take 10−15 years, making it imperative that the currently available drugs are deployed in a way most likely to maximize their lifespan by decreasing the likelihood that resistance will develop. The key strategy put forward to do this is to use available drugs in combination to prevent the emergence and spread of resistance. Once a drug-resistant mutant has arisen, preventing spread of resistance is difficult. Spread is facilitated by the exposure of malarial parasites to sub-therapeutic levels of antimalarial drugs, that kill sensitive parasites but allows parasites with a resistance mutation to survive and reproduce. Ensuring that drugs are taken in at a sufficient dose and for a sufficient duration reduces this risk. Drug pressure is higher where a drug with a long half-life is taken because the drug remains in the patient’s blood at low levels for weeks, exposing any newly introduced malarial parasites to sub-therapeutic levels (Bloland, 2001).

This is particularly likely to occur in high transmission areas where people are not only infected more frequently, but also take antimalarial drugs frequently whether or not they are having malaria. Theoretically,this form of drug pressure can be reduced by using drugs with a

shorter half-life and by restricting the use of the first line drug to patients with confirmed malaria: i.e., only treating those with a definitive diagnosis.

CONCLUSION

This study has established the incidence of *falciparum* malaria in Ogun State, Southwestern Nigeria. Moreover, a baseline for both genetic polymorphisms and drug sensitivity profiles of *P. falciparum* isolated from Ogun State Nigeria has been established. This data should serve as a baseline for future studies to monitor the changes in antimalarial drug resistance in this part of the country. To prevent resistance to antimalarial drugs, a high degree of vigilance is required; the level of antimalarial drug sensitivity of *P. falciparum* should be closely monitored while compliance to antimalarial drug use should be encouraged.

CONTRIBUTIONS TO KNOWLEDGE

This research work has contributed to knowledge in the following ways.

1. The incidence of malaria (*Plasmodium* infection) in Ogun state, South Western Nigeria has been established. This would help in planning prevention/control operations, resource allocation, and upgrading medical facilities in hospitals to treat severe and complicated cases.
2. The resistance pattern of *Plasmodium falciparum* isolates from Ogun State to existing antimalarial drugs established in this study would tailor drug choices specific to geographic regions.
3. The molecular diversity of *P. falciparum* strains in Ogun State, Southwestern Nigeria has been established. This Knowledge would help to type and characterize the *P. falciparum* strains in Nigeria and compare their diversity with the rest of the world.
4. Results from molecular studies on the drug resistance genes of *P. falciparum* obtained in this study would provide advance information on the emergence of drug resistance in the field. This knowledge should facilitate the ability to begin to anticipate genomic responses to drugs yet unseen by the parasite.
5. Some of the factors which contribute to the development of resistance of *Plasmodium* to antimalarial drugs have been established. This should lead to the development of strategies and policies to control the use of existing antimalarial drugs and forestall resistance to new drugs.
6. Antimalarial efficacy of some local herbs used as herbal drugs has been established. This is expected to lead to the discovery of novel antimalarial drug of local origin.

RECOMMENDATION

There is an urgent need to find and develop alternative drugs against multidrug-resistant *P. falciparum.* One group of alternative antimalarial drugs comprises artemisinin (qinghaosu) and its derivatives. Artemisinin is a traditional Chinese medicinal herb derived from *Artemisia annua.* Newer antimalarial drugs and approaches to overcome parasite resistance are needed to deal with the expanding problem of drug resistance which continues to challenge malaria control efforts based on early diagnosis and treatments. Only a limited number of antimalarial drugs are currently at an advanced stage of clinical development. There must therefore be a renewed interest in plant products. An attractive option for poor and developing countries is the exploitation of the possible therapeutic effects of their local herbs. Efforts must be made to reduce drug pressure which can lead to the selection of drug-resistant parasites.

Since there was positive correlation between artesunate and mefloquine; artesunate quinine, drugs containing these combinations should be recommended for Ogun State, the study area.

