CHAPTER ONE

1.0   **INTRODUCTION**

1.1   Background of the Study

Malaria has played an important role in the history of mankind because it is a constant threat to human health and it affects economic development and growth in areas highly affected by the disease. The disease is more lethal in children 0-5years, pregnant women and in people from non-malaria endemic areas. An estimated 247 million cases, nearly a million deaths, mostly in children less than five years old were attributed to malaria (WHO, 2008a). Nigeria accounts for a quarter of all malaria cases in the World Health Organization's (WHO) African Region and transmission occurs all-year round in the Southern States of the country (WHO, 2008a). In Nigeria, malaria is directly responsible for over one million deaths of children below school age and one quarter of an average family income is spent on the treatment of malaria (Etuk and Umoh, 2001).

Despite the attempts to eradicate malaria in Africa between 1955-1978, the disease resurged during the 1980s and 90s alongside with rapid spread of resistance to mainstay antimalarial medicines, for example, chloroquine and sulphadoxine-pyrimethamine (Bjorkman and Bhattarai 2005). However, the advent of a new millennium has been accompanied by increased awareness and expressed commitment by political leaders in Africa and by the international community to Roll Back Malaria. This has been clearly outlined in the Millennium Development Goals, the Abuja Declaration and in the Global Malaria Action Plan (WHO, 2003a; WHO, 2008b). This new era has coincided with access to powerful tools to control the disease, for example, efficacious combination treatment based on artemisinin-derivatives as well as improved vector control with long lasting insecticides treated nets and a revival of indoor residual spraying. Integrated, wide scale, high coverage interventions with these tools have recently shown

persuasive evidence of marked reduction in overall child mortality and burden of disease in endemic areas of Africa (Bhattarai *et al.*, 2007).

Reports on malaria prevalence in children in Nigeria are divergent in the literatures. These differences could be as a result of the period of the year when the study was conducted (Salako *et al.*, 1990) or on the quality of malaria microscopy that was employed. In Ogun State, South-West Nigeria, a prevalence of 67.5% was reported among 0-5years old children (Ibidapo, 2005) and in Kano State Nigeria, a prevalence of 32.9% was reported in children within the age of 1-14years (Adeleke, 2007). In Uyo, South-South Nigeria, a malaria prevalence of 87.2% was reported in 0-≤5years old children (Opara *et al.*, 2006) while a prevalence of 56.9% was reported in a Primary Health Care Centre in Jos, North Central Nigeria (Ikeh and Teclaire, 2008). Over a decade ago, a 27.1% malaria prevalence in infants 0-6months of age was reported in Lagos (Afolabi *et al.*, 2001). There is a dearth of current data on children presenting with malaria fever in health facilities in Lagos, especially in the light of increased malaria interventions.

Genetic and genomic advances have paved the way for discoveries into the origins and spread of antimalarial drug resistance and the underlying molecular mechanisms. Researchers can now use data from genome sequencing projects to identify genetic regions linked to resistance phenotypes. The genes of *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*), which encodes the transmembrane protein *Pfcrt*, and the *Plasmodium falciparum* multi-drug resistance (*Pfmdr1*) gene, which encodes a P glycoprotein homologue 1 (Pgh1), have been linked to CQ and other 4-aminoquinolones drugs resistant parasites (Fidock *et al.*, 2000; Duraisingh and Cowman, 2005; Sharma, 2005). Furthermore, specific point mutations in the parasite's dihydrofolate reductase (*Dhfr*) and dihydropterate synthase (*Dhps*) genes are associated with SP

resistance (Cowman *et al.*, 1988; Peterson *et al.*, 1988). *Dhfr* mediates pyrimethamine resistance while *Dhps* is responsible for sulphadoxine resistance.

Despite the well established evidence of chloroquine (CQ) resistance, the failing drug remained the medicines of choice for the treatment of malaria in a number of settings in Africa (including Nigeria) for many years due to lack of suitable alternatives and affordable drugs. Later, sulphadoxine-pyrimethamine (SP) replaced CQ in most sub-Saharan countries in Africa. The use of CQ and SP as the first line drug was discontinued in 2005 in Nigeria (FMOH, 2005). However, CQ and SP are widely used despite the change to the ACTs. As people continue to use these antimalarial medicines because of its affordability, even after the change in policy, there is the likelihood that the frequency of CQ and SP resistant genes in the general population could continue to escalate to further compromise the already poor efficacy of the medicines (CQ, SP, Amodiaquine etc). Therefore, it becomes imperative and urgent to determine the occurrence of CQ and other 4-aminoquinolones resistant genes (*Pfcrt* and *Pfmdr1*) and SP resistant genes (*Pfdhps* and *Pfdhfr*) in malaria confirmed febrile children who may or may not have taken CQ and SP at presentation in Lagos, Nigeria. An *in-vivo* Drug Therapeutic Efficacy Trial (DTET) conducted among children in Nigeria showed that in South-West Nigeria including Lagos, CQ and SP resistance was 51.1% and 24.4% respectively (FMOH, 2005).

1.2   Statement of the Problems

Malaria prevalence has been reported to drop to 247 million from the highly quoted 300-500 million cases globally and in a number of countries (WHO, 2009). The reported decline in malaria prevalence correlated with the deployment of several malaria control measures such as: Long Lasting Insecticides Nets (LLIN), Indoor Residual Spraying (IRS) and case management with Artemisinin Combination Therapy (ACTs) (WHO, 2009). Malaria prevalence reports in

Nigerian children vary widely across the various zones ranging from 20.8% to 87.2% (Ekpenyong and Eyo, 2008 - Opara *et al*., 2006). This variation in malaria prevalence could be due to the season when the study was done, the population of children and the quality of methods employed. The issue of methodology is significant due to the employment of poor malaria microscopy processes. This has been seen as a challenge in the report of malaria both in routine diagnosis in the health facilities and in research where microscopists have low mlalaria microscopy skills required for accurately detecting, staging, speciating and quantifying the parasites. Reports from some countries currently indicate a shift in the prevalence of malaria in children from less than five years old children to those, 5 years and above (Bouyou-Akotet *et al*., 2009; Delacollette *et al*., 2009; Okebe *et al*., 2010). However, it is not clear among children less than and above five in Nigeria. In addition, there is a dearth of current data that could be used as base-line for monitoring the trend of malaria with time as various malaria control measures are deployed in communities through increased funding from Global Fund.

CQ and SP are affordable and accessible and thus frequently used especially by patients that cannot afford the expensive ACTs (Ogungbamigbe *et al*., 2005) and where ACTs are available through government programme, there are issues on access and stock out. The more CQ and SP are used, the higher the chances of resistant genes circulating in the population the greater the level of threat on the partner drugs (such as amodiaquine, mefloquine and lumefantrine) in ACTs. The determination of resistance to CQ and SP using genetic markers have been reported in children treated with CQ and SP in Lagos (Olukosi *et al*., 2005), in Ibadan (Happi *et al*., 2006) and in Osogbo (Ojurongbe *et al*., 2007). However, these studies did not provide expanded haplotype information by genotyping for Single Nucleotide Polymorphisms (SNPs) on resistance to antimalarials and especially how it could impact on partner drugs to ACTs especially the aminoquinolines.

The results of molecular genotyping and characterization of mutations for SNPs have been used for drug resistance monitoring. Molecular genotyping for SNPs could predict emerging or existing drug resistance patterns. For example, the return of CQ sensitive (wild type) parasites was demonstrated in Malawi, Tanzania and Kenya after the total removal of CQ from the population (Laufer *et al.*, 2006; Temu *et al.*, 2006; Mwai *et al.*, 2009). There is therefore, concern that the continous use of CQ and SP as monotherapies in Nigeria could result in the increase of resistant *Plasmodium falciparum* parasites in the population and this could impair the effectiveness of the partner drugs to ACTs especially, the 4-aminoquinolines.

1.3 Aim of the Study

The aim of the study was to report the level of malaria and to describe *Pfcrt* haplotypes, point mutations in *Pfmdr1* genes and the frequency of *Pfdhfr* and *Pfdhps* haplotypes in children with uncomplicated *Plasmodium falciparum* malaria in Lagos, Nigeria.

1.4 Specific Objectives

The specific objectives of this study were to:

I.   Determine the level of malaria and compare the prevalence and intensity in children 0-≤5 years with those >5 years that presented with fever or history of fever in health facilities in Lagos.

II. Describe the occurrence of *Pfcrt* haplotypes and mutations in *Pfmdr1* genes in children with uncomplicated malaria.

III. Determine the point mutations and the frequencies of *Pfdhfr* and *Pfdhps* haplotypes in children with uncomplicated malaria.

5

1.5   Significance of the Study

This study was conducted to provide data on the current level of malaria in children 0-≤5years and >5-12years of age as current information is unavailable. The last data available in the literatures was published in 2001 among children <6 months old. After these time, malaria control has been expanded using Long Lasting Insecticides Nets (LLIN), Indoor Residual Spraying (IRS) and case management with Artemisinin Combination Therapy (ACTs). In addition, malaria control measures had targeted children <5years and pregnant women and there is dearth of data on the level of malaria in these age groups. This study is relevant at this time to to provide us knowledge on the level of malaria in these age groups and also to provide baseline data.

This study is important as it will provide us information on the level of *Plasmodium falciparum* chloroquine resistant strains circulating in Nigeria children. The study will provide molecular genotyping for SNPs to determine malaria parasite resistance to CQ and SP medicines in Nigeria. Resistance to CQ and SP using genetic markers has been reported in children to determine frequency of mutations in some already known codons when CQ and SP are still antimalarial medicines recommended in Nigeria. However, these studies did not provide expanded haplotype information by genotyping for Single Nucleotide Polymorphisms (SNPs) on resistance to antimalarials and especially how it could impact on partner drugs to Artemisinin especially the aminoquinolines. This study was done to provide haplotype data for Nigeria, West Africa and Africa on frequency of mutations to *Plasmodium falciparum* resistant genes to *Pfcrt, Pfmdr1*, *Dhfr* and *Dhps* for SNPs. The frequency of mutations to the antimalaria medicines that will be assayed (CQ and SP) will provide practical evidence to reduce access to monotherapies such as amodiaquine, CQ, SP and mefloquine.

1.6 Limitation of the Study

The rigorous follow up of the children to determine the outcome of the treatment as secondary end points was not done.

1.7 Operational Definition of Terms

Alleles- one of two or more alternative forms of a gene at corresponding sites (loci) on homologous chromosomes, which determine alternative characters in inheritance.

Blood smears- a blood test used to provide information diagnosis and morphology of *Plasmodium* species.

Blood spots- Drop of blood on filter paper for molecular diagnosis for mutations in *Pfcrt*, *Pfmdr1*, *Dhfr* and *Dhps* genes.

Codons - the arrangement of nucleotides in the polynucleotide chain of a chromosome governing transmission of genetic information to proteins seen in resistant genes.

Dihydrofolate reductase (*Dhfr*)- enzyme catalyzing the conversion of folate to 5,6,7,8-tetrahydrofolate, which is the key carrier of one-carbon units in purine and pyridime synthesis, the pathway for the breakdown of histidine and the synthesis of *S*-adenosylmethionine from *S*-adenosylhomocysteine.

Dihydrofolate reductase (*Dhfr*) genes- They are point mutations on a *Dhfr* genes genetic constitution that have been shown to confer resistance to pyrimethamine in codons 51, 59, 108 etc.

Dihydropteroate synthase (*Dhps*) genes- They are point mutations on a *Dhps* genes genetic constitution that have been shown to confer resistance to sulphadoxine in codons 431, 581, 437 etc.

Dihydropteroate synthase (*Dhps*)- is a pterin created from para-aminobenzoic acid (PABA) by the enzyme *Dhps*. It is an important intermediate in folate synthesis.

Genotyping- the entire genetic constitution of a malaria parasite; also, the alleles present at one or more specific loci of a malaria parasite.

Haplotype- a set of alleles (an alternative form of a gene that can occupy a particular place on a chromosome) of a group of closely linked genes which are usually inherited as a unit e.g. ACIRNVI, ACICNVI, ISGKGA, IFGKAS, etc.

High Power Field (HPF)- it is the maximum objective lens in a binocular microscope (100x) used in relation to microscopy references the area visible under the maximum magnification power of the objective being used.

Parasitaemia- is the presence of malaria (*Plasmodium*) parasites in the blood expressed as p/µl of blood.

Plasmodium falciparum **chloroquine resistance transporter** (*Pfcrt*)- a primary genetic mechanism conferring resistance to chloroquine.

Plasmodium falciparum **multi-drug resistance** (*Pfmdr1*)- an additional genetic mechanism conferring resistance to chloroquine, mefloquine, halofantrine and quinine.

Plasmodium falciparum **multi-drug resistance** (*Pfmdr1*) **fragment 1,2 and 3-** they are point mutations on *Pfmdr1* gene that are known to confer resistance to chloroquine, mefloquine, halofantrine and quinine.

Prevalence- is the number of cases in a defined population at a specific point in time

Quantitative polymerase chain reaction (qPCR)- a PCR based technique that is used to amplify and simultaneously quantify a targeted DNA molecule.

Single nucleotides polymorphisms (**SNP**)- (genetics) genetic variation in a DNA sequence that occurs when a single nucleotide in a genome is altered; SNPs are usually considered to be point mutations that have been evolutionarily successful enough to recur in a significant proportion of the population of a species.

1.8   List of Abbreviation

'A' Slide-   Archived Slide

'R' Slide-   Read Slide

ACT- Artemisinin Combination Therapy

ACT- Artemisinin Combination Therapy

ART-SP-   Artesunate-Sulphadoxine-Pyrimethamine

CDC- Centers for Disease Control

CMUL-   College of Medicine University of Lagos

CQ-   Chloroquine

CT- Threshold cycle

DHFR-   Dihydrofolate reductase

DHPS-   Dihydropteroate synthase

DNA- Deoxyribonucleic acid

DTET-   Drug therapeutic efficacy trial

FR1- Fragment 1 *Pfmdr1* gene

FR3- Fragment 3 *Pfmdr1* gene

FR4- Fragment 4 *Pfmdr1* gene

IMCI- Integrated Management of Childhood Illness

IRS-   Indoor Residual Spraying

LLIN- Long Lasting Insectiscide Nets

LSHTM- London School of Hygiene and Tropical Medicine

PBS- Phosphate buffer solution

PCR- Polymerase chain reaction

PFCRT-   *Plasmodium falciparum* chloroquine resistance transporter

PFDHFR-   *Plasmodium falciparum* **d**ihydrofolate reductase

PFDHPS- *Plasmodium falciparum* dihydropteroate synthase

PFMDR1- *Plasmodium falciparum* multi-drug resistance

qPCR- Quantitative polymerase chain reaction

RBC- Red Blood Cell

RDT- Rapid Diagnostic Test

SNP - Single nucleotides polymorphisms

SOP- Standard Operating Procedures

SP- Sulphadoxine-Pyrimethamine

TBE- Tris-borite buffer

TDRL- Tropical Disease Research Laboratory

CHAPTER TWO

2.0   LITERATURE REVIEW

The Malaria Parasite

Malaria is a parasitic disease caused by members of the genus *Plasmodium*. Classically, four species have been considered to infect humans, i.e. *Plasmodium falciparum, P vivax, P malariae* and *P ovale*. However, *P knowlesi* which has monkeys as natural host has been proposed as the fifth human malaria parasite (Cox-Singh *et al.*, 2008; White, 2008). The fifth human malaria species differ in morphology, life cycle and clinical presentation/severity. *P. falciparum* is the most predominant parasite species in Nigeria accounting for about 98% of malaria cases in the country (FMOH, 2005) and is the most virulent and drug resistance associated human malaria parasite and as such responsible for a great majority of the global malaria related morbidity and mortality. *P. malariae* and *P. ovale* usually occur as a mixed infection with *P. falciparum*. Thirty-seven *Anopheles* species have been documented in the country as vectors that transmit malaria. Among these, *Anopheles gambiae* is the main vector, but *An. funestus* and *An. arabiensis* are also commonly encountered, while *An. melas* is found in the coastal areas (FMOH, 2005).

Life Cycle of the Malaria Parasite

The life cycle requires the presence of three players, i.e. the parasite, (agent), the *AnIopheles* mosquito (vector) and man (host). However considering that the sexual recombination of the parasite takes place within the gut of the mosquito, the arthropod vector by definition also acts as the definite host. Consequently, man is defined as an intermediate host.

Malaria Prevalence in Sub-Saharan Africa

Malaria is one of the most prevalent human infections worldwide. Over 90% of all malaria cases occur in Africa and ninety percent of death occurs in sub-Saharan Africa. Sixty percent of malaria deaths worldwide occur in the poorest 20% of the population (Suh *et al.*, 2004). Malaria disease is causing nearly a million deaths, mostly of children under 5 years (WHO, 2008a). About 91% or 230 million (175–300 million) of them were due to *P. falciparum*. The vast majority of cases (86%) were in the African Region. The percentage of cases due to *P. falciparum* exceeded 75% in most African countries but only in a few countries outside Africa (WHO, 2008a). All age groups may be at risk of severe disease during malaria epidemics in non endemic regions, which occur either when there are changes in the physical environment (caused by climatic variation, agricultural projects etc), increased number of mosquitoes to transmit the disease or when there is population displacement.

Malaria has been reported to be associated with 30-60% of all fevers (Brinkmann and Brinkmann, 1991). Murphy and Breman (2001) showed that the annual incidence of malaria febrile episodes is 1.6-5.4 per African child < 5 years old; this shows that 150.9 − 509.4 million febrile malaria episodes occur in children that are at risk each year. In malaria endemic areas, children suffer an average of six bouts of malaria infection which makes it a common cause of school absenteeism (WHO, 2000). At least one third of primary school children in endemic rural areas miss a week of school time due to malaria disease (RBM, 1999). Malaria is the most prevalent infectious disease in sub-Saharan Africa and these prevalence rates differ from country to country. Mortality is predominantly among children that are five years and below (WHO, 2010).

Malaria Prevalence in Nigeria

Nigeria accounts for a quarter of all malaria cases in the WHO African Region. Transmission in the south occurs all-year round, and is more seasonal in the north. Almost all cases are caused by *P. falciparum* but most are unconfirmed (Salako *et al.*, 1990; WHO, 2008a). Malaria has remained a major public health problem in Nigeria and it is responsible for 30% of childhood mortality and 11% maternal mortality. More than 70% out patient visit in Nigeria is as a result of malaria. Indeed, the disease has impacted negatively on the country's economy with a reported loss of One Hundred and thirty-two billion naira (132 billion Naira) as cost of treatment and loss in man hours (FMOH, 2005). In Nigeria, malaria is directly responsible for over one million deaths of children below school age yearly and one quarter of average family income is spent on the treatment of malaria (Etuk and Umoh, 2001).

Malaria is endemic throughout Nigeria and constitutes the leading cause of death in children. Although, it affects all ages, cases in children under the age of five are more likely to be serious, reflecting their relative lack of immunity to the diseases compared with adults. It is characterized by a stable, perennial transmission in all parts of the country. Transmission is however higher in the wet season than in the dry season. In small part of the southern geographical region of the country, transmission is high all year round. While in the remaining part of the country, the duration of the transmission is 3-10months from February to December (FMOH, 2005).

Although, malaria is considered homogenous throughout Nigeria, minor differences occur among the different ecological zones of the country. In the forest zone of southern Nigeria, malaria transmission is perennial and morbidity is 30-50% of febrile cases spread throughout the year. The wet savannah of the middle belt zone has regular long seasonal transmission (about

seven months) with morbidity increasing during the rainy season. There is the steppe savannah of the far north where there is short seasonal transmission (Salako *et al.*, 1990).

In Enugu State, South-East, Nigeria, it was reported, that, of two thousand children case notes reviewed, 7.7% of the children were diagnosed for malaria (Okafor and Oguonu, 2006). The prevalence of *Plasmodium species* among blood donors in Imo state another South-East Nigeria showed that 77.4% of the blood donors were positive for malaria parasites and *P falciparum* was the main species of malaria parasites reported in the study (92.5%) (Mbanugo and Emenalo, 2004).

Gellert *et al.*, (1998) reported malaria prevalence in paediatrics patients in Maiduguri, Northeastern part of Nigeria. Highest malaria prevalence was seen in October (25.8%) and the lowest in March (3.96%). The work also reported that higher mortality was associated with coma, convulsions, hepatosplenomegaly, pulmonary congestion, jaundice, haemoglobinuria, bladder paralysis, anuria and anaemia.

Malaria prevalence in 300 paediatric age group studied in Jos had a prevalence of 27-29.5% in which 18% had mean parasite density greater than critical value of 10,000 per microlitre (Ikeh *et al.*, 2002). Another malaria prevalence study in children in Ilorin, North-Central part of the country, reported that out of the children studied, the proportion of children with severe malaria was significantly higher among the under fives compared to those over five years. 33.7% of the children studied had severe malaria with cerebral malaria carrying the greater risk of fatality (Olarewaju and Johnson, 2002). In Delta State, South-South, Nigeria, a malaria prevalence of 30.4% was reported where blood donors were examined for malaria parasites (Nmor and Egwunyenga, 2004).