There is little existing knowledge about actual quality of drugs provided by different providers in Nigeria and in many sub-Saharan African countries. Such information is important for improving malaria treatment that will help in reducing the development of resistance to the antimalarial drugs in use.

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APPENDICES

APPENDIX I

Preparation of Culture Medium (Stock)

One packet of RPMI which contains 25mM of HEPES buffer was dissolved in 960ml of triple distilled water. 2gm of glucose was added and dissolved. 40µg/ml of Gentamycin Sulphate

was added to prevent contamination. The solution was sterilized by passing it through a Millipore filter of 0.22µm porousity and was stored at 4^0C as 96ml aliquots in glass media bottles.

Preparation of 5% Sodium Bicarbonate

5gm of Sodium bicarbonate was dissolved in 100ml of double distilled water. The solution was filtered through a Millipore filter of 0.22µm porosity and stored at 4^0C

Preparation of Incomplete Culture Medium (washing medium)

4.0 ml of 5% Sodium Bicarbonate was added to 96ml of stock RPMI 1640. This was prepared fresh when needed.

3.8.5 Preparation of Complete Culture Medium

To prepare 100ml of the medium, 90ml of RPMI 1640 solution containing 5% $NaHCO_3$ was mixed with 10ml of human serum. The medium was then stored at 4^oC. Complete culture medium is a 3:1 mix (vol/vol) of RPMI 1640 medium containing 4- (2-hydroxyethyl) -piperazine-ethanesulfonic acid i.e. HEPES (25 mM), $NaHCO_3$ (25 mM), and Waymouth medium (Flow Laboratories, Irvine, United Kingdom) supplemented with 12% (vol/vol) human type AB Rhesus negative serum and hypoxanthine (10 µg/liter).

Preparation of Giemsa Stain

To prepare 400ml of Giemsa stain, 3g of Giemsa powder was dissolved in 200ml of glycerol. 200ml of methanol was added. The solution was be incubated in water bath for two hours with gradual shaking at five minutes interval (Cheesebrough, 2001).

APPENDIX II

Questionnaire

A survey of the predisposing factors to the development of antimalarial drug resistance in *Plasmodium falciparum* in South Western Nigeria

1. Residential area: Town/City/Village ……..
2. Sex M F

3. Age 18 – 25 26 – 35 36 – 45 45 and above

4. Educational Background:
Non formal Quranic Primary Secondary Post Secondary

5. Main Occupation:
Teaching Trading Student Artisan Housewife
Farming Civil Servant Others Specify

History of malaria disease/infection and management:

6. How often do you have malaria –
Once in a month once in three months
Once a year Twice a year Others Specify

8. Where do you seek Medical advice when you experience symptoms of malaria?
From: Doctors/Hospitals Nurse/midwife Pharmacists Chemists Self medication others List them

9. Thick appropriately, any antimalarial drug that you have used before
Chloroquine Amodiaquine Quinine Fansider Maloxine

Amalar Malamox Halfan Lornart Alaxin Artesunate
Artemether arteplus others specify

10. Which of the above drugs do you take frequently (mention)
…… ……..
……

11. Do you take these drugs based on doctors' prescription?
Yes No

12. If yes do you take the full regimen as directed by the doctor?
Yes No.

13. When do you stop medication?
    a) When I feel okay
    b) When I complete dosage
    c) Anytime I like
    d) When I finish what my money can buy
    e) Others (specify)

14. What factors influence you to stop the medication?
    a) Price of drugs
    b) Dislike for drugs
    c) Fear of overdose once the fever is down
    d) When I feel cured
    e) Personal/religious beliefs
    f) Others (specify)

15. Do you take all drugs prescribed by the doctor?
    Yes No All drugs except the vitamins
    Just the ones I feel are important
16. Do you belief there is a need for Doctors' prescription?
    Yes No
17. Do you take antibiotics in combination with above antimalarial drugs? Yes No