14

The frequency of asymptomatic malaria parasitaemia was investigated by Salako *et al.* (1990) in South-Western Nigeria, where it was detected that lower parasite rate occurred in January while the highest was in July (corresponding to the mid-dry and wet seasons respectively). Out of the clinically diagnosed patients, 57.4% were found to have parasitologically proven malaria. Among secondary school students in urban and rural areas of Ibadan, 8% and 27% were reported for urban and rural malaria prevalence respectively (Ademowo *et al.*, 1995). A total of 446 infants in the first 6 months of life who presented at an urban children's hospital with complaints of any illness whatsoever were recruited into a study with aim of determining the contribution of malaria to infants morbidity in a malaria endemic urban area (Lagos State) in Nigeria. Overall, 107 (24.0%) infants were clinically diagnosed as having malaria (Afolabi *et al.*, 2001).

Clinical Features of Malaria in Infants and Children

Infants are vulnerable to malaria from the age of approximately three months, when immunity acquired from the mother is wearing off. Hospital-based studies showed that in areas of intense transmission, most cases of severe malarial anaemia, blood transfusions and deaths occur in infants (Schellenberg *et al.*, 1999; Kahigwa *et al.*, 2002) and children less than five years old (Biemba *et al*, 2000).

Malaria causes anaemia through haemolysis and increased splenic clearance of infected and uninfected red blood cells and cytokine induced dyserythropoeisis (Nagel, 2002; Ekvall, 2003). A single, overwhelming episode of malaria, or repeated episodes due to reinfection or failure to adequately clear parasitaemia as a result of antimalarial drug resistance (Bjorkma, 2002) may result in life-threatening anaemia, metabolic acidosis and if untreated deaths. Half of the childhood death due to malaria in Africa was probably attributed to severe anaemia (Murphy and Breman, 2001).

Uncomplicated or Mild Malaria

The erythrocytic cycle in falciparum malaria is usually synchronized, thus the initial stage of infection; fever occurs in day one and day 3 (and thus is a tertian fever). In advance infections the pattern of fever becomes less regular, and could be continuous. Nausea, vomiting, diarrhoea, and abdominal pain may accompany fever (Murphy and Breman, 2001). In an uncomplicated infection, signs are few, with the notable absence of lymphadenopathy or rash, but may include splenomegaly and mild jaundice. If the course of treatment is incomplete or if the parasites are resistant to the treatment given, then the parasites may recrudesce and once more cause a patent infection (Murphy and Breman, 2001).

Severe/Complicated Malaria

Severe malaria as defined by the WHO, refers to a parasitaemic person with one more of the following: prostration (inability to sit up without help), impaired consciousness, respiratory distress or pulmonary oedema, seizures, circulatory collapse, abnormal bleeding, jaundice, haemoglobinuria or severe anaemia (haemoglobin < 50 g/L or hematocrit <15%) (WHO, 2000).

Prostration and altered consciousness occur frequently in both children and adults with severe disease: respiratory distress, seizures and severe anaemia are common in children (WHO, 2000). Children who live in endemic area are at high risk of complications. Complications generally involve the central nervous pulmonary, renal and hematopoietic system. Hypoglycaemia occurs because of parasite consumption of glucose and treatment with quinine. Acidosis is another common metabolic derangement. Severe anaemia, acute renal failure respiratory failure, intravascular haemolysis and coagulopathies and shock may develop (Suh *et al.*, 2004).

Severe anaemia is the most common complicated of chronic malaria in children, with haematocrit not infrequently approaching 15%. Massive splenomegaly causing abnormal pain

may be associated with both bone marrow and immune dysfunction. Nephrotic syndrome has also been attributed to falciparum malarial in endemic areas (Taylor and Strickland, 2000). Severe anaemia is more common in areas of high transmission, while cerebral malaria or impaired consciousness' is more common in areas of low or unstable transmission (Snow *et al.*, 1997). However, in a study among paediatric patients, both severe malaria anaemia and impaired consciousness may be an important complication even in areas of high transmission (Idro *et al.*, 2006). The study also showed that severe malaria under high transmission affected much younger children compared to areas with moderate or low transmission (Idro *et al.*, 2006). On the contribution of malaria to infants morbidity in a malaria-endemic urban area of Nigeria, it was showed the percentage of infants with anaemia increased as the parasites density increased; and malaria was reported to be the most common cause of anaemia among the infants (Afolabi *et al.*, 2001).

Impaired consciousness is reported to be one of the manifestations of severe malaria in children under 5years of age; increasing with rising transmission intensity and parasite load while respiratory distress occurs with similar frequency in areas with very low, moderate and very high transmission (Idro *et al.*, 2006). The median age of presentation with severe malaria decreases with increasing transmission intensity. Heavy *P falciparum* malaria parasitaemia may be important in the development of seizures, severe anaemia and impaired consciousness, but may not be necessary in the development of respiratory distress (Idro *et al.*, 2006).

Fever

Fever is a cardinal symptom of malaria though it may be absent in congenital and neonatal malaria. Classically, it is described as paroxymal high fever with varying intervals in between episodes (Lokeshwar, 2000). Fever reduces appetite, and exacerbates malnutrition. It is the most

common clinical feature of malaria in the human host. The major clinical features of falciparum malaria are not related to the level of the fever. Clinical features range from mild problems (such as low-level fever and headache) to more severe problems (such as prostration coma and acidosis). Severity is not necessarily associated with higher parasitaemia but often relates to the immune status (Winstanley *et al.*, 2004).

In the first attack of *P. falciparum*, fever is usually irregular rather than occurring with a regular repeating pattern as seen with a tertian fever in subsequent attacks and there are usually no relapses unlike with *P. ovale* and *P. vivax* where hypnozoites are formed (Theresa, 2005). Fever is not always present and rigors may or may not be present. The temperature may rise above $41^{o}C$, over several days, and the fever is produced as the schizonts mature, at 48hours intervals usually for *P falciparum*. The different malarias produce fevers of different frequency depending on how long it takes to complete schizogony in erythrocytes (Theresa, 2005).

Fever or a recent history of fever (usually above $38^{0}C$) is almost always present, but rarely in the classic tertian (occurring every 48 hours) or quartan (every 72 hours) pattern (Winters and Murray, 1992). Fever and splenomegaly are the most frequent physical findings in a study of malaria presentation. Less often, hepatomegaly, jaundice and abdominal tenderness are noted (Suh *et al.*, 2004). In Niamey, Niger, the incidence of fever was reported as 91% of malaria cases in children. These symptoms were often combined and the most common combination was fever and digestive disorders (Gay Andrieu *et al.*, 2005). Great majority of disease episodes in young children studied were associated with fever and about half were attributed to falciparum malaria (Muller *et al.*, 2003). Fever and malaria episodes were frequently accompanied by diarrhoea, vomiting and cough. In another finding, fever was shown to be the main clinical presentation for malaria in endemic areas (Runsewe-Abiodun *et al.*, 2006).

Cerebral Malaria

Approximately 7% of children who survived cerebral malaria (a severe form of the disease, characterized by coma and convulsions) are left with permanent neurological problems and epilepsy. The earliest symptom of cerebral malaria in children is usually fever, which is followed by failure to eat or drink. Most but not all children with cerebral malaria are febrile, rectal temperatures ranges from 36 to 41°C or even higher. The signs of 'cerebral malaria' are reduced consciousness and coma. Loss of consciousness can develop very rapidly, most of the patients being in deep coma by the time they reach the hospital.

The pathology of cerebral malaria is linked with the sequestration of parasitized erythrocytes in postcapillary venules of the cerebral circulation (Newton *et al*., 2000), although the functional disturbances that lead to an encephalopathy are poorly understood. In Nigeria as with high stable malaria endemicity, severe malaria occurs commonly in children aged six months to five years and in pregnant women. The most common and most fatal form of severe malaria is said to be cerebral malaria, followed by anaemia (Salako *et al*., 1990).

Murphy and Breman, (2001), in their review reported that 10-17% of survivors of cerebral malaria experience some form of neurological impairment, one quarter of which persist greater than six months. The annual incidence of neurological sequelae was calculated from the cerebral malaria survival rate (4.9 survivors per 1,000 children less than 5years).

Children who recover from cerebral malaria usually do so within 48 hours, although a significant minority suffers from neurological sequelae. Up to 15% of children may have hemiplegia, ataxia, dysphasia, hearing difficulty, visual problems including cortical blindness, or epilepsy. Over half of these children make a full recovery, but there is a residue of serious disability caused by

cerebral malaria, including abnormal cognitive function and behaviour (Newton and Krishna, 1998; Holding and Snow, 2001).

The highest specific prevalence of cerebral malaria was found in children aged >12 months. Sixty five percent of cases of cerebral malaria occurred in children aged between 12-48months (Dzeing-Ella *et al.*, 2005). Cerebral malaria is often associated with other serious systemic complications. Seizures are a prominent feature of cerebral malaria. Children may suffer simple febrile seizures, have prolonged or multiple seizures, or have features suggesting a focus of epileptic activity, including localized motor seizures. Diagnosis has showed that in cerebral malaria generalized seizures may occur with minimal physical signs, such as twitching of fingers, conjugate or nystagmoid deviation of the eyes, or hypoventilation with excessive salivation (Miller *et al.*, 1971).

Findings had shown that most deceased children with malaria diagnosis showed a typical signs and symptoms of cerebral malaria before death (Muller *et al.*, 2003). This finding is however, not in agreement with the opinion of severe anaemia being the most common cause of malaria deaths in areas of high transmission intensity but could probably reflect the influence of a more seasonal distribution of transmission (WHO, 2000). In cerebral malaria, as unconsciousness deepens, the patient fails to localized pain and may demonstrate abnormal posturing (decorticate rigidity, decerbrate rigidity and opisthotonus) papillary changes, absent corneal reflexes and abnormal respiratory patterns including hypoventilation and periodic breathing (Newton and Warell, 1998).

Anaemia

It is an important and commonly life threatening complication of falciparum malaria in children. It occurs as a result of destruction of parasitised red blood cell by the spleen, tumour necrosis factor mediated depression of erythropoiesis and immune mediated haemolysis. The rate of development and degree of anaemia depend on the severity and duration of parasitaemia. Acute and repeated malaria infections can lead to anaemia in sub-Saharan African children (Newton *et al.*, 1998). Malaria induced anaemia are classified by WHO diagnostic guidelines: mild anaemia is defined as haemoglobin concentration <110 g/L or haematocrit <33%; severe anaemia is haemoglobin <50 g/L or haematocrit <15% (Newton *et al.*, 1997). Mild or severe anaemia accompanied by *P. falciparum* infection is designated uncomplicated and severe malarial anaemia, respectively. However, the connection between malaria and anaemia is difficult to define because there are cases of people experience parasitaemia in the absence of malaria disease, whereas some become anaemic as the parasites are cleared (Newton *et al.*, 1998).

Although, other concurrent health conditions like malnutrition, Human Immunodefiecency Virus (HIV) and hookworm infection lead to anaemia in children, evidence suggest that, in endemic countries, malaria is one of the most important factors. Antimalarial drug resistance exacerbates the situation by increasing the proportion of children who fail to adequately clear parasitaemia after treatment, and who consequently remain anaemic (Murphy and Breman, 2001). It has been estimated that severe malarial anaemia causes between 190,000 and 974000 deaths each year among children less than 5years (Murphy and Breman, 2001). Malnutrition, haemoglobinopathies and other disorders are known to contribute to anaemic in Africa and thereby making the differential diagnosis of malarial anaemic difficult. (Newton *et al.*, 1997; Newton *et al.*, 1998).

In severe anaemia, malaria gives ample reasons for both increased destruction and reduced production of red cells. Red blood cells are destroyed as parasites complete their growth cycle, although, some parasites may be removed from erythrocytes as immature ring forms by phagocytic cells (Angus *et al*., 1997). Infected erythrocytes may also be phagocytosed by macrophages following opsonization by immunoglobulin and/or complement components other effector cells and mechanism, may include antibody-dependent cytotoxicity and natural killer cells (Casals-Pascual and Roberts, 2002). The survival of uninfected erythrocytes is reduced and the activity and number of macrophages are increased in malaria infection. Moreover, the signals for recognition of uninfected erythrocytes for removal by macrophages are enhanced (Casals-Pascual and Roberts, 2002). Reticulocytopenia has also been observed in numerous clinical studies of malaria anaemia. The histopathological study of the bone marrow of children with malaria anaemia shows erythroid hyperplasia, with dyserythropoiesis (Abdallam et al., 1980; Helleberg., *et al* 2005).

Severe anaemia was the most frequent feature of severity in a study done in Gabon, but it was associated with decreased mortality (Dzeing-Ella *et al*., 2005). A similar study in Ghana showed a better outcome in children with anaemia where findings confirmed that severe malaria anaemia has a lower case fatality rate than other complications of severe malaria (Mockenhaupt *et al*., 2004).

Severe malaria anaemia was common in area with very high transmission intensity and parasite load (Idro *et al*., 2006). Gay-Andrieu *et al*., (2005) in their finding reported that severe anaemia is associated with severe cases of malaria in less than two years old children. The severity of malarial anaemia were said to have probably enhanced by the very precarious nutritional

condition of the infant population. Anaemia was reported to be the most serious and prevalent paediatric malaria complication in Nigeria (Okogun and Amadi, 2005).

Respiratory Distress and Metabolic Acidosis

Respiratory distress affects children that of younger age, mostly less than 35months (Marsh *et al.*, 1995). It is defined by tachypnea, by deep gasping breathing and use of secondary muscles of respiration and usually represents metabolic acidosis (English *et al.*, 1996). Excessive lactic acid leads to acidosis. Some children presenting with respiratory distress are dehydrated and may be resuscitated with saline. However, it has been discovered that the majority of children presenting with respiratory distress are severely anaemic, have a metabolic acidosis secondary to reduced oxygen-carrying capacity (English, 2000).

Respiratory distress is associated with severe malaria (Gay-Adrieu *et al.*, 2005). More respiratory distress was reported in severe malaria and significantly associated with both hyperlactataemia and cerebral malaria (Dzeing-Ella *et al.*, 2005). Metabolic acidosis and hyperlactataemia have been reported to be associated with severe malaria (Krishina *et al.*, 1994).

Hyperlactataemia was defined in a study as a blood lactate concentration higher than the conventional cut-off (>5 mmol/L) (Dzeing-Ella *et al.*, 2005). Hyperlactataemia is a frequent and serious complication of severe malaria in childhood (Krishina *et al.*, 1994; Planche *et al.*, 2003), which may be due microcirculatory sequestration of parasitised erythrocytes resulting in increased production of lactate by anaerobic glycolysis (Newton and Krishna, 1998).

Hypoglycaemia

This is diagnosed as blood glucose <40 mg/dL (2.2 mmol/L) and it may accompany cerebral or other forms of malaria (Solomon *et al.*, 1994; Marsh *et al.*, 1995). It is commonly recognized as a complication of falciparum malaria.

Hypoglycaemia occurs as a result of decrease intake, increased glucose, utilization, antimalarial mediated reduction, glycogen depletion or impaired gluconeogenesis. Hypoglycaemia is common in children and pregnant women with severe diseases (Molyneux *et al.*, 1989). Estimates of the prevalence of hypoglycaemia have been reported in Africa. It ranges from 8% to 34% (Walker *et al.*, 1992; Taylor *et al.*, 1998). Hypoglycaemia complicates malaria in patients with severe disease, especially young children, in patients given quinine or quinidine, and in pregnant women. In severe childhood malaria hypoglycaemia results from impaired gluconeogenesis and increased tissue demand for glucose (Agbenyega *et al.*, 2000) and quinine induced hyperinsulinaemia. It is therefore of importance that blood glucose concentrations should be monitored in all children hospitalized for malaria especially those who receive quinine.

Hypoglycaemia is an important complication of falciparum malaria. The clinical picture includes deteriorating consciousness, generalized seizures, extensor posturing, shock and coma. The diagnosis is easily overlooked because all the clinical features also occur in cerebral malaria itself. Deterioration in consciousness may be the only sign and it is therefore important to confirm by biochemical test (Taylor *et al.*, 1998).

Seizures

It is a common feature of cerebral malaria in both children and adult. Children may have febrile seizures from which they recover quickly without prolonged encephalopathy (Crawley *et al.*,

2000) or have prolonged and multiple seizures, or have features suggesting a focus on epileptic activity, including localized motor seizures (Crawley *et al*., 1996).

When severe malaria was compared with age and parasite density in Uganda, It was reported in the study that in high transmission areas, seizures were most prevalent in children 1-2 years with proportion of those with seizures declining rapidly among 3-4years olds. In areas with either very low or moderate transmission, seizures were most common in children 2-3years of age and the decline in the proportion of children with seizures was less precipitous than in high transmission areas. It was suggested in the study that high parasite density and young age may predispose children to seizures and that children in very high transmission areas may be most at risk (Idro *et al*., 2006).

Comparisons between the different types of malaria parasites provided further evidence to suggest that *Plasmodium falciparum* parasites may be epileptogenic. Seizures are more common in falciparum than in vivax malaria despite a similar febrile response (Wattagoon *et al*., 1994). The sequestration of such high parasite loads in cerebral vessels may be responsible for initiating pathological mechanisms that may result in seizure disorders (Gimenez and Barraud, 2003).

Convulsions occur in the majority of children with cerebral malaria. Generalised convulsions are more common than partial seizures (Gilles, 1991). Possible causes of these convulsions include cerebral hypoxia associated with cerebral malaria, fever, hypoglycaemia, and other metabolic disturbances, such as lactic acidosis, antimalarial drugs and Reye's syndrome in children (Taylor *et al*., 1998).

It has been shown that generalized seizures may occur with minimal physical signs, such as twitching of fingers, conjugate or nystagmoid deviation of the eyes or hypoventilation with

excessive salivation. Such patients may recover consciousness after anticonvulsant therapy (Crawley *et al.*, 1996).

Immune Response

In areas of endemicity, the age specific distribution of malaria-associated morbidity and mortality is a function of malaria transmission pressure and the rate of development of naturally acquired immunity. In areas of intense malaria transmission, children 3-18 months old have the most malaria-associated morbidity and mortality, and older children who survive have typically acquired sufficient immunity to be protected from severe disease (Bloland *et al.*, 1999; McElroy *et al.*, 2000).

Charlwood *et al.*, (1998) showed that incidence of malarial infection increases with entomological inoculation rate (EIR), until saturation at approximately one infectious bite/person/night, the dynamics of the relationship between exposure and malaria-associated morbidity and mortality has been a subject of debate. Community-based studies from Senegal suggest that transmission intensity affects the age distribution of malaria attacks rates but not the total number of lifetime attacks (Trape and Rogier, 1996).

Another study of malaria-associated mortality rates in relation with different transmission intensities proposes that malaria-associated mortality plateaus or declines above certain transmission level (Snow and Marsh, 1995). It has also been reported that individuals living in endemic regions develop immunity to infection and disease with age and increased experience with blood stage parasitaemia (Branch *et al.*, 1998). It is however of importance to understand how protection against malaria develops in young children and how this can be booted.

The roles of placental malaria on infants' malaria morbidity and mortality have also been assessed. Evidence from reports demonstrates that placental malaria can increase the risk of malaria infection and morbidity in the first year of life (Le Hesran *et al*., 1997; van Eijk *et al*., 2003). A study reported that placental malaria infection diminished the development of the severe malaria epitopes in the first year of life, the age at which most of the severe malaria-associated morbidity occurs in areas of holoendemicity (Bonner *et al*., 2005).

Diagnosis

A high degree of suspicion and rapid diagnosis are essential to optimize outcome. Diagnostic tests for malaria include standard thick and thin blood smears, rapid antigen detection tests, direct observation of stained blood specimen to sophisticated and staining techniques (Quantitative Buffy coat ®, acridine orange method), PCR (Polymerase chain reaction) and antibody tests. Thick and thin peripheral blood smears, stained with Giemsa stain (or, alternatively, Wright's or Field's stains), remain the "gold standard" for routine clinical diagnosis. Malaria smears permit both species identification and quantification (expressed as a percentage of erythrocytes infected as parasites per microlitre) of parasites.

Rapid malaria tests which require minimal skill to perform and interpret have been developed to overcome the problems of malaria smears. The most practical of these are the rapid antigen detection tests (RDTs) which detect parasite proteins in finger-prick blood samples (Moody, 2002). Most of these tests will differentiate between *P .falciparum* and non-falciparum infections through the detection of histidine-rich proteins and/or parasite lactate dehydrogenase. These tests have shown mixed results in multiple trials, but several seem to compare favourably with blood smears when performed by trained personnel under laboratory conditions (Moody, 2002). A non-immune, febrile child who may have been exposed to *P .falciparum* represents a medical

emergency; an aggressive inpatient evaluation should be performed, regardless of the child's appearance (Stauffer and Fischer, 2003).