18. Which antibiotics do you take in combination with antimalarial drugs? (Mention)……

19. Do you follow doctors' prescription strictly? Yes No
20. Do you take any local herbal preparation to cure malaria? Yes No
    Mention the local name of the herb……..
21. Do you get a medical Laboratory diagnosis before administration of drugs?
    Yes No Sometimes

22. Do you take antimalarial drugs as prophylaxis/preventive against malaria?
    Yes No If yes, mention the drugs you take to prevent malaria……

23. How effective is the antimalarial drug you commonly use in case of malaria
    Highly ineffective 1 2 3 4 5 extremely effective (circle as appropriate)

24. Which of these do you consider most effective for the cure of malaria?

Local herbal preparation Doctors' prescribed drugs

Traditional healers others (specify)

25. Which method do you use to prevent mosquito bite/transmission of malaria infection?

Window/door nets Insecticide treated bed nets

Insecticide spray (specify) Mosquito repellent creams mosquito coils others (specify)

APPENDIX III

ASSENT FORM

Title of study:

***IN-VITRO* AND MOLECULAR STUDIES ON THE RESISTANCE OF *P. falciparum* TO ANTIMALARIAL DRUGS IN OGUN STATE, SOUTHWESTERN NIGERIA**

Date

Dear participant,

This is to seek your assent despite the explanation given to you and your parent/guardian on the above project. You are free to decline to participate even if your parent/guardian has agreed on your behalf. If you agree that your blood be taken for the tests required in the study Please signify by nodding your head.

HEAD NODDED……..

HEAD NOT NODDED…….

Witness;

Signature…….

Thank you,

Olasehinde, Grace I.
Principal investigator

APPENDIX IV

CONSENT FORM

***IN-VITRO* AND MOLECULAR STUDIES ON THE RESISTANCE OF *P. falciparum* TO ANTIMALARIAL DRUGS IN OGUN STATE, SOUTHWESTERN NIGERIA**

Date

Dear participant,

You are invited to participate in the above research study, which has been designed to determine the incidence of malaria, the resistance patterns of the malaria parasite and the possible factors that may contribute to the development of resistance to antimalarial drugs by the parasites in South Western Nigeria

You will be subjected to malaria screening test which requires 2mls of blood from you through venepuncure; this same blood will be processed to get the DNA there from. The DNA will be screened for novel resistance genes using molecular methods. You will also be required to complete a questionnaire and it will take you about 10minutes.

The information generated from this survey will be of direct benefit to you as it aims at prevent incessant use of drugs, and will probably contribute to general knowledge on how to proffer public health intervention that will help control malaria in this part of the country.

Meanwhile, the study will not disclose any confidential information pertaining to you but the information generated can be used for public health intervention as regards the disease in south western Nigeria.

Your decision to participate is voluntary. You are free to decline to participate in this study, or to withdraw your information, at any time.

Before you complete the questionnaire or decide to participate in this study, please ask questions on any aspect of the study that is not clear to you. If you have any additional questions later, you can contact Olasehinde Grace on 08055439005.

Address :

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Ogun State, Nigeria
e-mail; golasehinde@yahoo.com

…….. ……..