Malaria Treatment

The objectives of an antimalarial treatment policy are to: ensure rapid cure of the infection; reduce morbidity and mortality, prevent the progression of uncomplicated malaria into severe and potentially fatal disease; prevent the emergence and spread of drug resistance; and prevent malaria in travellers (WHO, 2008a).

During the 1980s the chloroquine resistance spread to Africa and in the 1990s most countries changed their first line therapy from CQ to SP. However, in contrast to CQ which remained efficacious in decades, resistance SP was reported already after a few years. The development and expansion of resistance to these mainstay antimalarials caused a major increase in morbidity and mortality of *P falciparum* malaria in Africa (Bjorkman and Bhattarai, 2005). Knowledge from combination therapy against cancer, HIV and tuberculosis, encouraged the suggestion on antimalarial combination therapy (White, 1999). The rationale for combination therapy is that the probability of the parasite to develop resistance to both drugs is the product of probability to develop resistance to each of the drugs. This is provided that the two drugs have different mode of action, as compared with SP. With the rediscovery of the Chinese traditional remedy artemisinin (*Qinghaosu*), the concept of ACT was launched, which is recommended as first line therapy against umcomplicated *P falciparum* malaria (WHO, 2009).

Antimalarial Drugs

Malaria is both preventable and curable through prompt and effective chemotherapy. However, the arsenal of antimalarial drugs is limited. These include the aminoquinolones (CQ,

amodiaquine and primaquine), the arylaminoalcohols (mefloquine, lumefantrine, halofantrine, quinine), the antifolates (SP, chlorproguanil-dapsone), the atovaquone, the artemisini derivates (AS, artemether, dihydroartemisinin) and antibiotic (tetracycline, clindamycin).

Chloroquine

Chloroquine is a 4-aminoquinoline that has been used extensively for the treatment and prevention of malaria. CQ has marked and rapid shcizonticidal activity against all infections of *Plasmodium* and it has gametocytocidal against immature *P. falciparum* gametocytes and other forms of other species. Widespread resistance has now rendered it virtually useless against *P. falciparum*. It has been shown that the primary driving force for drug accumulation is binding to ferriproptoporphyrin IX (heme), a byproduct of haemoglobin degradation. The formation of CQ heme complexes is important for drug activity. The CQ binds to heme, preventing detoxication of heme by crystallization into malaria pigment (or haemozoin). The CQ-heme complex that retains its cytotoxicity potential thus accumulates to a level capable of killing the parasite.

Phenotypically, CQ resistance is characterized by reduced cellular accumulation of the drug. This phenotype can be partially reversed by agents such as verapamil, so called resistance-reversing agents (Winstanley *et al.*, 2004). Point mutations in the gene encoding a food vacuole transporter protein (*Pfcrt*) have been linked to CQ resistance and this correlate with reduced CQ efficacy (Winstanley *et al.*, 2004). In presence of *Pfcrt* mutations, mutation in the second transport genes (*Pfmdr* 1) further modulates resistance *in-vitro*.

CQ is rapidly and almost completely absorbed from the gastrointestinal tract when taken orally. Absorption is also very rapid following intramuscular and subcutaneous administration (White *et al.*, 1988). It is extensively distributed into body tissues, including the placental and breast milk.

About 60% of CQ is bound to plasma proteins and the drug is eliminated slowly from the body via the kidneys, with estimated elimination half life of 6-10 days. CQ is metabolized in the liver, mainly to monodesethylchloroquines, which has similar activity against *P. falciparum*.

Chloroquine is generally well tolerated, but when concentrations in plasma exceed 250μg/ml, unpleasant symptoms such as dizziness, headache, and gastrointestinal disturbances may occur. The limiting adverse effects are unpleasant taste, which may upset children and pruritus, which may be severe in dark-skinned patients (Mnyika and Kihamia, 1991).

Formulations of CQ are in tablet, syrup and injectable form. Tablets containing 75mg, 150mg and 300mg base (as diphosphate or sulphate) are available. Syrup containing 50mg base (as diphosphate or sulphate) in 5ml is the standard. The injection is available as solutions containing 40mg base per ml in 5ml ampoule.

Chloroquine is no longer recommended in Nigerian as a first line medicine because of the high resistance. The recommended dosage is 25mg/kg of body weight orally over 3 days for both children and adults. The regimen in use in many areas consists of 10mg/kg on first and second days and 5mg/kg on the third day.

Amodiaquine

Amodiaquine is a 4-aminoquinoline antimalarial drug similar in structure and mode of action to CQ. Like CQ it also possess antipyretic and anti inflammatory properties. It is effective against some CQ-resistant strains of *P. falciparum*. Review of studies on treatment of uncomplicated falciparum malaria conducted in Africa showed that amodiaquine proved significantly more effective than chloroquine in clearing parasites, with tendency for faster clinical recovery.

Amodiaquine hydrochloride is readily absorbed from the gastrointestinal tract. It is rapidly converted in the liver to the active metabolite desethylamodiaquine, which contributes nearly all of the antimalarial effect (Winstanley *et al.*, 2004). Both amodiaquine and desethylamodiaquine have been detected in the urine several months after administration. It has similar adverse effect to that of chloroquine.

Amodiaquine is associated with less pruritus and is more palatable than CQ, but is associated with much higher risk of agranulocytosis and hepatitis when used for prophylaxis (Hatton *et al.*, 1986). It is available in both tablet and suspension form. Tablets containing 200mg amodiaquine base as hydrochloride or 153.1mg base as chlorohydrate. Amodiaquine suspension contains 10mg/ml of base as hydrochloride or chlorohydrate. It has half life of 10hours following intravenous administration, while desethylamodiaquine has half life of 18-20housr. It is no longer used for prophylaxis or even as an alternative treatment because of risk of severe reactions.

Sulfadoxine-Pyrimethamine (SP)

The antifolates are nearly always used in fixed-ratio combinations. The SP is the commonly used antifolates. Available combinations include the SP and sulfalene-pyrimethamine, the former being more widely available. The use of this drug for uncomplicated falciparum malaria in many parts of Africa has been stopped due to widespread in malaria resistance to SP in most part of the world. SP combinations are highly active blood schizonticides against *P.falciparum* but are less effective against other *Plasmodium* species. There is no cross-resistance with the 4-aminoquindines, mefloquine, quinine, halofantrine or the artemisinin derivatives. It combinations have been successfully used in areas with highly developed *P.falciparum* resistance to CQ and during malaria epidemics. Compliance is high since they offer single-dose therapy. There is

evidence that folic acid administered concurrently with SP can antagonize the action of sulfadoxine (Winstanley *et al.*, 2004).

Pyrimethamine is well absorbed after oral or intramuscular administration. It is mainly concentrated in kidneys, lungs, liver and spleen and about 90% bound to plasma proteins. Peak plasma concentrations occur 2-6hours after an oral dose. It is metabolized in the liver and slowly excreted via the kidneys. The plasma half life is around 4 days. The elimination half-life in children with malaria averages 81 and 124 hours after oral and intramuscular injection respectively (Winstanley *et al.*, 1992). Sulfadoxine is readily absorbed from the gastrointestinal tract. Peak blood concentrations occur about 4 hours after an oral dose. The terminal elimination half life is 4-9 days. Around 95% is bound to plasma proteins. It is widely distributed to body tissues and fluids, passes into fetal circulation and is detected in breast milk. The drug is excreted in urine, primarily unchanged (WHO, 2010).

The SP group interferes with DNA synthesis by depleting the pool of tetrahydrofolate, an important cofactor. The pyrimethamine is part of group of molecules that acts as competitive inhibitors of the enzyme *Dhfr*. While sulfadoxine is another group of molecules that acts as competitive inhibitor of the enzyme *Dhps*. Pyrimethamine and sulfadoxine both have long elimination half-lives, this equates with a strong selective pressure for resistance (Watkins and Mosobo, 1973). Mutations in the *Dhps* gene correlate with *invtro* sulfonamide chemosensitivity.

Pyrimethamine is well tolerated. Administration for prolonged periods may cause depression of haematopoiesis due to interference with folic acid metabolism. Administration of pyrimethamine with other folate antagonists such as cotrimazole trimethoprim, methotrexate or with phenytoin may exacerbate bone marrow depression. Formulations are available in tablet, suspension and

injectable form. Tables contain 500mg sulfadoxine and 25mg pyrimethamine. Ampoules contain 500mg sulfadoxine and 25mg pyrimethamine in 2.5ml injectable only solution (WHO, 2010).

Quinine

Quinine acts principally on the mature trophozoites stage of parasite development and does not prevent sequestration or further development of circulating ring stages of *P falciparum*. It also acts on the sexual stages of *P vivax, P malariae* and *P ovale*, but not mature gametocytes of *P falciparum*. It does not kill pre-erythrocytic stages of malaria parasites. Parenteral quinine is the drug of first choice for severe malaria, and oral quinine is an option for treatment of uncomplicated malaria. It can be combined with another drug especially where some degree of quinine resistance may be present. It is a reasonable option for treatment of travelers returning to non-endemic areas who develop malaria, since the drug-resistance pattern of the parasites may not be known (WHO, 2010).

Injectable quinine given by intramuscular route can be a valuable initial treatment for a patient with uncomplicated malaria, repeatedly vomiting and unable to take oral drugs. Quinine can be used as a second line treatment for patients who fail to respond to standard first line therapy. When used in this way it should be accompany by another drug. It is usually combine with tetracycline or doxycycline, but these are contraindicated drugs in children and pregnant women. Clindamycin can however, be used for these group. The drug is approximately use under supervision of a qualified person. It is similar to mode of action in CQ. Quinine interferes with parasite metabolism of heme, a toxic product of haemoglobin digestion (WHO, 2010).

Quinine pharmacokinetics is altered significantly by malaria infection, with reductions in apparently volume of distribution and clearance in a proportion to disease severity (Krishna and

White, 1996). In children less than 2 years of age with severe malaria, concentrations are slightly higher than in older children and adults (Winstanley *et al.*, 2004). It is a rapidly and almost completely absorbed from the gastrointestinal tract and peak plasma concentrations occur 1-3 hours after oral administration of the sulfate or bisulfate (Winstanley *et al.*, 2004). It is well absorbed after intramuscular injection in severe malaria (White, 1995). Plasma protein binding, mainly to alpha 1-acid glycoprotein is 80% in healthy subjects but rises to around 90% in patients with malaria. It is widely distributed throughout the body including cerebrospinal fluid, breast milk and placental. Excretion is increased in acid urine. In adults with uncomplicated malaria, elimination half life is 16 hours, longer than in healthy person (11hours) and 18 hours in severe malaria (White, 1995).

Different tablets and injectable formulations of quinine salts are available. The most common are quinine hydrochloride, quinine dihydrochloride, quinine sulphate and quinie bisulphate. The tablets contain 300mg base. Injections contain 300mg in 2ml ampoules. Both intramuscular and intravenous infusion can be used. For intramuscular administration, dilution should be sterile water or saline (and not dextrose), 60mg/ml for children and 120mg/ml for adults. Recommended by this route is 10mg/kg of body weight, repeated after 4 hours and then 8 hourly for adults and 12 hours in children. A loading dose not recommended by this route. Quinine in children is given with a loading dose of 20mg dihydrochloride salt/kg of body weight diluted in 10ml isotonic fluid/kg by intravenous infusion over 4 hours, then 12 hours (8 hours for adults) after loading dose give maintenance dose of quinine, 10mg salt/kg over 2 hours. It should be repeated every 12 hours (8 hours for adults) from the beginning of previous infusion until the patient can swallow, the quinine tablets, 10mg salt/kg 8 hourly to complete 7days course or treatment, or a single dose of 25mg/kg sulfadoxine and 1.35mg/kg pyrimethamine in areas where

is still sensitive. Because of the known risk of hypoglycaemia the infusion should be in 5% dextrose (WHO, 2010).

Mefloquine

Mefloquine is a 4-aminoquinoline methanol and is related to quinine. It is potent long acting blood schizonticide active against *P falciparum* resistant to 4-aminoquinoline and SP combinations. It is not gametocytocidal and is not active against hepatic stages of malaria parasites. Mefloquine is very effective in combination with artesunate. It is similar to quinine (WHO, 2010).

Mefloquine is well absorbed from the gastrointestinal tract, but there is marked inter individual variation in time required to achieve peak plasma concentration. Mefloquine is highly protein bound (98% in plasma) and is widely distributed throughout the body. The pharmacokinetics can be altered by malaria infection with reduced absorption and accelerated clearance (Krishna and White, 1996). It has a long elimination half life varies between 10 and 40 days in adults but tending to be shorter in children and pregnant women. Mefloquine is metabolized in liver and excreted mainly in the bile and faeces. Tablets contain 274mg mefloquine hydrochloride, equivalent to 250mg mefloquine base. It is not available in parenteral dosage forms (WHO, 2010).

Halofantrine

Halofantrine is a phenanthrene methanol, a blood shcizonticide, active against all malaria parasites. Halofantrine is not active against gametocytes or the hepatic stages of malaria parasites because of its cost, variable bioavailability and cross-resistance to mefloquine. It has no place in malaria control (WHO, 2010).

Halofantrine is largely insoluble in water. It systemic absorption is variable, but increases up to six folds in presence of fatty foods. The elimination life varies but generally between 24-48 hours. The functional elimination half life is 4-5 days. The major route of elimination is the faeces. It is not recommended in children less than 10kg. It is available in tablet form containing 250mg halofantrine hydrochloride (233mg base) and paediatric suspension form containing 100mg halofantrine hydrochloride (93.2mg base) in 5ml i.e. 20mg salt/ml (WHO, 2010).

Lumenfantrine

Lumenfantrine belongs to arylaminoalcohol group of antimalarials, which also includes quinine, mefloquine and halofantrine. It has similar mechanism of action. It is available in combination with artemether. Lumefantrine is incompletely bioavailable from the gut and is eliminated half life of 1-6 days. Lumefantrine is used only in combination with artemether for treatment of uncomplicated multiresistant falciparum malaria. Absorption increases by 108% after a meal and is lower in patients with acute malaria than in convalescing patients. Peak plasma levels occur approximately 10 hours after administration. The terminal elimination half life is around 3 days (WHO, 2010).

Primaquine

Primaquine is 8-aminquinolones and is effective against intrahepatic forms of all types of malaria parasites. It is used to provide radical cure of *P.vivax* and *P. ovale* malaria in combination with a blood schizonticide for the erythrocytic parasites. It is also has gametocyticidal against *P vivax*. Primaquine is readily absorbed from the gastrointestinal tract. Peak plasma concentrations occur around 1-2 hours after administration and then decline with elimination half life of 3-6 hours. Primaquine is widely distributed into the body tissues. It is rapidly metabolised in the liver. The major metabolite is carboxyprimaquine, which may

accumulate in the plasma with repeated administration. It is contraindicated in pregnancy and in children less than 4 years of age because of the risk of haemolysis and in active rheumatoid arthritis and lupus erythematous (WHO, 2010).

Proguanil

Proguanil is metabolise via the polymorphic cytochrome P_{450} enzyme CY_2C_{19} to the active metabolite, cycloguanil. The parent compound has weak intrinsic antimalarial activity through uncommon mechanism. It is not used alone because resistance may develop. Proguanil has marked effect on primary tissue stages of *P.faciparum, P vivax,* and *P ovale*. It does not affect hypnozoites and therefore does not have antireplase activity (WHO, 2010).

Proguanil exhibit weak blood schizonticidal activity. A combination of proguanil with atovaquone, a hydroxynaphthoquinone, has been shown to be effective against multidrug resistant *P falciparum*. Proguanil is a dihydrofolate reductase inhibitor acting primarily through its major metabolite, cycloguanil. It has been used for chemoprophylaxis in combination with CQ in areas of with low prevalence of CQ-resistant *P falciparum* (WHO, 2010).

Absorption is rapid, peak plasma concentrations of proguanil and its active metabolite, cycloguanil, being achieved within 4 hours of administration. It is metaboilised in the liver to the active cyclologuanil and peak plasma levels of cycloguanil occur 1 hour after those of the parent drug. Elimination half life in proguanil and cycloguanil is 20 hours (WHO, 2010).

Atovaquone-proguanil is thought to inhibit mitochondrial respiration. Atovaquone synergizes with proguanil (not with proguanil activite metabolite, cycloguanil) (Canfield *et al*., 1995) and is formulated in fixed ratio tablet. Atovaquone is poorly and variably absorbed and is entirely

eliminated unchanged via the bile into the gut. The elimination half life is long (50-70 hours). Atovaquone-proguanil is used for treatment and prophylaxis of multidrug-resistant falciparum malaria. It is expensive and the drug has little relevance to public health in tropical countries.

Atovaquone

Atovaquone is hydroxynapthoquinone antiparasitic drug active against all *Plasmodium* species. It also inhibits pre-erythrocytic development in the liver, and oocyst development in the mosquito. It can be combined with proguanil for treatment of malaria.

Chlorproguanil

Chlorproguanil is biguanide and is given as the hydrochloride salt. It actions and properties are similar to those of proguanil. It is available only in combination with a sulfone such as dapsone (co-formulated as Lapdap).

Dapsone

Dapsone is a sulfone widely used for treatment of bacterial and parasite infections. For malaria, it is given in combination with antimalarial. It is co-formulated with chlorproguanil. Dapsone inhibits plasmodial dihydropteroate synthase.

Dapsone is almost completely absorbed from the gastrointestinal tract, with peak plasma concentrations occurring 2-8 hours after an oral dose. Dapsone is 50-80% bound to plasma proteins. It is widely distributed to body tissues, breast milk and saliva. Dapsone is mainly excreted excreted in the urine, only 20% as unchanged drug (WHO, 2010).

Artemisinin and its Derivatives

This include: artemether, artesunate and dihydroartemisinin. It is a potent antimalarial compound extracted from *Artemisia annua* (sweet wormwood). It is a potent and rapidly acting blood schizonticide and is active against all *Plasmodium* species. It kills all stages from rings to schizonts, and it also kills gametocytes of *Plasmodium* including gametocyte stage which is only sensitive to primaquine. It is well tolerated and is used in management of uncomplicated malaria in combination with other drugs. A major disadvantage is its relatively high cost (WHO, 2010).

Artemisinin and its derivatives are rapidly hydrolysed *invivo* to dihydroartemisinin. The elimination half life is 1 hour (White, 1994). Peak plasma concentrations occur around 3hours and 11hours following oral and rectal administration respectively. Artemisinin and its derivates are safe and remarkably well tolerated (WHO, 2010).

Antibiotics used as antimalarials drugs

Doxycycline

Doxycycline is derived from oxytetracycline and has identical spectrum of activity. It differs from tetracycline in that it is more completely absorbed and more lipids soluble. It also has a longer plasma life. Doxycycline, like tetracycline can be used for therapy in combination with quinine. Doxycycline should not be used alone for treatment of malaria because of its low action. It is readily and almost completely absorbed from gastrointestinal tract. Peak plasma concentrations are reached around 2hours after oral administration. Doxycycline is bound to plasma protein (80-90%) and has a biological half life of 15-25 hours. It is mainly excreted in faeces (WHO, 2010). It is widely distributed in tissues and body fluids. Other antibiotic use for malaria treatment include; Tetracycline and Clindamycin.

Management of uncomplicated malaria in children

Effective case management of uncomplicated malaria relies heavily on the availability of effective and accessible drugs. Management of uncomplicated malaria is the principal way in which antimalarial drugs are employed and this has greatly reduced morbidity and mortality.

In the setting of a country where malaria is endemic, the choice of drug is determined mainly by availability cost and resistance patterns. Most countries have a committee that coordinate national effort and establish policy. Resistance to CQ is common throughout the tropics and most countries have changed their first-line drug according to WHO guildlines (WHO, 2000). In some places where CQ has been replaced, SP has generally been the next choice of drug. Unfortunately SP resistance is becoming more alarming and is becoming more prevalent in Africa (Winstanley *et al.*, 2004).

In Nigeria, CQ and SP have been the first and second the antimalarial drugs respectively but evidence from local research spanning a period of two decades show that the therapeutic efficacy of these two drugs have deteriorated due to high levels of *P. falciparum* resistance in all parts of the country (Falade *et al.*, 1997; Ekanem *et al.*, 2000).

Artemisinin derivations are extremely effective and safe. However, they must be used in combination with a long acting agent to prevent recrudescence of disease (Stauffer and Fischer, 2003). The artemisinin derivates reduce the parasite biomass by around 4log units for each asexual cycle, and this makes them most rapidly efficacious antimalarial drugs in use. The rapid reduction in the parasite biomass plays major role when artemisinin derivatives are combined with another antimalarial drug (Winstanley *et al.*, 2004). The key goal of artemisinin-based

combination treatment (ACT) is to enhanced cure rates and delay development of parasite resistance to components drugs (White, 1999).

Both combinations (artesunate+sulphadoxine and artesunate+amodiaquine) were found to be highly efficacious in the sampled children in Sudan (Van den Broek *et al.*, 2005). Rapid parasite clearance and fever reduction with the rise in haemoglobin values and reduction of the proportion of anaemic children after treatment confirms that the malaria parasites were effectively removed from the blood and the red blood cell levels rose after treatment. Children aged 6-59 months with signs and symptoms of uncomplicated malaria were randomized into four treatment groups and followed up for a maximum of 28 days showed sensitivity of 100% for amodiaquine+artesunate; 97.5% for artemether-lumefantrine, 60% for SP and 25% for CQ (Koram *et al.*, 2005). Artemether-lumefantrine was also examined in two different groups in Zambia. The drug was given to two dominant groups categories of 5 to 9kg and 10 to 14kg and was reported to be safe and effective in the two groups (Zurovac *et al.*, 2007).

Management of complicated malaria in children
Effective management of severe malaria syndromes is expensive and relies heavily on hospital and human resources. Patients with complicated malaria need immediate parenteral antimalarial drugs and aggressive supportive therapy.

The main antimalarial drugs used for severe falciparum malaria are quinine and the artemisinin class. Management of severe malaria is of importance in order to save life, clearance of parasite and fever clearance. A drug regimen should be chosen that is appropriate for the known local pattern of drug resistance and drug dosage should be calculated by body weight rather than estimated, especially when administering quinine, which have small therapeutic indices.

In severe malaria antimalarial drug should be given parenterally wherever possible, oral dosing is best avoided because there may be gastric stasis and drug absorption may be unreliable (Winstanley *et al*., 2004). Oral therapy is initiated as soon as it can be tolerated by the patient. The only contraindication to use of quinine is reliable evidence of serious quinine allergy (Warell, 1999). Efficacy of intravenous quinine and intramuscular artemether is similar under hospital condition (WHO, 2004). However, due to the more complicated dosing schedule of intravenous quinine and the requirement for monitoring of cardiac function and glucose levels, artemether is considered for severe malaria where intravenous administration is impossible (WHO, 2003b).

Artemisinin can be used where sensitivity to quinine is declining (Tran *et al*., 1996). Suppositories of artemisinin and artesunate have proved effective in children with cerebral and other forms of severe malaria in China and Southeast Asian countries (Krishna *et al*., 2001). A three day course of dihydroartemisinin suppository in combination with SP was found to be as efficacious as a three day course of intramuscular artemether plus SP in management of moderately severe malaria in Southwestern Nigeria (Falade *et al*., 2007).

Available ACTs in the Nigerian Markets and their Combinations

Artemisinin combination therapy is the drug of choice in view of its effectiveness and prompt action against all forms of malaria species. Besides, the high rate and increasing antimalarial drug resistance to former first and second line drugs (CQ and SP) has compounded malaria therapy in the country. Artemisinin combined drugs are the recommended mode of treatment of uncomplicated malaria, because of its prompt and effective action and quick resolution of illness. They reduce progression of illness to complicated malaria, thereby reducing malaria disease burden. These combinations take advantage of rapid blood schizonticidal action of the

artemisinin and the long duration of action of the partner compound to effect rapid cure with low level of recrudescence. The advantages of artemisinin derivatives that make them ideal combination partners are: rapid reduction of parasite biomass (>10 times that of monotherapies); rapid resolution of clinical symptoms; effective action against multi-drug resistant *P.falciparum*; no documented resistance as yet with the use of artemisinin and its derivatives; few clinical adverse reactions; and reduction of gametocyte carrier rate which may reduce transmission (WHO, 2010).

Currently available ACTs in the country are: artemether-lumefantrine, artesunate+amodiaquine, artesunate+mefloquine, and artesunate+SP. Recommended drug of choice for uncomplicated malaria in Nigeria is artemether-lumefantrine. However, other ACTs may be used where artemether-lumefantrine is not available. ACTs use in children less than 3 months of age (<5kg) and in the first trimester in pregnancy has not been ascertained for a categorical recommendation (FMOH, 2005). Dihydroartemisinin+piperaqinetrimethoprim is currently being evaluated and is not yet available as a formulation manufactured under good manufacturing practices, and has not yet been evaluated sufficiently in Africa and South America.

Antimalarial Drug Resistance

Drug resistance is a result of at least one mutation and its selection. WHO defines antimalarial drug resistance as the ability of the parasite to survive and/or multiply despite the administration and absorption of a medicine given in a dose equal to, or higher than those normally recommended but within the tolerance of subject, with the caveat that the form of the drug active against the parasite must be able to gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action (WHO, 1986). However, drug resistance is usually not an absolute phenomenon, rather a process, or a chain of events, where exposed

parasites through genetic alterations develop increased level of tolerance against particular drug/groups of drug until apparent clinical treatment failure is reached. Development of tolerance is usually at the cost of the parasite fitness (Hasting and Watkins, 2006). This hypothesis is strengthen by observations that withdrawal of drug exposure in an area resulted in decline prevalence of mutataed parasites towards a ''wild type' parasite (Kublin *et al.*, 2003).

Mechanism of Antimalarial Drug Resistance

Genotyping of the full *Plasmodium falciparum* genome have identified about 5300 genes distributed on the 14 chromosomes (Gardner *et al.*, 2002). Parasite might alter its response to an antimalarial drug through an ability to regulate gene expression. Most frequent mutations associated with drug resistance are SNPs, insertions/deletions and gene amplifications. SNPs can be located either in the coding area of a gene which might alter the protein structure, or in the regulatory areas surrounding the gene which might alter the gene expression. Multiple unlinked events might be necessary, forming a specific combination of mutations (haplotype).

CQ acts in the lysosome or food vacuole of the parasite where it interferes with the detoxification process of the haemoglobin derived products. In CQ sensitive parasites, the drug accumulates at the site of its action *i.e.*, food vacuoles, but the resistant parasites efflux the drug continuously. During this process, the membrane associated proteins of the food vacuole probably play a significant role. Though, exact molecular mechanism of CQ resistance remains elusive, three such membrane proteins have been identified, namely, Pgh1, a ~330 kDa protein and *Pfcrt*. Their respective genes *Pfmdr1*, *cg2* and *Pfcrt* have been cloned and sequenced (Foote *et al.*, 1990; Su *et al.*, 1997; Fidock *et al.*, 2000). However, CQ resistance could well be a multigenic phenomenon since the resistance to this drug has arisen very slowly despite its continuous usage

in the field for several decades. This might indicate that we need to search for additional target molecules in the parasite besides those that have been described (Mu *et al.*, 2003).

Pfcrt

Resistance to CQ, the most widely used and affordable antimalarial drug, has contributed to increased mortality and morbidity caused by *P. falciparum* infections. CQ resistance in *P. falciparum* is conferred by mutations in the parasite *Pfcrt*, a putative transporter localized in the digestive vacuole (Fidock *et al.*, 2000; Das and Dash, 2007; Tinto *et al.*, 2008). A single mutation, Lysine to Threonine at codon 76 (K76T), led to *in-vitro* resistance in all progeny of a genetic cross between CQ sensitive and CQ-resistant parental clones and among a set of geographically diverse parasite isolates (Djimde *et al.*, 2001; Sidhu *et al.*, 2002).

Mutation at codon 76 (Lys to Thr) has been found in almost all the CQ resistant parasite lines and clinical isolates (Fidock *et al.*, 2000; Basco and Ringwald, 2001; Chen *et al.*, 2001). Therefore, it has been proposed as a molecular marker to monitor the CQ resistance in field isolates. While it is true that K76T mutation is associated with CQ resistance, this mutation is not absolute. Because large number of CQ responders are also found to harbour this mutation and it is highly prevalent in Indian isolates (Vathsala *et al.*, 2004; Vinayak *et al.*, 2003). This raises several issues like the involvement of host response such as the status of immune system which can clear the parasite irrespective of its being CQ resistant or not (Djimde *et al.*, 2003). Similarly, the drug absorption and metabolic rate of individuals will also affect the outcome of CQ treatment. There is yet another possibility that other mutations in *Pfcrt* are also involved to give rise to this resistance. Else, more than one gene is involved in making the parasite to become CQ resistant. Besides K76T mutation in *Pfcrt*, mutations at codon 72, 74, 75, 97, 220, 271, 326, 356 and 371 have also been found to be associated with CQ resistance. There are

reports that mutation in codon 220 (Ala to Ser) in *Pfcrt* is associated with CQ resistance in African countries but not in the Philippines (Chen *et al*., 2003).

CQ has been the most frequently used drug for the first-line treatment of malaria infections in many tropical countries including Nigeria and it is effective against all forms of malaria, relatively easy to manufacture and chemically stable and thus it is readily stored and transported even under extreme climatic conditions (Ginsburg, 2005). Despite the change in National malaria drug policy to ACT in Nigeria, CQ still remains the most common antimalarial drug widely prescribed in the treatment of falciparum infection in certain areas of the country (Ogungbamigbe *et al*., 2005). Several countries in West Africa, including Nigeria, have changed their recommended first-line treatment for uncomplicated malaria to ACTs. In the year 2005, Nigeria changed first-line treatment for uncomplicated malaria to ACTs. However, high cost (US$ 6.20-11.7 per adult course) (Yeung *et al*. 2004) and non-availability of this drug has been limiting the effective use of this drug, rather the cheap and readily available CQ is continually being used. The result of the Therapeutic Efficacy of Anti-malarial Drugs in Nigeria showed that CQ have efficacy of 3.7%, 9.1%, 53.2%, 77.3%, 40.9%, 50.8% in South-East, South-South, North-Central, North- West, South-West and North-East respectively (FMOH, 2005).

Based on the patterns of the *Pfcrt* mutations, haplotype data and microsatellite analysis, CQ-resistant *Pfcrt* alleles vary depending on geographic origin. In general, CQ-resistant isolates from Southeast Asia and Africa (Old World) possess *Pfcrt* alleles with multiple mutations across *Pfcrt*, corresponding to the amino acid haplotype CVIET (residues 72 to 76), whereas most CQ-resistant parasites (SagtVMNT (SVMNT1; Asia, South America, and Tanzania), StctVMNT (SVMNT2; South America) have SVMNT haplotype (Fidock *et al*., 2000; Mehlotra *et al*., 2001; Chen *et al*., 2003; Nagesha *et al*., 2003; Durrand *et al*., 2004; Alifrangis *et al*., 2006; Ursing *et*

al., 2006). CQ-sensitive strains are characterized by a CVMNK haplotype, regardless of geographic origin. CQ-resistant *P. falciparum* (CRPF) malaria isolates in sub-Saharan Africa and Southeast Asia share the CVIET haplotype, and it has been assumed that CQ resistant *Plasmodium falciparum* parasites spread from Southeast Asia via the Indian subcontinent to the African continent (Wellems and Plowe, 2001; Wellems, 2002; Wellems, 2004; Bray *et al.*, 2005). Sutherland *et al,* (2007) observed that the use of CQ/proguanil prophylaxis were more likely to carry parasites with resistant alleles of *Pfcrt* than were patients who had been using antimalarials other than CQ.

A high prevalence of *Pfcrt* 76T gene known to be associated with CQ resistance parasites have been reported in Nigeria (Ojurongbe *et al.* 2007). A recent study of *Plasmodium falciparum* CQ resistant parasites (*Pfcrt*) showed that more 76T allele was identified (99.1%), while *Pfcrt* 76K was found in a single (0.9%) isolate (Kanchana *et al.*, 2009). High prevalence of *Pfcrt* with molecular marker K76T in Indian were discovered (91%), as well as in Uganda (98%) and Suriname (100%). All isolates from Suriname contained the CQ-resistant SVMNT haplotype typical of South American isolates, and 98% of isolates from Uganda possessed the CQ-resistant CVIET haplotype characteristic of Southeast Asian/African strains (Keen *et al.*, 2007). In Sudan high prevalence of *Pfcrt* CVIET haplotype (93%) was found (A-Elbasit *et al.*, 2008). Gadalla *et al.*, (2010), also reported *Pfcrt* CVIET haplotype in Sudan of recent. However, following the withdrawal of CQ in some countries, there was significant decreased in the frequency of *Pfcrt*-76 mutant (Kublin *et al.*, 2003; Mita *et al.*, 2003; Mwai *et al.*, 2009). The presence of SVMNT haplotype typical of South American isolates and one previously undescribed haplotype (CVM**DT**) was detected in Angola (Gama *et al.*, 2010).

Pfmdr1

Polymorphisms in the *Pfmdr*1 gene have been shown by transfection to modulate higher levels of CQ resistance and also to affect mefloquine, halofantrine, and quinine resistance (Djimde *et al.*, 2001; Sidhu *et al.*, 2002).

This gene is located on chromosome 5 of *P. falciparum*, encodes a P glycoprotein of 160 kDa which plays a role in drug efflux (Wilson *et al.*, 1989; Foote *et al.*, 1990). There had been contrary reports on the role of this protein in CQ resistance (Haruki *et al.*, 1994). Earlier reports provided evidence for the increased copy number of this gene in some resistant parasite lines so as to synthesize large amount of protein to counter the drug pressure (Foote *et al.*, 1990).

It has been reported that the expression of the *Pfmdr1* gene is regulated differently *i.e.*, higher level of *Pfmdr1* transcription under the influence of CQ (Myrick *et al.*, 2003). However, certain mutations in the *Pfmdr1* gene at codons 86, 184, 1034, 1042 and 1246 have been proposed to be associated with CQ resistance (Su *et al.*, 1997; Reed *et al.*, 2000). Among these, the mutation from asparagine to tyrosine at codon 86 has been used widely (Fidock *et al.*, 2000). PCR based molecular methods have been used to detect this mutation in the *in vitro* and *in vivo* tested parasites for CQ sensitivity as well as in the field isolates of different countries with variable range of CQ resistance. The mutations most often cited as potential contributors to CQ resistance are *Pfmdr1* N86Y and D1246Y (Reed *et al.*, 2000). In Nigeria, T76 and Y86 have been discovered in children who failed CQ treatment (Happi *et al.*, 2003).

The reports have not been very convincing although N86Y mutation in *Pfmdr1* seems to be playing some role in the CQ resistance, it is not essential (Awad-el-Kariem *et al.*, 1992; Huaman *et al.*, 2004). Some field studies supported the linkage of this mutation with CQ resistance while

others did not (Adagu *et al*., 1995). *P. falciparum* isolates from Malaysia, Indonesia, Guinea-Bissau, Nigeria and Sub- Saharan Africa showed N86Y mutation among CQ resistant parasites (Basco *et al*., 1995; Cox-Singh *et al*., 1995; Adagu *et al*., 1995; Adagu *et al*., 1996). But studies from Uganda, Laos, Cameroon, South Africa, Brazil and Peruvian Amazon reported that this mutation was not predictive of treatment outcome (Dorsey *et al*., 2001; Pillai *et al*., 2001; Huaman *et al*., 2004). Based on the gene knock out and transfection studies, it has been proposed that *Pfmdr1* alone is not sufficient to provide CQ resistance but it may help in the developmental process of drug resistance.

Certain mutations in the *Pfmdr1* gene at codons 86, 184, 1034, 1042 and 1246 were proposed to be associated with CQ resistance (Reed *et al*., 2000). There are indications that *Pfmdr1* N86 is associated with resistance to lumefantrine that is widely used in combination with artemether (Sisowath *et al*., 2005), and also with decreased sensitivity to artemisinins (Duransingh *et al*., 2000). Selection for mefloquine resistance has been associated with a decreased resistance to CQ and an amplification of the *Pfmdr1* gene (Reed *et al*., 2000). This amplification of *Pfmdr1* gene copy numbers may also be associated with the observed higher *in-vitro* IC50 (50% inhibitory concentration) for MQ in resistant *Plasmodium falciparum* isolates (Price *et al*., 2001). A novel substitution of phenylalanine for asparagine at codon 86 of *Pfmdr1* (N86F) was reported of recent in Swaziland (Dlamini *et al*., 2010).

Dhfr and *Dhps*

The use of SP as the first line drug alongside with CQ was discontinued with the implementation of current malaria treatment policy that recommends the use of ACTs. Despite this change in policy, SP is still widely prescribed and used as monotherapy in the country. Factors such as decreased susceptibility of the malaria parasites to drugs such as CQ and SP have contributed to

the increase in children malaria globally. *Plasmodium falciparum* drug resistant parasites have been the major obstacles in the control of the disease and this has increased malaria-related morbidity and mortality (Trape, 2001; Bjorkman and Bhattarai, 2005). This has amplified the cost of malaria control, as combination therapy are often more expensive. SP was the first line agent for uncomplicated malaria in many African countries until its changed due to the development of resistance (Wongsrichanalai *et al.*, 2002; EANMAT 2003). Most African countries have since adopted ACT as their official first line drug for uncomplicated malaria. However, SP is still used in Africa due to the limited distribution of ACT (Nosten and White 2007). SP therapeutic efficacy in Nigeria showed that SP have efficacy of 14.9%, 8.5%, 82.7%, 94.2%, 75.6%, 64.8% in South-East, South-South, North-Central, North- West, South-West and North-East respectively (FMOH, 2005).

The requirement for alternative drugs has led to the extensive use of the SP combination drug in the treatment of uncomplicated malaria. CQ, amodiaquine, Mefloquine and SP resistance has forced malaria endemic countries to change to ACTs as first-line treatment for uncomplicated malaria. The ACT drugs, currently being implemented in Sub-Saharan Africa combine various artemisinin analogues with novel drugs (lumefantrine) or with already widely used drugs such as SP, amodiaquine and mefloquine. ACTs are highly efficacious and are likely to decrease the rate of development of resistance, and thus expand the useful lifetime of the drugs (White, 1999). Artemisinin derivates are acting and are eliminated rapidly after treatment and the development of resistance to ACTs will probably depend on the already existing background level of resistance to the partner drugs in the parasite population, as it has been observed in areas with high levels of resistance to SP and amodiaquine (Rwagacondo *et al.*, 2003; Mutabingwa *et al.*, 2005).

P. falciparum resistance to SP is due to point mutations in the *Pf-dhfr* and *Pf-dhps* genes (Triglia *et al.*, 1998; Basco, 2003). In *Pf-dhfr*, point mutations changing Asn51 to Ile (N51I), Cys59 to Arg (C59R), Ser108 to Asn (S108N), and Ile164 to Leu (I164L) have been shown to confer resistance to pyrimethamine (Sirawaraporn *et al.*, 1997; Gregson and Plowe, 2005). S108N is usually observed first, and parasites that carry *Pfdhfr* alleles with mutations at N51I and/or C59R and I164L, resulting in double, triple, or quadruple mutants, are increasingly resistant to pyrimethamine. Mutations in the *Dhfr* gene at position 50 (Cys to Arg) and a five amino acid repetitive insert between positions 30 and 31 have also been found to be highly prevalent in Bolivia, where SP resistance is high (Plowe *et al.*, 1997; Cortese and Plowe, 1998). A novel leucine mutation at position 140 in the *Dhfr* gene was also found in the isolate VP8, which also has the characteristic changes at positions Val-16 and Thr-108, which confer cycloguanil resistance (Zindrou *et al.*, 1996). Sulfadoxine resistance also depends on mutations in its target, the *Dhps* domain of the bifunctional protein dihydro-hydroxymethylpyrophosphokinase- *Dhps*, at codons 436 (S436A/F), 437 (A437G), 540 (K540E), 581 (A581G), and 613 (A613S/T) (Brooks *et al.*, 1994; Wang *et al.*, 1997). Normally, multiple *Dhps* mutations result in synergistic effect on SP resistance (Meyer *et al.*, 2002).

Currently, SP combination with artesunate is the first-line treatment policy in some African countries. The prevalence of combinations of SNP in the *P. falciparum Pfdhfr* and *Pfdhps* genes have been correlated with resistance to SP *in vivo* (Kublin *et al.*, 2002; Kyabayinze *et al.*, 2003). In Africa, the *Pfdhfr* triple mutation, N51I+C59R+S108N, with wild type at codons 50 (C50) and 164 (I164), is combined into a highly prevalent and resistant haplotype, CIRNI. In a study, children were discovered to be having high prevalence of mutation in *Dhfr* genes *dhfr*59R and *dhfr*108N (Schoepflin *et al.*, 2008). For *Pfdhps* the double mutation, A437G + K540E, with wild type at codons 436 (S436), 581 (A581) and 613 (A613) are combined into the resistant haplotype

SGEAA. The combination of the two; CIRNI and SGEAA, have been suggested as a molecular marker of *in vivo* resistance (Kublin *et al*., 2002). Five *Dhps* (S436A, S436F, S436C, A437G, and K540E) and three *Dhfr* mutations were found in Tanzania (Malisa *et al*., 2010).