Signature of participant Date

APPENDIX V

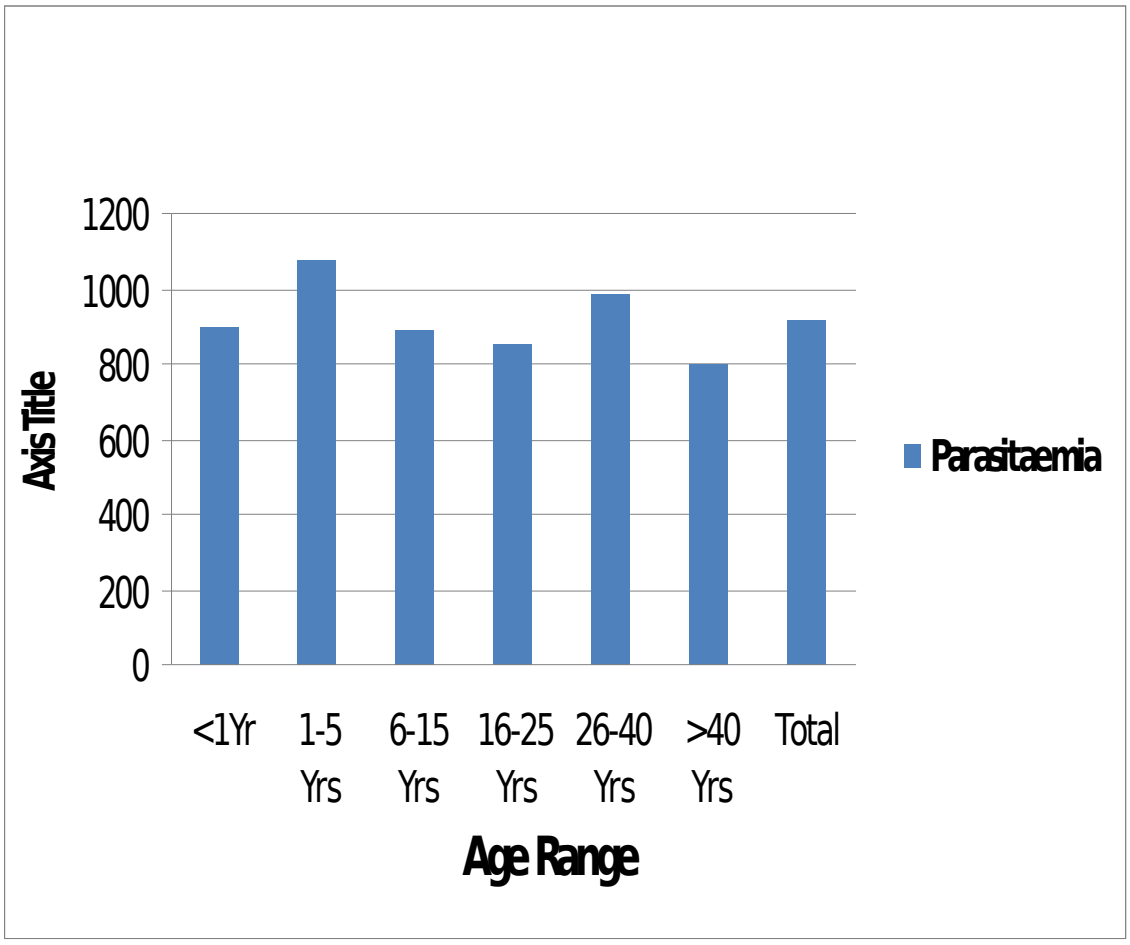


Fig 4.1. Parasitaemia Rate by Age

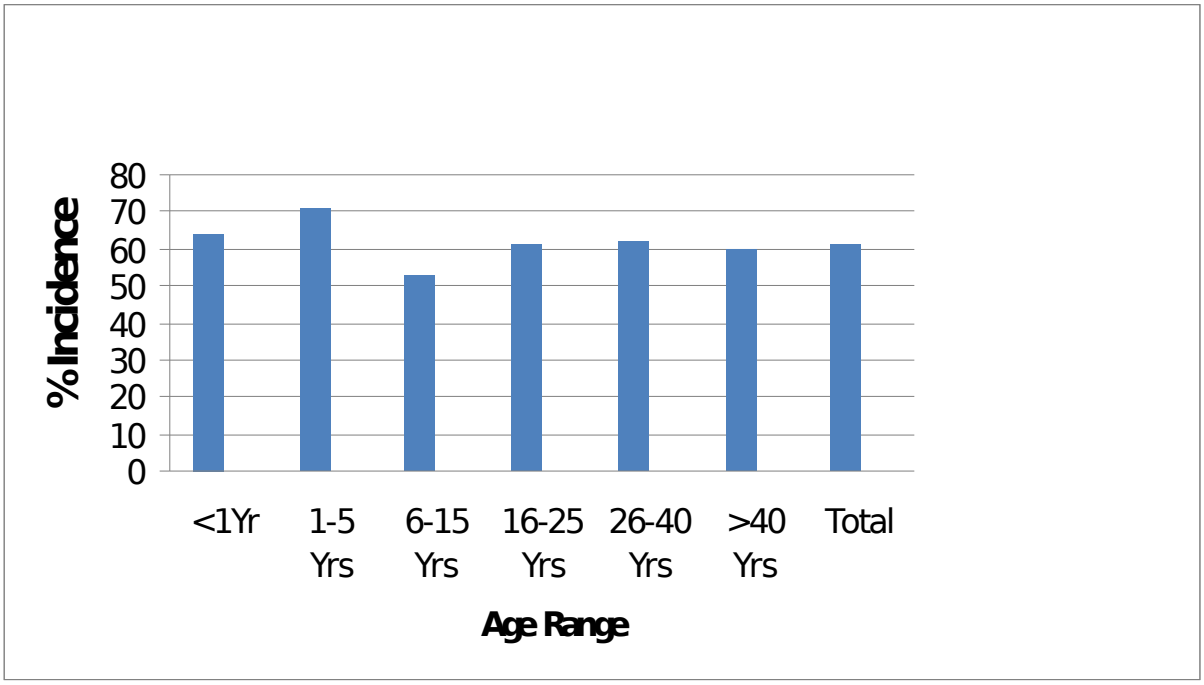