At molecular level, the emergence and magnitude of resistance to SP can be monitored by measuring the prevalence of SNPs in the *P falciparum Pfdhfr* gene, responsible for pyrimethamine resistance (Cowman *et al*., 1988) and the *Pfdhps* gene, responsible for sulfadoxine resistance (Brooks *et al*., 1994). An increase in the proportion of mutant alleles of *Pfdhfr* and *Pfdhps* and a increasing parasitologic resistance at mild and moderate levels has usually preceded the decrease in clinical effectiveness of SP. Initial clinical failures to SP usually become evident when an isolate carries a triple mutant (N51I + C59R + S108N) in the *Pfdhfr* gene with or without additional mutations in *Pfdhps* (Nzila *et al*., 2000; Talisuna *et al*., 2003; Talisuna *et al*., 2004). The *Dhfr* triple (Asn-108/Ile-51/Arg-59) mutants or the *Dhps* double mutants (Gly-437/Glu-540) were independently associated with SP treatment failure in children aged less than 5 years, but not in older children. The *Dhfr* and *Dhps* quintuple mutant (*Dhfr* triple mutant+*Dhps* double mutant) was the genotype most strongly associated with SP treatment failure in both younger and older children in Nigeria (Happi *et al*., 2005).

The sequence analysis of the isolates of known drug susceptibility profile revealed that a single *Dhfr* mutation or double *Dhfr* mutations alone will not cause SP treatment failure but double *Dhfr* mutations plus a single *Dhps* mutation or triple *Dhfr* mutations or quadruple *Dhfr* mutations will certainly provide a higher level of drug resistance (Wang *et al*., 1997). The increased level of sulphadoxine drug resistance has been shown to be associated with the higher number of mutations in *Dhps*. Also, similar to S108N mutation in *Dhfr*, the A437G is the key point

mutation in *Dhps* which allows the parasite to reduce its susceptibility towards sulphadoxine (Ahmed *et al*., 2004).

P. falciparum with the IRNI haplotype have been reported in isolates from Cameroon, Ghana, Guinea, Kenya, Liberia, Malawi, Mozambique, Nigeria, Sierra Leone and Uganda. A novel mutation was identified among the sample from Nigeria. The presence of Valine at codon 431 (I431V) of *Pfdhps* occurred in Nigerian origin (40%), and in one other isolate of unknown origin (Sutherland e*t al*., 2009). A rare occurrence of mutations at positions 581 and 613, in 5 Nigerian isolates was also reported. These all carried the novel I431V substitution and so were of the quintuple mutant haplotype VSGKGS, suggesting there may be linkage of the 431, 581 and 613 codons in some Nigerian parasite populations. Two isolates carrying the *Pfdhps* haplotype VSGKAA were also found. The results verify the occurrence of the VAGKGS and VAGKAA haplotypes in Nigeria (Sutherland e*t al*., 2009). The pyrimethamine-resistant isolates haplotypes deduced are ACIRNI, ACIRSI, ACNRNI, and ACNCNI and the deduced haplotypes ACNCSI in the pyrimethamine-sensitive isolates (Gama *et al*., 2009). The aim of the present study was to describe the prevalence in *Pfdhfr* and *Pfdhps* mutations SNPs in children with uncomplicated malaria.

CHAPTER THREE

3.0   MATERIALS AND METHODS

3.1.1   Study Area

This study was conducted at the St. Kizito Primary Health Centre, Lekki, and Massey Street Children's Hospital Lagos Island, Lagos State, Southwestern Nigeria – a holoendemic area for malaria. The St. Kizito Primary Health Centre, Lekki, receives patients from different areas of Lagos State. Massey Street Children's Hospital is located in a high-density area of Lagos Island and it receives patients referred from surrounding Primary Health care Clinics and other hospitals within Lagos State.

3.1.2   Study Population and Designs

The study group consisted of a total of 1,211 children (553 males and 658 females), aged 0-12years who presented with fever or history of fever in the last 24 hours at the Outpatient's Department of the Health facilities studied. The study design was cross-sectional and the enrolled children were grouped into three subgroups for the purpose of data analysis, namely: **Age Group I,** made up of children less than one year (1-3months, 4-6months, 7-9months and 10-12months), **Age Group II** (0-≤5years and >5-12years), and **Age Group III** (>1-12years).

The study participants were enrolled into the study if they met the following inclusion criteria: age 0 to 12 years, documented fever at presentation or history of fever in the last 24 hours, absence of danger signs of complicated/severe malaria and known serious chronic disease and willingness of the Parent or Guardian to provide written assent and consent. Those that presented with signs of complicated malaria namely: convulsions/coma, postration, severe vomiting, etc were excluded and these were managed appropriately or referred.

3.1.3 Ethical Considerations

Ethical approval for this study was obtained from the Research, Grants and Experimentation Committee, of the College of Medicine, University of Lagos, Idi-Araba, Lagos and the Lagos University Teaching Hospital, Idiaraba, Lagos. Assent was obtained from the children with corresponding consent from their Parents/Guardian before they were enrolled. All patients studied received appropriate standard of care after their blood was examined.

3.1.4 Sample Collection

Immediately after clinical examination by the managing Physician, each child was sent to the laboratory from where finger prick blood was collected onto microscope slide for microscopy and blood spots were made on filter paper (Whatmann No 3) for molecular genotyping. These slides were transported to the International Centre for Malaria Microscopy, Tropical Disease Research Laboratory, Department of Medical Microbiology and Parasitology, College of Medicine (CMUL), University of Lagos, Idi-Araba, Lagos.

3.1.5 Sample size

The sample size of this study was determined using the Epinfo software (Epi-Info 6.0 from CDC) based on the prevalence of malaria infection in children and the population of the Lagos State. The minimum sample size at 95% CI is 217.

3.2.1 Equipment and Reagents

1) Microscope Slides.

2) Giemsa stain.

3) Methanol (Sigma-Aldrich, USA).

4) Absolute Ethanol (Sigma-Aldrich, USA).

5) Binocular light microscope (Olympus, Japan).

6) Whatman No 3 filter paper (Whatman International, Ltd., Maidstone, UK).

7) Chelex-(Bio-Rad, London).

8) 0.5µg/ml Ethidium Bromide (Sigma-Aldrich, USA).

9) Agarose (Sigma-Aldrich, USA).

10) 3M Sodium acetate solution (Sigma-Aldrich, USA).

11) Standard DNA ladder (Fermentas, Germany).

12) Dd2, 3D7 and 7G8 positive DNAs from MR4 (www.mr4.org, Manassas, VA, USA).

13) Real Time PCR-Corbett Rotorgene 3000 (Corbett, Sydney, Australia).

14) Peltier Thermal Cycler (PTC-225) (MJ Research Thermo Scientific, USA).

15) ExoSap IT (Exonuclease 1 and Shrimp Alkaline Phosphate, Bioline, London, UK).

16) BigDye reaction with ABi BigDye V3.1dye terminator cycle with AmpliTaq® DNA Polymerase (Applied Biosystems, UK).

17) 5x Sequencing buffer.

18) 3M Sodium acetate (pH 4.6).

19) ABI 377 Automated Sequencer, Peltier Thermal Cycler DNA Engine DYAD^{TM} from MJ Research, Thermo Scientific, USA).

3.2.2 Preparation of 1X PBS (Phosphate buffered saline)

One tablet of PBS (Sigma) was added to 200ml of distilled water and this was stirred with an automated magnetic stirrer until the tablet was completely dissolved.

3.2.3 Preparation of 0.5% saponin in 1X PBS

To 100ml of 1X PBS, 0.5g of saponin was dissolved in the solution.

3.2.4   Preparation of Chelex suspension

Chelex-100 resin (Bio-Rad, London) in 100-200 mesh sodium form was suspended in nuclease free water. A chelex suspension of 6% weight/volume was prepared in nuclease free water.

3.3.1   Laboratory Investigations

The laboratory investigations were done in two phases at the following institutions:

I. Thr International Centre for Malaria Microscopy, Tropical Disease Research Laboratory (TDRL) at the Department of Medical Microbiology and Parasitology, CMUL, University of Lagos, Nigeria.

II. Department of Immunology, London School of Hygiene and Tropical Medicine, (LSHTM). UK.

3.3.2   Phase One: Laboratory Diagnosis of Malaria Parasites (Microscopy)

Thick and thin blood smears were made on the same slide from finger prick of each child using a sterile lancet. Two slides were made for each child. The first slide was the read ("R") slide (that is the slide to be read), while the other slide was the slide to be archived ("A") as part of the quality assurance process of the International Centre for Malaria Microscopy, Tropical Disease Research Laboratory, Department of Medical Microbiology and Parasitology, CMUL, University of Lagos, Lagos. The thin films were fixed with methanol after airdrying for 45 minutes. The thin and thick films were then stained with 3% Giemsa at a pH of 7.2 for 45 minutes. The staining process was quality controlled to ensure that the morphology of the malaria parasites in positive slides were distinct and clear.

The parasite density was measured as the number of parasites per 500 leucocytes on a thick film and this calculated as parasites per microlitre of blood assuming an average white blood-cell

count of 8000 per μl of blood (Greenwood and Armstrong, 1991). The parasite density of blood was expressed as:

Parasite density per μl of blood = $\dfrac{\text{No of Parasite Count X 8000}}{\text{No of Leucocytes/ WBC count}}$

The thin films were used to speciate the *Plasmodium* parasite. Stained slides were examined under the light microscope using the x100 objective lens (immersion oil). A slide was considered negative after 100 high power fields (HPF) have been examined. As part of the Standard Operating Procedures (SOPs) for slide reading in the TDRL, CMUL, another Microscopist was made to re-read each slide and only parasite counts with less than 20% discordance between the first and second reader were accepted. Parasite counts with >20% discordance was re-read by a third reader, who served as the tie breaker. The stage of the parasite, species and counts were reported for each child. The children enrolled in this study received standard routine care.

3.3.3   Phase Two: Molecular Genotyping

3.3.3.1   Extraction of DNA from Samples Collected on Filter Paper

Deoxyribonucleic acid (DNA) were extracted from a cohort of malaria parasites slide positive children of the 251 malaria positive children using Chelex as described by Wooden *et al*., (1993) for *Pfcrt*, *Pfmdr*1, *Dhps* and *Dhfr* amplification. Briefly, parasite genomic DNA was extracted from the dried blood spots on filter papers. The blood spots were lysed in 5% saponin in 1 x PBS and incubated at 37°C overnight. After the overnight incubation, the saponin and debris were removed after centrifugation using a vacuum pump. The resultant DNA pellets were washed twice in buffered saline and then suspended in 6% chelex 100 resin. This suspension was heat-sealed in deep 96-wells, incubated in boiling water for 20-25 minutes and then centrifuged for the resin to precipitate. Approximately 100μl of supernatant containing DNA was removed and stored at -20°C.

3.3.3.2 **Genotyping of the *P. falciparum* chlroquine resistant tranporter (*Pfcrt*) locus**

The *Pfcrt* gene is located on chromosome 7 and encodes for a protein named *Pfcrt* (Wellems *et al.*, 1991). A total of 119 parasites DNA obtained from a cohort of the parasite positive blood were assayed for the *Pfcrt* genes.

The *Pfcrt* gene was genotyped using a quantitative PCR (qPCR) that used double-labelled probes with a different fluorophore that represented each of the three most common *Pfcrt* alleles (Sutherland *et al.*, 2007). Essentially, this was made up of the amplification primers *Pfcrt* forward (TGG TAA ATG TGC TCA TGT GTT T) and the *Pfcrt* reverse (AGT TTC GGA TGT TAC AAA ACT ATA GT). The amplification was performed in a Corbett Rotorgene 3000 Real Time PCR (Corbett, Sydney, Australia). The three double labelled probes used represented the wild-type and the two most common resistance-associated haplotypes at codons 72-76 of *Pfcrt*. The probes were: crt CVMNK (Wild type), 5'FAM-TGT GTA ATG AAT AAA ATT TTT GCT AA-BHQ1 (3D7 DNA, positive control), crt C**V**IET (resistant), 5' JOE-TGT GTA **ATT GAA ACA** ATT TTT GCT AA-BHQ1 (Dd2 DNA, positive control); and crt **S**VMN**T** resistant, 5'ROX-**AGT** GTA ATG AAT **ACA** ATT TTT GCT AA-BHQ2 (7G8 DNA, positive control). The probes were also designed to bind to both mutant (76T) and wild-type (K76) alles at codon 76. A heterologous internal control was included in the assay to monitor both DNA extraction quality and potential PCR inhibition during the real-time PCR run. Furthermore, each run included a known positive control for each of the three *Pfcrt* haplotypes (CVMNK, SVMNT, and CVIET). The negative controls in all the reactions contained the reaction mixture without the parasite DNA. The probes were synthesized by a private company, MWG, Germany.

The validation and optimization method of the probes that was done in this study was described by Sutherland *et al.*, 2007. The control parasites DNA was obtained directly from MR4

(www.mr4.org, Manassas, VA, USA). The samples were considered positive for a particular genotype if a CT (threshold cycle) value of 35 cycles or fewer was obtained.

The master mix was prepared for a total of 72 lid cap Eppendorf tubes of 0.1mL each in a sterile hood with laminar air flow and the reaction was run in a Corbett Rotorgene 3000 thermocycler as follows: Hold at 95°C for 6 minutes; Step 1 at 95°C, hold for 15 seconds (45 cycles) and Step 2 at 55°C, hold for 60 seconds. The DNA sample (5μl) was amplified in a 20μl Master Mix containing 11.55μl Nuclease free water, 2.50μl 10 x NH_4 PCR buffer, 2.75 μl 50mM $MgCl_2$, 0.75μl 10mM dNTPs, 0.75μl 10μM primer mix *crt* F/R, 0.5μl 5μM *crt* probe CVMNK (FAM), 0.5μl 5μM *crt* probe CVIET (JOE), 0.5μl 5μM *crt* probe SVMNT (ROX), and 0.2μl 5U/μl Taq enzyme. To each tube (0.1mL), 20μl of the Master Mix was dispensed into the 72 tubes. Five microliters (5μl) of each DNA sample was added to each tube, with six of the seventy two tubes used as control (Chelex, water [negative control] and known positive samples; 7GB, FCR3 and Malay crt[+]). A total of 66 extracted DNA samples were used for each real time reaction.

3.3.3.3   *Pfmdr1*, *Pfdhfr* and *Pfdhps* Amplification

A nested-PCR technique was employed to amplify the DNA containing the major SNPs related to drug resistance for each target gene as: SNPs A16V/S, C50R, N51I, C59R, S108N, V140L plus I164L for *Pfdhfr*, S436A/ F/C, A437G, K540E, A581G plus A613T/S for *Pfdhps*, and SNPs N86Y, Y184F, S1034C, N1042D and D1246Y for *Pfmdr1*. All the amplifications were performed in 96 wells plate, placed into DNA Engine Tetrad PCR Peltier Thermal Cycler (PTC-225) (MJ Research Thermo Scientific, USA) for each reaction.

3.3.3.4   *Pfmdr1* Genotyping

This gene is located on chromosome 5 of *P. falciparum*, encodes a P glycoprotein of 160 kDa which plays a role in drug efflux. Mutations in the *Pfmdr1* gene at codons 86, 184, 1034, 1042 and 1246 have been proposed to be associated with CQ resistance (Foote *et al.*, 1989). A total of 81 parasite DNA obtained from some of the parasite positive children were assayed for *Pfmdr1* genes. These samples were amplified using Nested 2 PCR reactions for *Pfmdr1* fragment 1(FR1), fragment 3 (FR3) and fragment 4 (FR4). In each reaction, appropriate known positive (Dd2, 7GB and FCR3) and negative samples (DNA negative wells on each row) were used.

3.3.3.5   *Pfmdr1* Fragment 1 (FR1) Amplification

The *Pfmdr1* amplification was done using the protocol of Humphreys *et al.*, 2007. Briefly, amplification of the *Pfmdr1* FR1 Nest 1 reaction was performed with 5µl of DNA into a 20µl mixture containing 14.8µl Nuclease free water, 2.5µl 10xNH$_4$ PCR buffer, 1µl 50mM MgCl$_2$, 0.5µl 10mM dNTPs, 0.2µl BioTaq DNA polymerase and 0.5µl of 10mM forward primers AGGTTGAAAAAGAGTTGAAC (FN1/1) and 0.5µl 10mM reverse primers ATGACACCACAAACATAAAT (REV/C1) was used to amplify the 578bp region. The reactions were done with an initial hold at 94ºC for 3 minutes, followed by 30 cycles of denaturation at 94ºC for 30seconds, annealing at 45ºC for 1minute, initial extension at 72ºC for 1 minute, and final extension at 72°C for 5 minutes and at a hold time of 5minutes at 15ºC. Then, 1µl of the first PCR amplification were added to 24µl of a second mixture containing 18.8µl Nuclease free water, 2.5µl 10xNH$_4$ PCR buffer, 1µl 50mM MgCl$_2$, 0.5µl 10mM dNTPs, 0.2µl BioTaq DNA polymerase and 0.5µl of the second set of 10mM forward primers ACAAAAAGAGTACCGCTGAAT (MDR2/1) and 10mM reverse primers AAACGCAAGTAATACATAAAGTC (NEWREV1) was used to amplify the 534bp region comprising SNPs N86Y and Y184F. The reaction was done with *Pfmdr1* FR1 Nest 1 cycling

condition. The obtained PCR products were analysed by ethidium bromide-stained agarose gel electrophoresis.

3.3.3.6 *Pfmdr1* Fragment 3 (FR3) Amplification

The *Pfmdr1* FR3 PCR master mix was prepared step-wise beginning with Nest 1 and then Nest 2 reaction following the same procedure as described in FR1 amplification (section 3.3.3.5), but with a different forward (GCATTTTATAATATGCATACTG [MDRFR3N1]) and reverse primers (GGATTTCATAAAGTCATCAAC [MDRFR3R1]) for Nest 1 and different forward (GGTTTAGAAGATTATTTCTGTAA [MDRFR3N2]) and reverse primers (GGATTTCATAAAGTCATCAAC [MDRFR3R1]) to amplify 234bp region for Nest 2 reaction comprising SNPs S1034C and N1042D. The FR3 was settled with the same cycling conditions for Nest 1 and 2 reaction (initial denaturation at 94ºC for 3 minutes, followed by 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 1minute, extension at 65ºC for 40 seconds, final extension at 72°C for 5minutes and hold time for 5minutes at 15°C). The obtained PCR products were analyzed by ethidium bromide-stained agarose gel electrophoresis.

3.3.3.7 *Pfmdr1* Fragment 4 (FR4) Amplification

The *Pfmdr1* FR4 Nest 1 and Nest 2 PCR was done following the same procedure, the same reagents and the same volume as described in FR1 amplification (section 3.3.3.5), but with different forward (CAAACCAATCTGGATCTGCAGAAG [MDRFR4N1]) and reverse primers (CAATGTTGCATCTTCTCTTCC [MDRFR4R1]) for Nest 1 and different forward (GATCTGCAGAAGATTATACTG [MDRFR4N2]) and reverse primers (CAATGTTGCATCTTCTCTTCC [MDRFR4R1]) was used to amplify the 194bp region for Nest 2 reaction comprising SNPs D1246Y. The FR4 was done with the same cycling conditions

described for Nest 1 and 2 *Pfmdr1* FR3 reaction (section 3.3.3.6). The obtained PCR products were analyzed by ethidium bromide-stained agarose gel electrophoresis.

3.3.3.8. *Pfdhfr* and *Pfdhps* Genotyping

Resistance to SP emanated from structural mutations that occurred in genes encoding the *Dhfr* enzyme target on chromosome 4 and *Dhps* enzyme on chromosome 8 (Conway, 2007). Pyrimethamine inhibits the *Dhfr* enzyme of *P. falciparum* and thus its folate biosynthesis pathway. However, the parasites can upregulate the translation of *Dhfr* under the influence of pyrimethamine to counter its effect leading to mutations seen in S108N, N51I, C59R, A16V and I164L (Le Bras and Durand, 2003). Sulphadoxine acts as a competitive inhibitor in the folate biosynthetic pathway of the parasite, inhibiting the enzyme *Dhps* thus interfering in the step of conversion of dihydropteridine pyrophosphate to dihydropteroate (Gregson and Plowe, 2005). However, the parasite has developed resistance to sulphadoxine leading to mutations in 436 (Ser to Ala/Phe), 437 (Ala to Gly), 540 (Lys to Glu), 581 (Ala to Gly and 613 (Ala to Ser/ Thr) (Triglia *et al.*, 1997). A nested PCR described by Pearce *et al.*, (2003) was used to amplify the *Dhfr* and *Dhps* genes.

3.3.3.9.1. *Pfdhfr* Genotyping

Five microlitres of each DNA were added to the 20µl *Dhfr* outer PCR mixture consisting of 14.8µl Nuclease free water, 1.5µl 10xNH$_4$ PCR buffer, 1µl 50mM MgCl$_2$, 0.5µl 10mM dNTPs, 0.2µl BioTaq DNA polymerase and 1µl each of 10mM forward primers TTTATGATGGAACAAGTCTGC (M1) and reverse primers CTAGTATATACATCGCTAACA (M7) was used to amplify the 650bp region. The reactions were done with an initial denaturation at 94ºC for 3 minutes, followed by 40 cycles of denaturation at 94ºC for 1minute, annealing at 52ºC for 2minutes, extension at 72ºC for 1

minute, and final extension at 72°C for 10minute. Then, 1µl of PCR product that was obtained from the first PCR amplification were added to 24µl of a second mixture containing 18.8µl Nuclease free water, 1.5µl 10xNH$_4$ PCR buffer, 1µl 50mM MgCl$_2$, 0.5µl 10mM dNTPs, 0.2µl BioTaq DNA polymerase and 1µl each of the second set of 10mM forward primers CTGGAAAAAATACATCACATTCATATG (M3) and 10mM reverse primers TGATGGAACAAGTCTGCGACGTT (M9) was used to amplify the 594bp region comprising SNPs A16V/S, C50R, N51I, C59R, S108N, V140L and I164L. The reaction was settled with initial denaturation at 94ºC for 3 minutes, followed by 40 cycles of denaturation at 94ºC for 1minute, annealing at 44ºC for 2minutes, extension at 72ºC for 1 minute, and final extension at 72°C for 10minutes. PCR products were analysed by ethidium bromide-stained agarose gel electrophoresis.

3.3.3.9 .2.   *Pfdhps* Amplification

The Nest 1 reaction was performed by adding 5µl of the amplified DNA into a 20µl mixture containing 14.8µl Nuclease free 20µl *dhfr* outer PCR mixture consisted of 14.8µl Nuclease free water, 1.5µl 10xNH$_4$ PCR buffer, 1µl 50mM MgCl$_2$, 0.5µl 10mM dNTPs, 0.2µl BioTaq DNA polymerase and 1µl each of 10mM forward primers GATTCTTTTTCAGATGGAGG (N1) and reverse primers TTCCTCATGTAATTCATCTGA (N2) was used to amplify the 770bp region. The reactions were settled with an initial denaturation at 94ºC for 3 minutes, followed by 40 cycles of denaturation at 94ºC for 1minute, annealing at 51ºC for 2minutes, extension at 72ºC for 1 minute, and final extension at 72°C for 10minutes. The nested reaction was completed by adding 1µl of the first PCR amplification to 24µl of a second mixture that contained 18.8µl Nuclease free water, 1.5µl 10xNH$_4$ PCR buffer, 1µl 50mM MgCl$_2$, 0.5µl 10mM dNTPs, 0.2µl BioTaq DNA polymerase and 1µl each of the second set of 10mM forward primers AACCTAAACGTGCTGTTCAA   (R1)   and   10mM   reverse   primers

AATTGTGTGATTTGTCCACAA (R2) to amplify 711bp region that comprises of SNPs for S436A/ F/C, A437G, K540E, A581G plus A613T/S. The reaction was settled with the same cycling condition used for *Dhps* Nest 1 reaction as described in section 3.3.3.9. The obtained PCR products were analysed by ethidium bromide-stained agarose gel electrophoresis.

3.4   Agarose gel Electrophoresis

Eight microlitres (8μl) of the amplified DNA (Nest 2 PCR products) was separated on a 1.5% (1.5g agarose powders to 100ml of 0.5X TBE) agarose gel (Sigma-Aldrich, USA) stained with 0.5μg/ml ethidium bromide. Briefly, the electrophoresis tank was filled with 0.5X Tris-borite [TBE] buffer that was diluted from 5 X TBE stock buffers. Eight microlitres (8μl) of 0.5μg/ml ethidium bromide was added to 1.5% agarose gel and mixed thoroughly. Two microlitres (2μl) of 5x loading buffer were added to 8μl of the amplified DNA. Eight microlitres (8μl) out of the mixture (10μl of the 5x loading buffer and the amplified DNA), was gently loaded into the wells of the submerged gel using a sterile disposable micropipette. DNA marker (DNA ladder) was then added into the first or last well on each row and a negative control (chelex negative) was added into a well on each row. The last three wells were used for positive controls (3D7, Dd2 and 7G8. The electrophoresis tank was then subjected to a voltage of 120V and 52mA for 1 hour. Separated DNA was visualized with UV transillumination and DNA band of interest was determined by comparing with a standard DNA ladder of known molecular weights (loaded on the first or last slot on each row).

3.5   Amplified DNA Purification and Sequencing

3.5.1 Purification

Five microlitres of the amplified DNA were cleaned up using aliquoted ExoSap IT (Exonuclease 1 and Shrimp Alkaline Phosphate, Bioline, London, UK) into a different sterile 96 wells plate and 2μl of ExoSap-IT was added to each well. The ExoSap-IT was kept on ice. The plate was

then incubated in the thermo cycler (Peltier Thermal Cycler [MJ Research Thermo Scientific, USAp]) for 15minutes at 37°C (reaction 1), and at 15 minutes at 85°C (reaction 2).

3.5.2 Sequencing

BigDye reaction using ABi BigDye V3.1dye terminator cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (Applied Biosystems, UK) was later performed on the purified PCR products using conditions and sequencing primer pairs as described by Humphreys *et al.*, 2007. Briefly, the reaction was seperated into forward and reverse reactions. Ten microlitres of the reaction mixture was used for each reaction. Nine microlitres of the prepared BigDye master mix, made up of 5.75µl of Nuclease free water, 0.5µl of the BigDye, 1.75µl of the 5x Sequencing buffer, 1.0µl each of the sequencing primers (Nest 2 forward and reverse primers for *Pfmdr1*, *Dhps* and *Dhfr*) and 1.0µl of the amplified DNA was added to the different reactions (forward and reverse reactions). The BigDye reaction was performed with the following profile: 96°C for 1 minute for activation of reagents, followed by 25 cycles at 96°C for 30seconds, 50°C for 15seconds and 60°C for 4minutes. The products were held at 4°C until it was ready to further purification. The rapid thermal ramp required for all heating and cooling was 1°C for 1second.

Further purification was done using Ethanol/Sodium acetate for each reaction (forward and reverse reactions). To each well, 90µl of the Ethanol/Sodium acetate mixture (24.5µl of Nuclease free water, 62.5µl of Absolute Ethanol (Analar), and 3µl of 3M Sodium acetate (pH 4.6) was added into the different plates, making a total of 100µl in each well. The plates were left on the ice for 20 minutes before spinning at 4000rpm for 30 minutes at 4°C. After the spinning, the plates were then inverted on a sterile tissue paper to drain quickly. The plates were again spun upside down on a sterile tissue paper at 50g for 1 minute. To each well, 150µl of 70% ethanol

was then added and the plates were again spun at 4000rpm for 10 minutes at 4°C. The plate was again inverted on a sterile tissue paper to drain quickly and spun upside down on a blue roll at 50g for 1 minute). Before adding 10μl of HiDi to each well, the plates were dried properly. The plates were then taken to freezer in a dedicated sequencing room. Plates were booked into folder using 9-digit bar code number on the sequencing plates. Samples were sequenced with the ABI 377 Automated Sequencer (Peltier Thermal Cycler DNA Engine DYAD^{TM}, MJ Research Applied Biosystems, Foster City, CA, USA).

3.6   Genotyping and Sequence Analysis

3.6.1   Genotyping

The *Pfcrt* haplotypes (CVIET, CVMNK and SVMNT) were genotyped if a particular DNA fluorescence at a CT (threshold cycle) value of 35 cycles or fewer.

3.6.2   Sequence Analysis

The sequenced DNA were analysed and aligned using SeqMan DNAStar software (Lasergene 8.0; DNAStar, Inc., Madison, WI) and Chromas software to check for mutations (The DNA sequence was compared with reference sequence of the *Pfmdr1, Pfdhfr* and *Pfdhps* portions of the *P. falciparum* 3D7 clone using BLAST similarity alignment (Washington University, USA). Appropriate control DNA samples with known *pfmdr1, pfdhfr* and *pfdhps* sequences were used in parallel with field collected parasite isolates in every step of the protocol.

3.7   Statistical Analysis

Data analysis was performed with Epi-info 2002 statistical package (CDC, Atlanta, Georgia, USA). Descriptive statistics and comparative analyses considered differences between the age groups: [**Age Group I** (1-3months, 4-6months, 7-9months and 10-12months], **Age Group II** (0-

≤5years and >5-12years), and **Age Group III** (>1-12years). The parasite density was analysed in the following groups: 1-500p/µl, 501-1,000p/µl, 1,001-10,000p/µl, 10,001-250,000p/µl and >250,000p/µl.

CHAPTER FOUR

4.0   **RESULTS**

4.1   Malaria Prevalence in Children

A total of 1,211 children, 0-12 years old were enrolled in the study {658(54.4%) males and 553(45.6%) females}. The mean age ± SD of the studied population and those positive for malaria parasite is 2.65 ± 2.83 (Table 1 and 2). Table 2 showed the parasite density of the malaria positive children. Of the 1,211 children examined, 800 (66.1%) were between 1 and 12 years while the remaining (33.9%) were less than 1 year old (Group I) (Table 3).

Two hundred and fifty one (20.7%) of the children examined microscopically were positive for malaria parasites, while the remaining 960(79.3%) were slide negative. The children, >1-12years (Age Group III category) had a malaria prevalence of 25.8% while the subtotal of children in the Age Group I category (0-1year) had a prevalence of 11.0% (P=0.001) and a mean parasite density of 26,607.7p/µl. In Age Group II, 174(16.9%) of the 0-≤5years children were positive for malaria parasites while children >5-12years had a prevalence of 42.1% (P=0.001). The children in the age range 1-3 months had a prevalence of 5.8% in the Age Group I category (χ^2 =5.8275, df=3, P=0.1203) (Table 3).

Table 1. Sex, Age and Temperature of the Children enrolled

Characteristics	Age Groups								
	1-3m	4-6m	7-9m	10-12m	0-≤1yr	0-≤5yr	>5-12yr	>1-12yr	0-12yr
Number	52(4.3%)	85(7.0%)	93(7.7%)	181(14.9%)	411(33.9%)	1028(84.9%)	183(15.1%)	800(66.1%)	1211
Sex									
Male	23(44.2)	31(36.5)	40(43.0)	83(45.9)	177(43.1)	485(47.2)	68(37.2)	376(47.0)	553(45.7)
Female	29(55.8)	54(63.5)	53(57.0)	98(54.1)	234(56.9)	543(52.8)	115(62.8)	424(53.0)	658(54.3)
Age									
Mean/±SD	0.02/0.01	0.05/0.01	0.08/0.01	0.10/0.01	0.39/0.45	1.67/1.49	8.22/2.03	3.82/2.83	2.65/2.83
Temp (^{o}C)									
Mean/±SD	37.80/0.97	37.72/1.12	38.06/0.83	38.17/1.10	37.90/0.99	37.72/1.00	37.25/1.09	37.51/1.04	37.71/1.29

**Table 2**. Sex, Age, Temperature and Parasite Density of 251 Malaria Positive Children

Characteristics			Age Groups						
	1-3m	4-6m	7-9m	10-12m	0-≤1yr	0-≤5yr	>5-12yr	>1-12yr	0-12yr
Number	3	6	9	27	45	174	77	206	251
Sex									
Male	2(66.7)	4(66.7)	8(88.9)	17(63.0)	27(60.0)	104(59.8)	43(55.9)	120(58.3)	147(58.6)
Female	1(33.3)	2(33.3)	1(11.1)	10(37.0)	18(40.0)	70(40.2)	34(44.1)	86(41.7)	104(41.4)
Age									
Mean/±SD	0.02/0.01	0.05/0.01	0.08/0.01	0.10/0.01	0.46/0.45	2.38/1.68	8.34/1.84	5.03/3.01	2.65/2.83
Temp (^{o}C)									
Mean/±SD	38.90/1.15	37.84/0.82	38.67/1.40	38.74/1.82	38.46/1.38	37.88/1.28	37.36/1.18	37.53/1.18	37.71/1.29
Parasite Density									
Mean	10,197.00	62.60	63,615.00	25,155.33	26,607.67	39,903.63	40,678.21	43,097.62	40,141.25
±SD	14,035.56	56.23	18, 2911.47	58,026.58	90,473.42	10, 5282.00	84,142.09	100,861.80	99,109.79

Table 3: Prevalence and Parasite Density of *Plasmodium* in the Different Age Groups

Age Group (mon/yrs)	No. Examined	No. Positive (%)	Parasitaemia Range	Mean Parasitaemia
Age Group I				
1-3months	52	3(5.8)	16 – 26,208	10,197.0
4-6months	85	6(7.1)	16 - 141	62.6
7-9months	93	9(9.7)	16 – 551,116	63,615.0
10-12months	181	27(14.9)	47 – 177,873	25,155.3
Subtotal (1-12months)	411	45(11.0)	16 – 551,116	26,607.7
Age Group II				
0-≤5yrs	1,027	174(16.9)	15 – 678,269	39,903.6
>5-12yrs	183	77(42.1)	16 – 432,077	40,678.2
Age Group III				
>1-12yrs	800	206(25.8)	15 – 678,269	43,097.6
(Total: 0-12yrs)	1,211	251(20.7)	15 – 678,269	40,141.2

Most of the malaria positive children (33.9%) had parasite density between 1-500p/μl while 4.8% of them had parasite density >250,000p/μl (χ^2 =1210, df=5, P=0.001) (Figure 1). Children in age Group III (>1-12years) had higher parasitaemia ([94.1%][parasite density range, 501-1,000p/μl]) than those in the age Group I (0-≤1 year)([70.6%] [parasite density range, 1-500p/μl]) (χ^2 =48.001, df=5, P=0.001) (Figure 2). Children in age range 0-≤5years, had higher parasitaemia (75.3% in parasite density range, 1-500p/μl and 52.9% in the parasite density range 501-1,000p/μl) than those in age range >5-12years (χ^2 =70.943, df=5, P=0.001) (Figure 3). Children in age range 4-6 months (100%) and in age range in 10-12 months (44.4%) in age Group I had parasite density range 1-500p/μl (P=0.001) (Figure 4).

Plasmodium falciparum was the dominant species encountered (96.5%). This was followed by *Plasmodium malariae* (1.2%) and *Plasmodium ovale* (0.4%). Mixed *Plasmodium falciparum* and *Plasmodium malariae* infection was 1.2%.
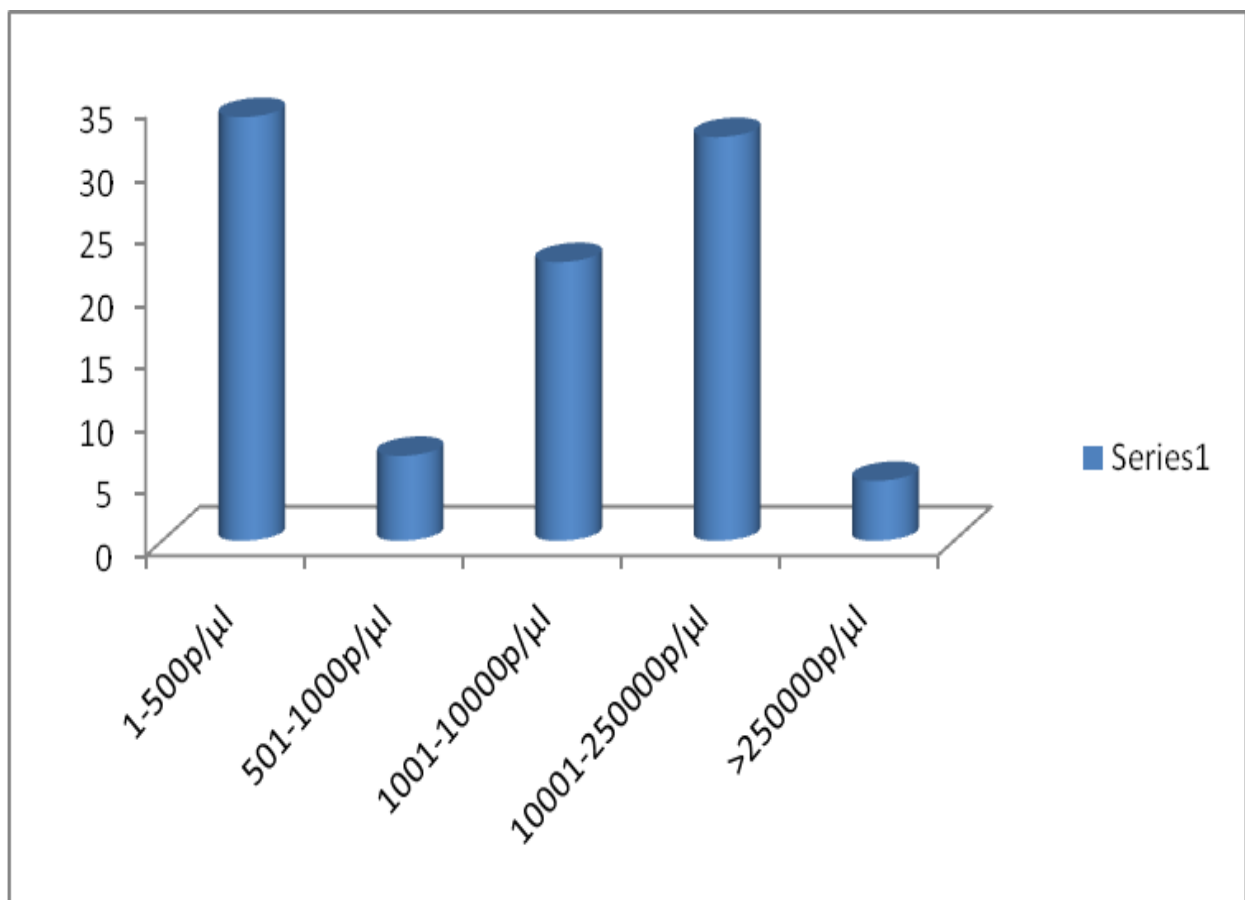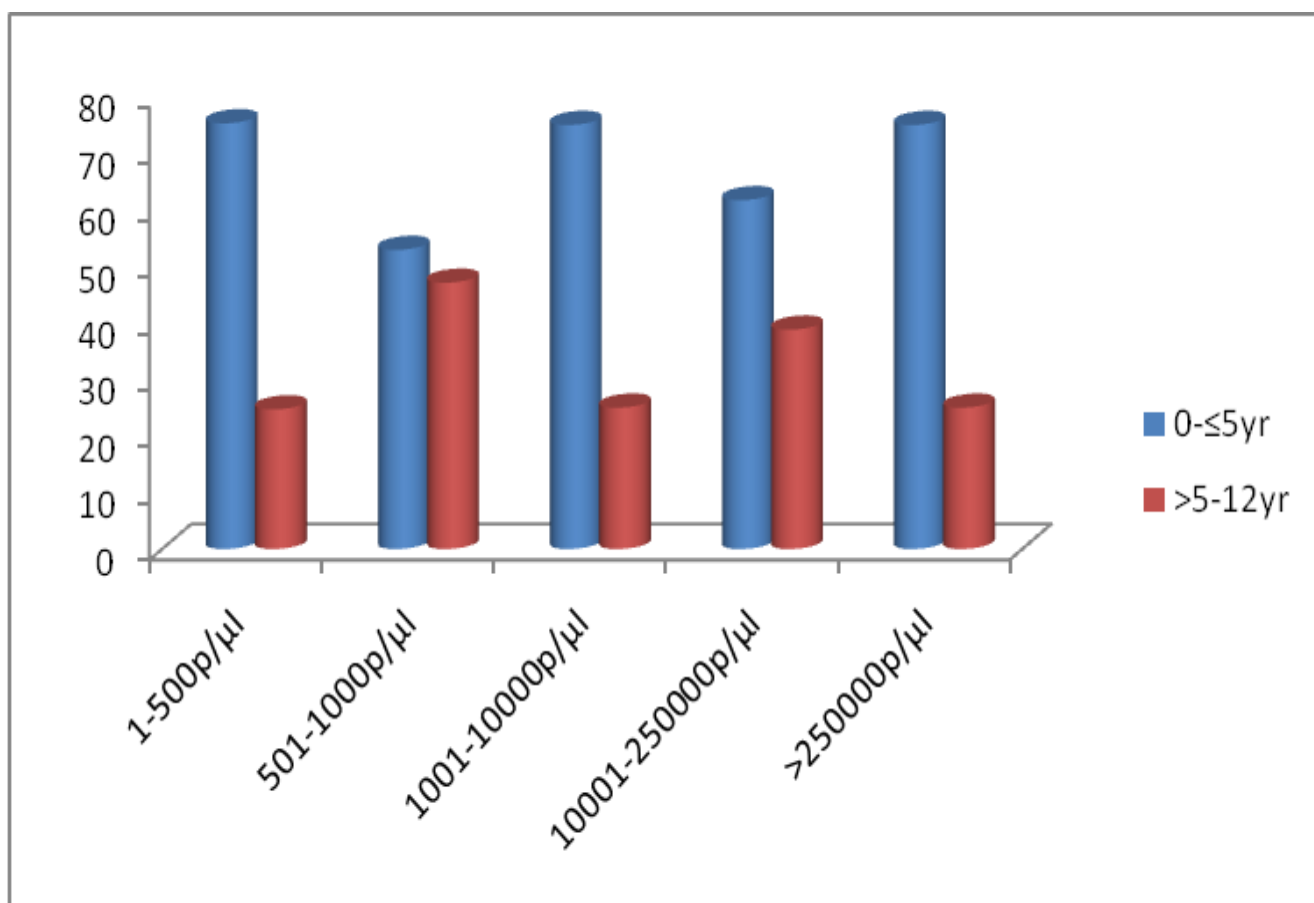
PARASITE DENSITY GROUPS