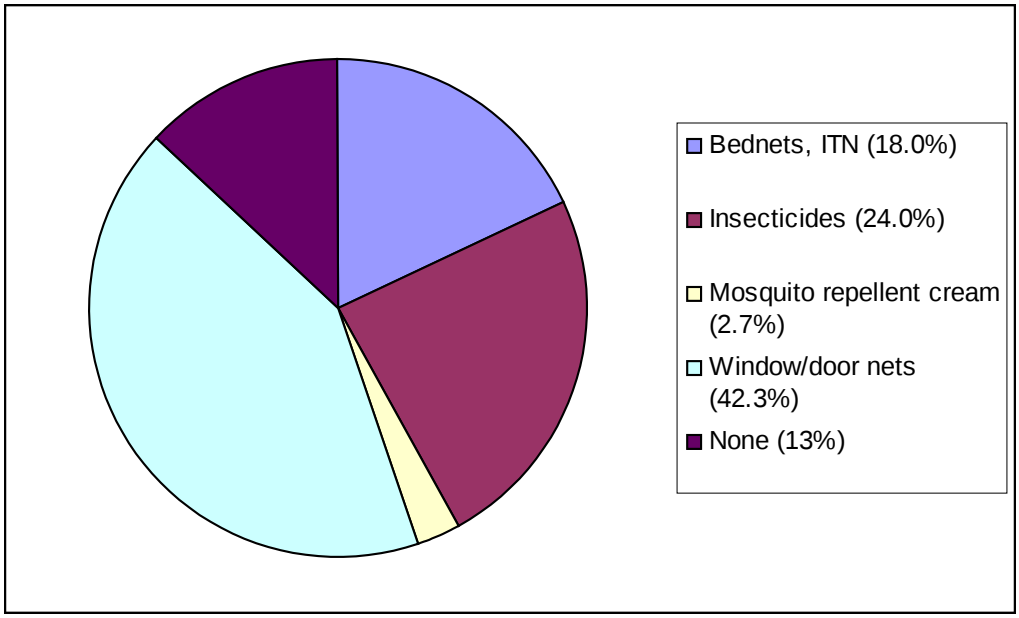


Fig 4.2. Percentage Incidence of *P. falciparum* Infection in Ogun State

APPENDIX VII



Mosquito – bite preventive methods employed by respondents.