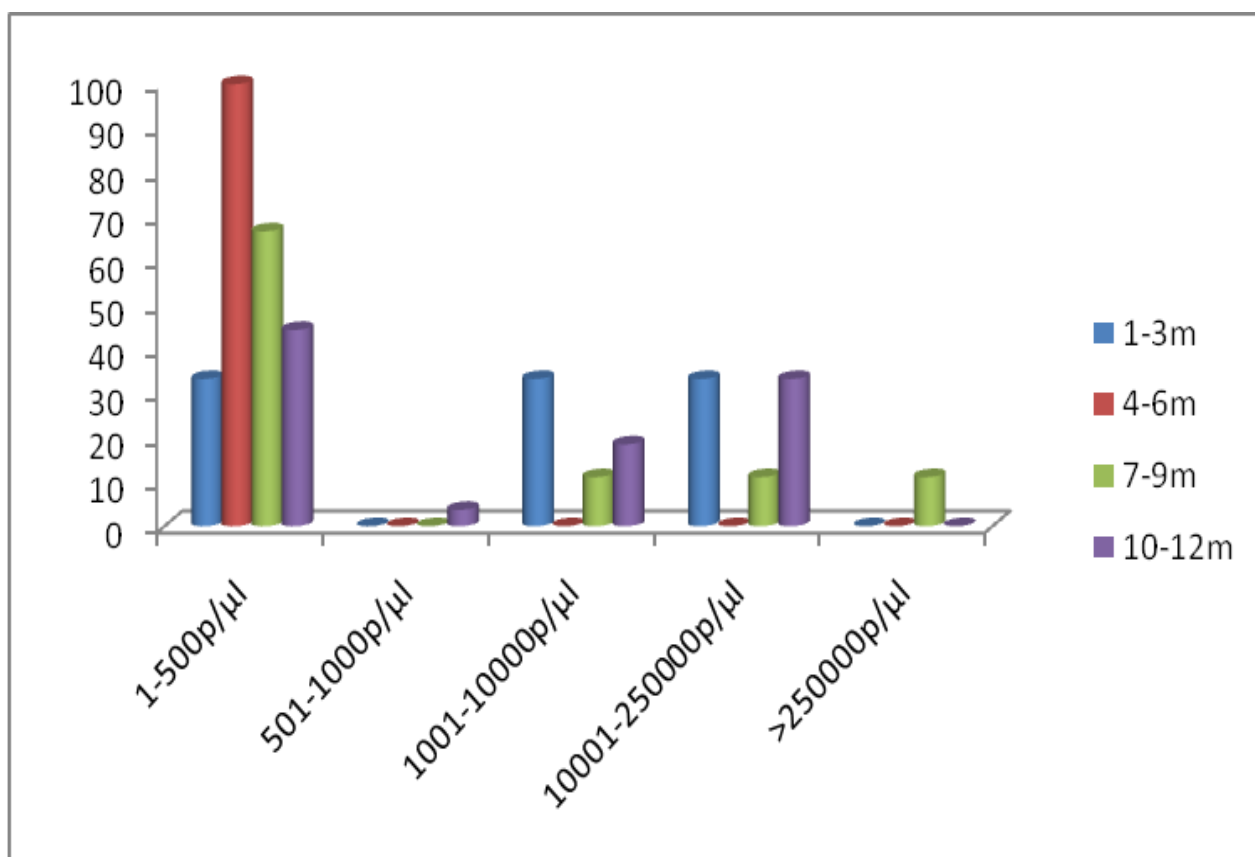FIGURE 1. DISTRIBUTION OF PARASITAEMIA IN THE EXAMINED CHILDREN (0-12yrs).
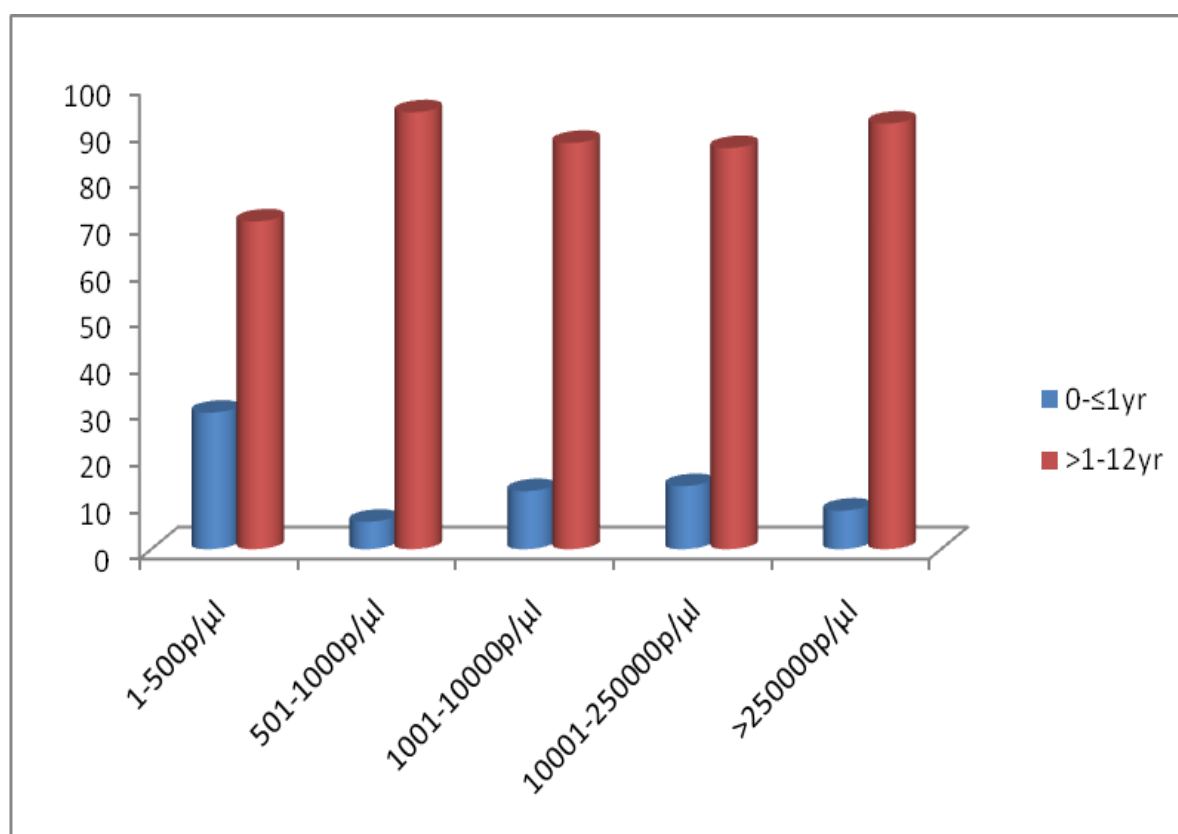
PARASITE DENSITY GROUPS

FIGURE 2. DISTRIBUTION OF PARASITAEMIA IN 0-≤1yr AND AGE GROUP III (>1-12yr)

PARASITE DENSITY GROUPS

FIGURE 3. DISTRIBUTION OF PARASITAEMIA IN AGE GROUP II (0-≤5yr) AND (>5-12yr)

PARASITE DENSITY GROUPS

FIGURE 4. DISTRIBUTION OF PARASITAEMIA IN AGE GROUP I (1-3m,4-6m,7-9m,10-12m)

4.2   Genotyping of the *Pfcrt* locus

Of the 119 isolates that were successfully assayed for *Pfcrt* genes at codon 72-76, 91.6% harboured parasites with the common African mutant haplotype (CVIET) and these reflected the geographical origin of the infections (Figure 5). The proportion of the wild type (CVMNK) among the isolates was (4.2%), while 5 isolates (4.2%) were a mixture of CVIET and CVMNK. The Southeast Asian/South American chloroquine-resistant haplotype SVMNT (mutant type) was not seen in all the isolated samples. The real time PCR results analysed using Rotor-Gene 6.1.93 software is presented in figure 6 showing the allelic distribution of all the haplotype with FAM representing the wild type, JOE representing the African mutant type and ROX representing the Asian mutant type. Figure 7 is showing the cycling data for the wild type (FAM), figure 8 is showing cycling data for the African mutant type (JOE) and figure 9 is showing the cycling data for Asian mutant type (ROX). Out the eleven children that were recorded to have taken CQ before coming to the hospital, four of them had the African mutant type CVIET.
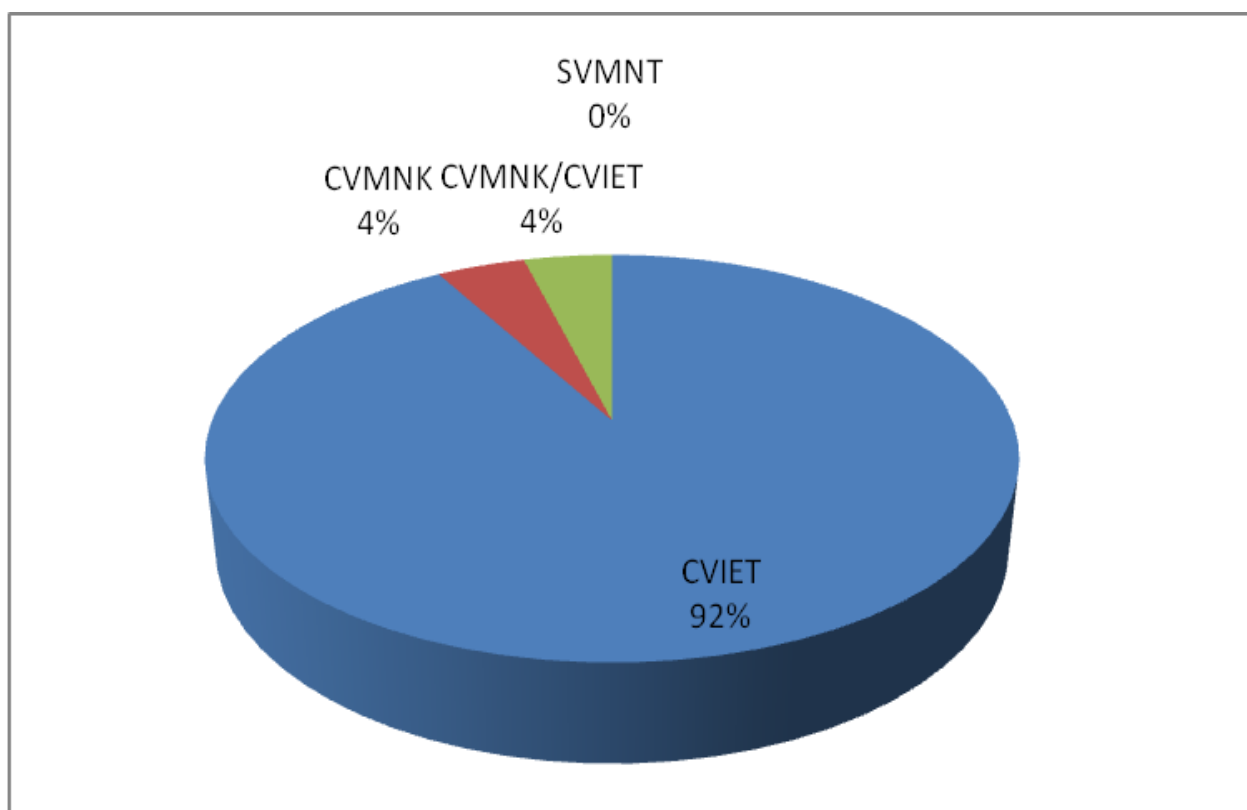
FIGURE 5. *Pfcrt* Haplotype Frequencies among 119 *P falciparum* Isolates

CVIET-   *Pfcrt* resistant haplotype (Asian and African Origin)
CVMNK-   *Pfcrt* wild haplotype
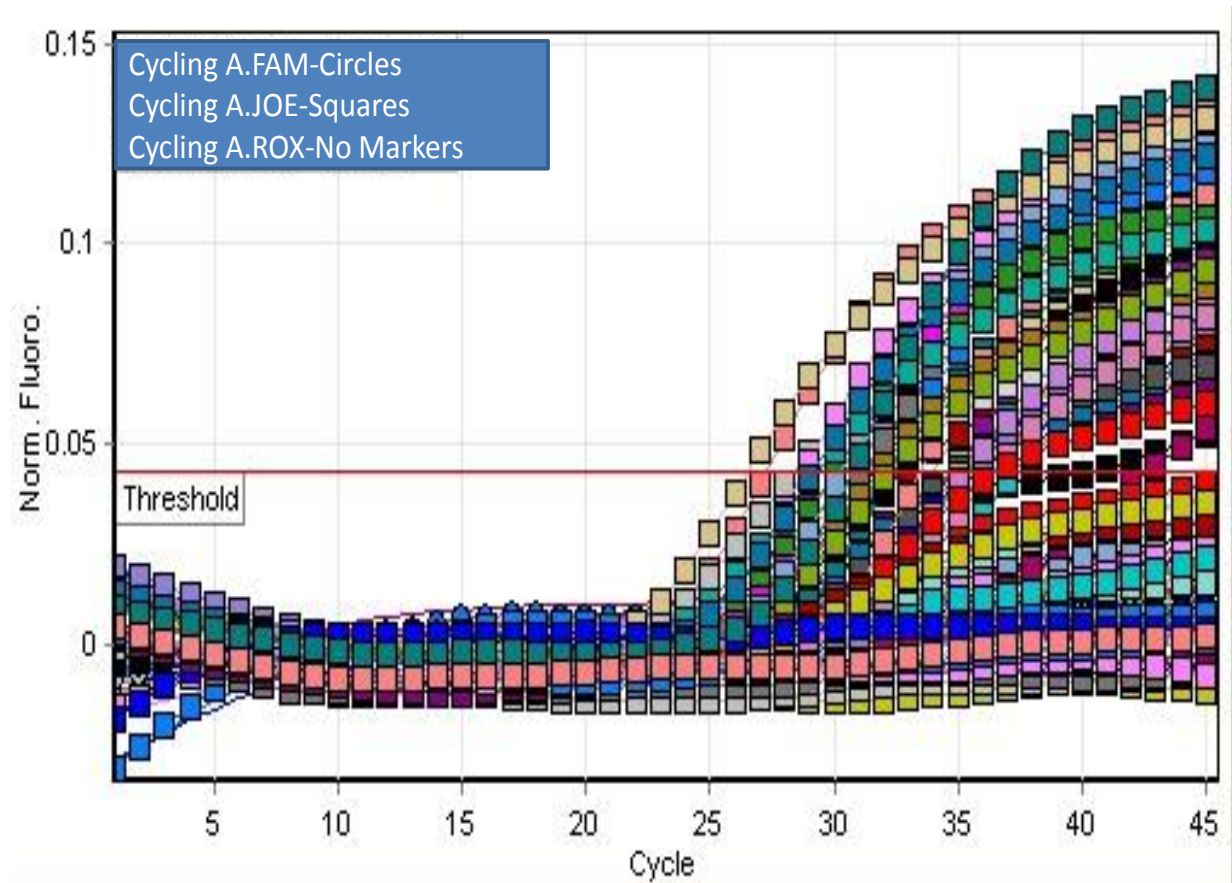SVMNT-   *Pfcrt* resistant haplotype (Asian and American Origin)

FIGURE 6. *Pfcrt* Allelic data for Cycling A.FAM, Cycling A.JOE, Cycling A.ROX

○ represent FAM, CVMNK (Wild Haplotype)
□ represent JOE, CVIET (Mutant Haplotype)

Note:
The multiple colours above the threshold line represent the DNA that fluorescence, while the DNA below the threshold represents the DNA that did not fluorescence.
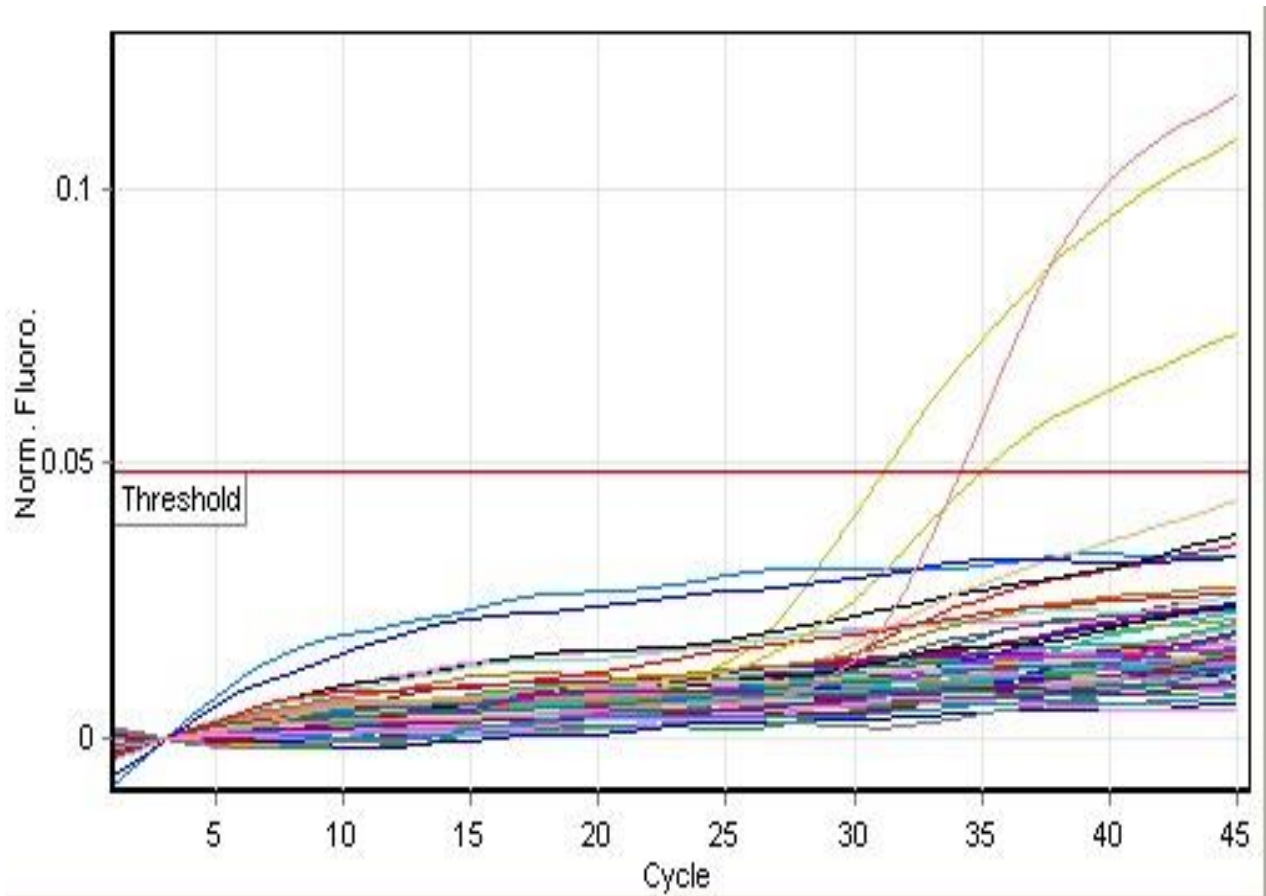
FIGURE 7. Wild (CVMNK) *Pfrct* Cycling for FAM (Wild haplotypes)

Legend:
The lines above the threshold represent DNA that fluorescence for FAM (CVMNK, Wild haplotype)
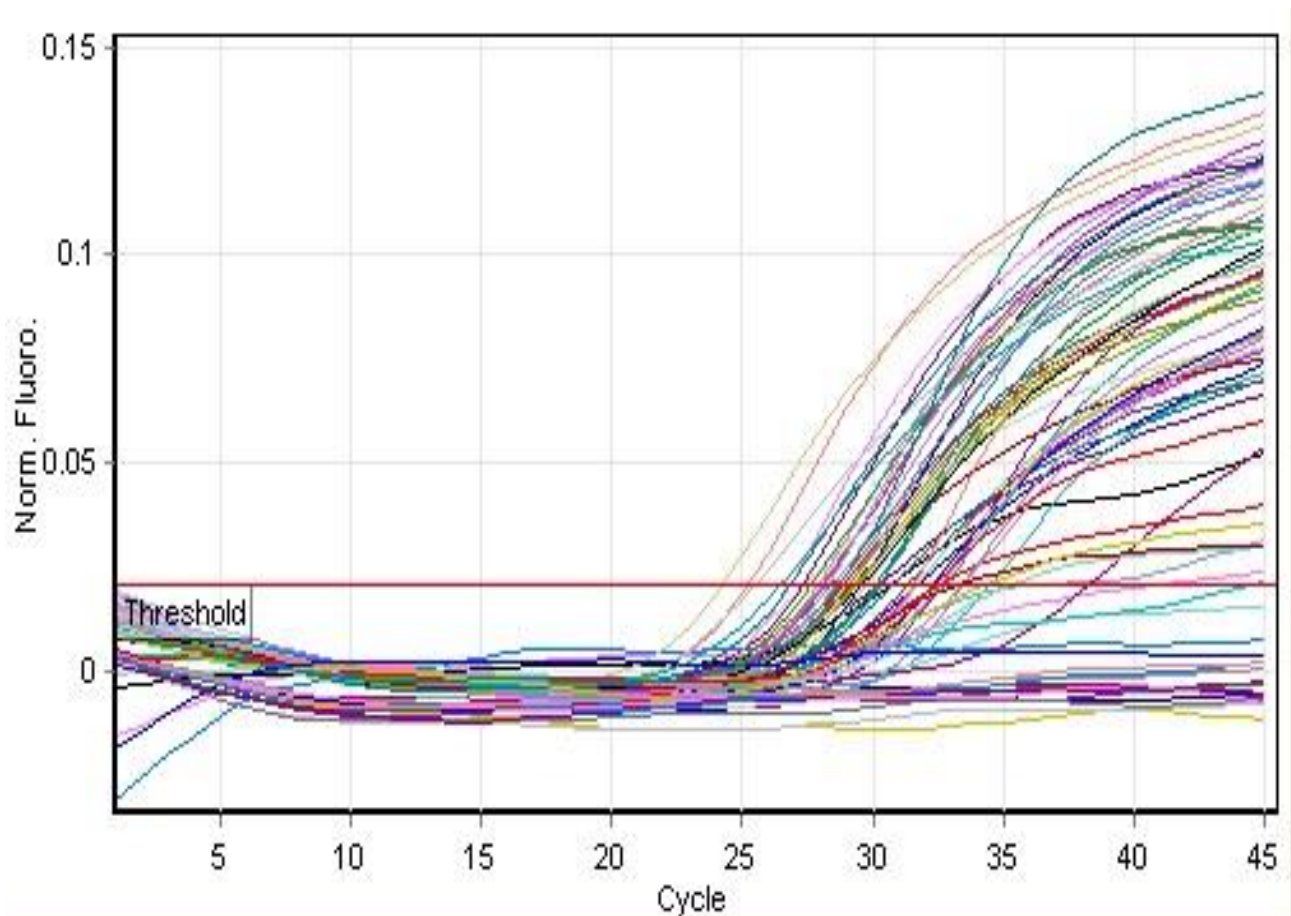The lines below the threshold represent DNA that did not fluorescence.

FIGURE 8. Mutant (CVIET) *Pfcrt* Cycling JOE (Mutant haplotypes)

Legend:
The lines above the threshold represent DNA that fluorescence (CVIET, Mutant haplotype)
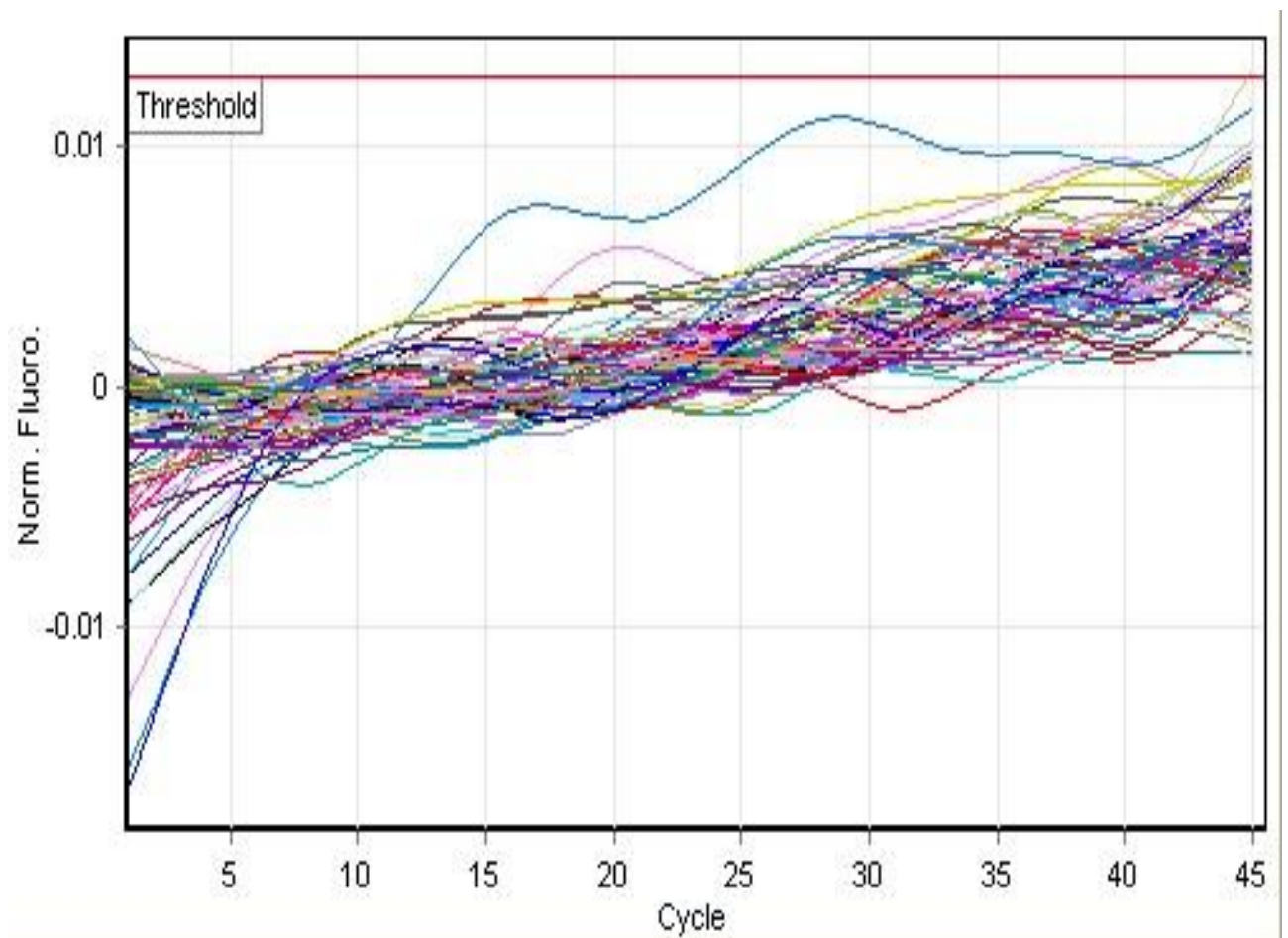The lines below the threshold represent DNA that did not fluorescence

FIGURE 9. Non- fluorescence (SVMNT) *Pfcrt* Cycling for ROX (Asian haplotypes)

Legend:
The lines below the threshold are the DNA that did not fluorescence (SVMNT)

4.3   Amplified *Pfmdr1* Alleles (FR1, FR2 and FR3)

The *Pfmdr1* fragments amplified were in 86, 184, 1034, 1042 and 1246 codons. Nested PCR was done on a cohort of 81 DNAs. Results of *Pfmdr1* FR1 amplified (codon 86 and 184) is shown in figure 10 with the band length of 534bp, *Pfmdr1* FR3 at codons 1034 and 1042 shown in figure 11 with band length of 234bp and *Pfmdr1* FR 4 with amplified codon in 1246 is shown in figure 12 with band length of 194bp.

4.3.1   Prevalence of Polymorphisms at the *Pfmdr-1* Loci

Sample of the sequenced amplified *Pfmdr1* genes chromatogram is shown in figure 13. Analysis of the 74 isolates of the *Pfmdr1* FR1 that were succesfully sequenced at codon 86 showed that the mutant type (Y86) had a prevalence of 62.2% (Table 4) and out of the 71 isolates that were succesfully sequenced at codon 184, the mutant type at this codon (F184) had a prevalence of 69.0% (Table 4). No mutation at *Pfmdr1* FR3 and *Pfmdr1* FR4 (Table 4).

4.3.2   Combined analysis of *Pfcrt* and *Pfmdr1* (FR1, FR3 and FR4) Haplotypes

A total of 21 isolates of *Pfcrt* and *Pfmdr1* (FR1, FR3 and FR4) were matched and annlysed. Of the isolates matched, 13 (61.9%) were in the group CVIET + YF + SN +D corresponding to *Pfcrt* mutant + *Pfmdr1* 86Y+184F + 1034S + 1042N and 1246D (Table 5).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

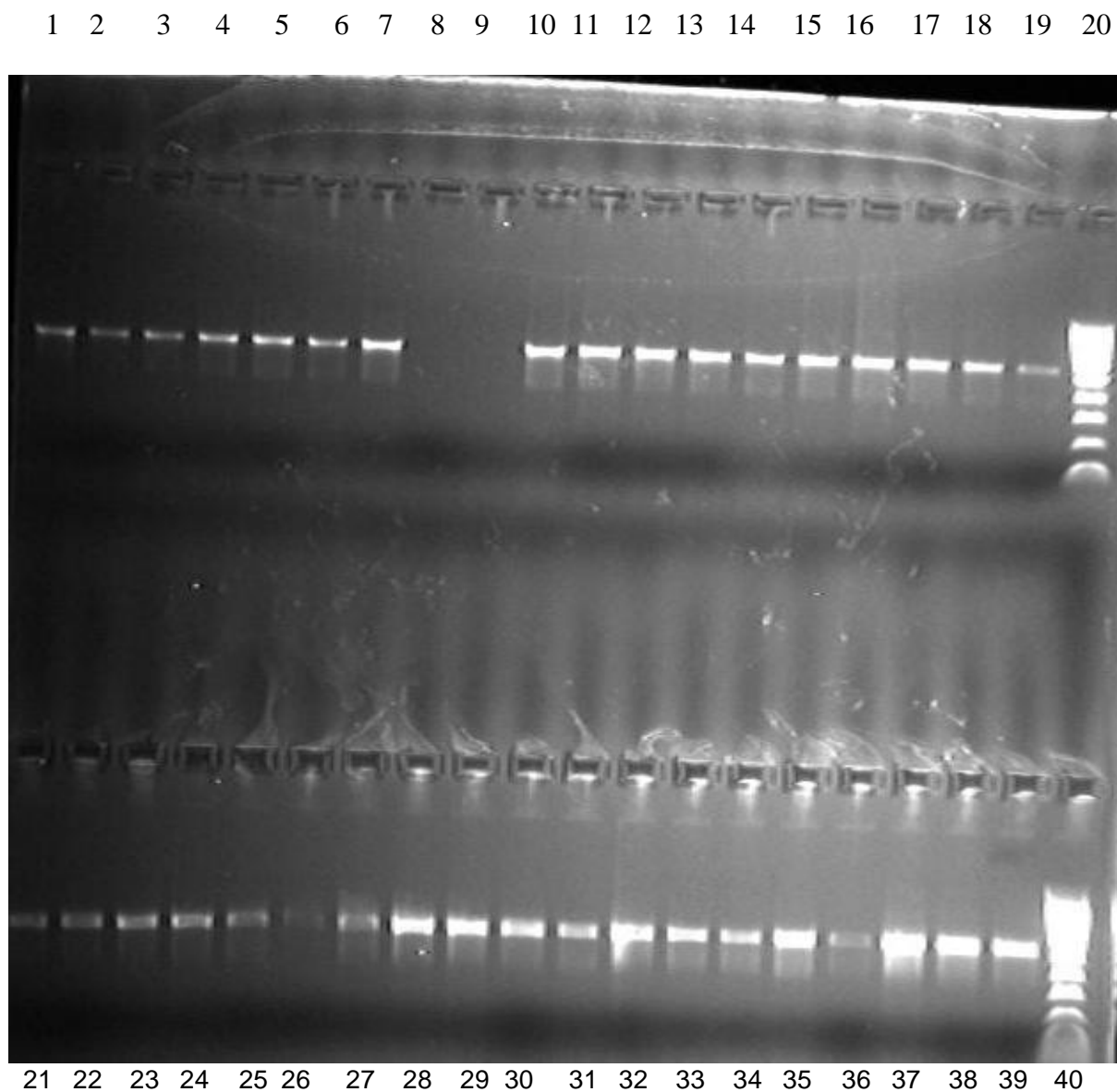21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

FIGURE 10. PCR products for *Pfmdr1* fragment 1.

Legend:
Lanes 8 and 9 represent chelex negative control without any DNA template. Lanes 37–39 represent positive control samples, Lanes 20 and 40 represent DNA marker (100-bp) and the rest of the lanes are the nested DNA for *Pfmdr1* FR1 (534-bp).
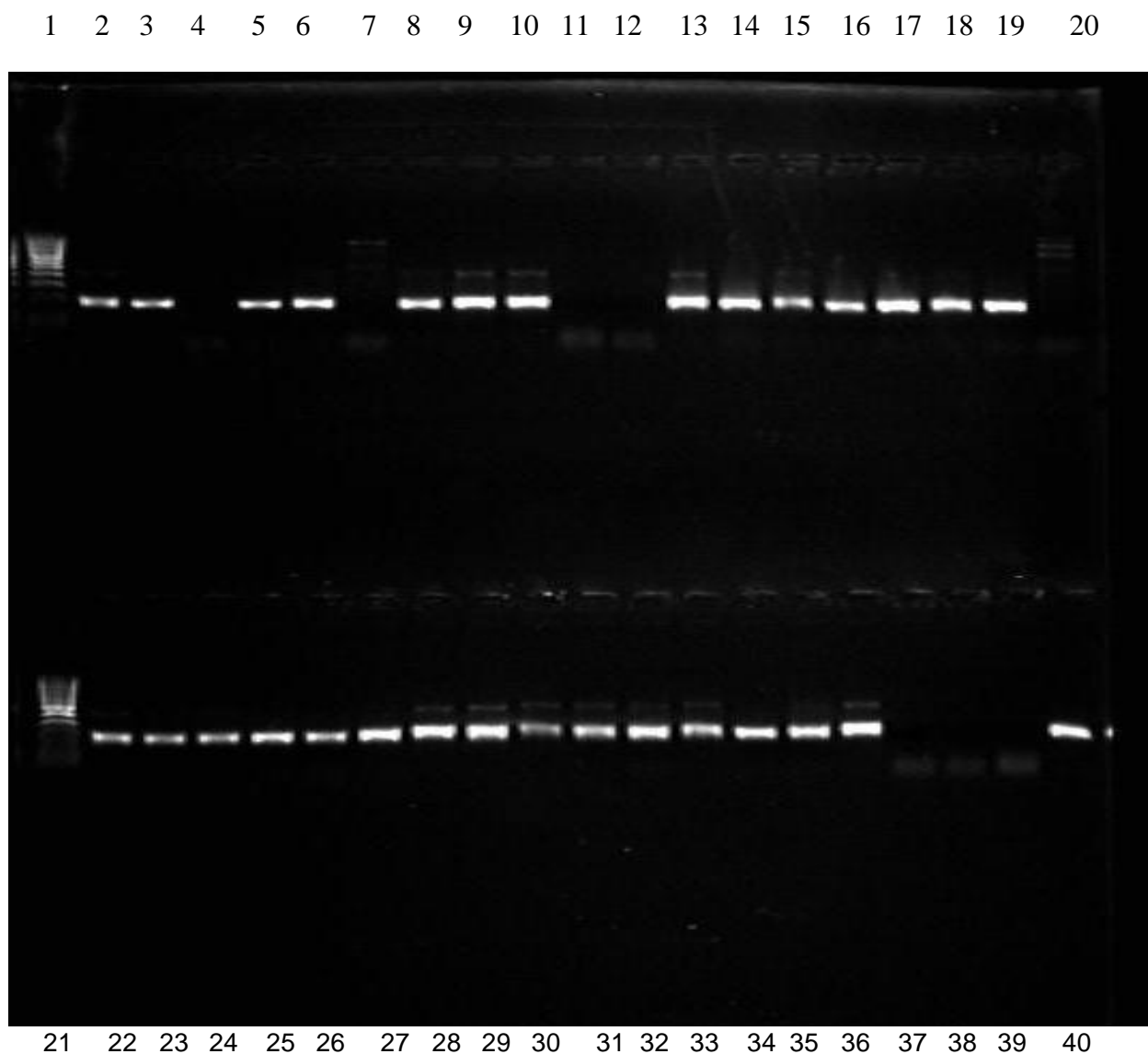
FIGURE 11. PCR products for *Pfmdr1* fragment 3.

Legend:
Lanes 4, 11, 12, 37, 38 and 39 represent chelex negative controls without any DNA template.
Lanes 40 represents positive control samples, Lanes 1 and 21 represent DNA marker (100-bp)
and the rest of the lanes are the nested DNA for *Pfmdr1* FR3 (234-bp).
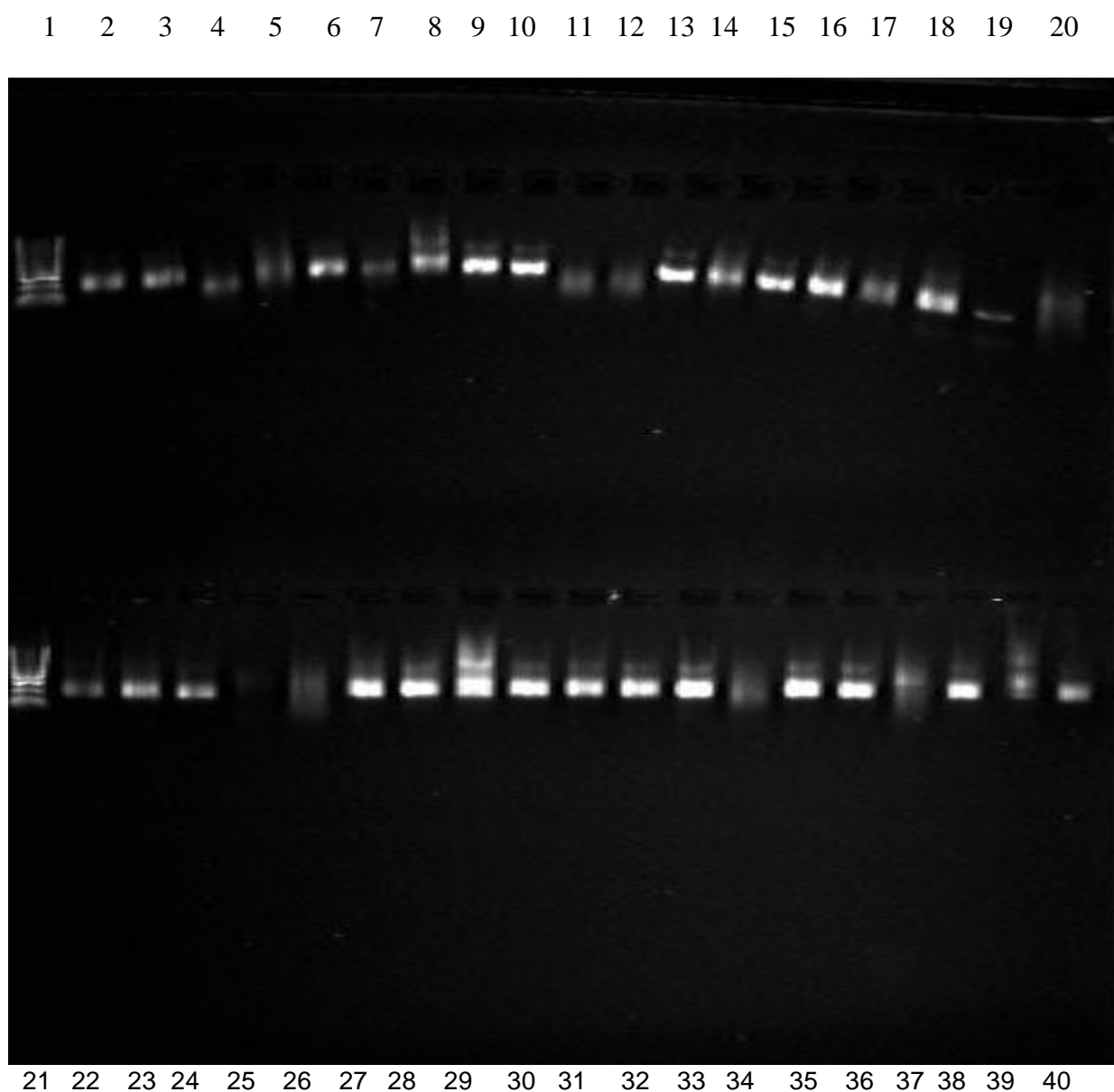
FIGURE 12. PCR products for *Pfmdr1* fragment 4.

Legend:
Lane 25 represents chelex negative control samples without any DNA template. Lanes 39,39 and 40 represent positive control samples, Lanes 1 and 21 represent DNA ladder (100-bp) and the rest of the lanes are the nested DNA for *Pfmdr1* FR 4 (194-bp).
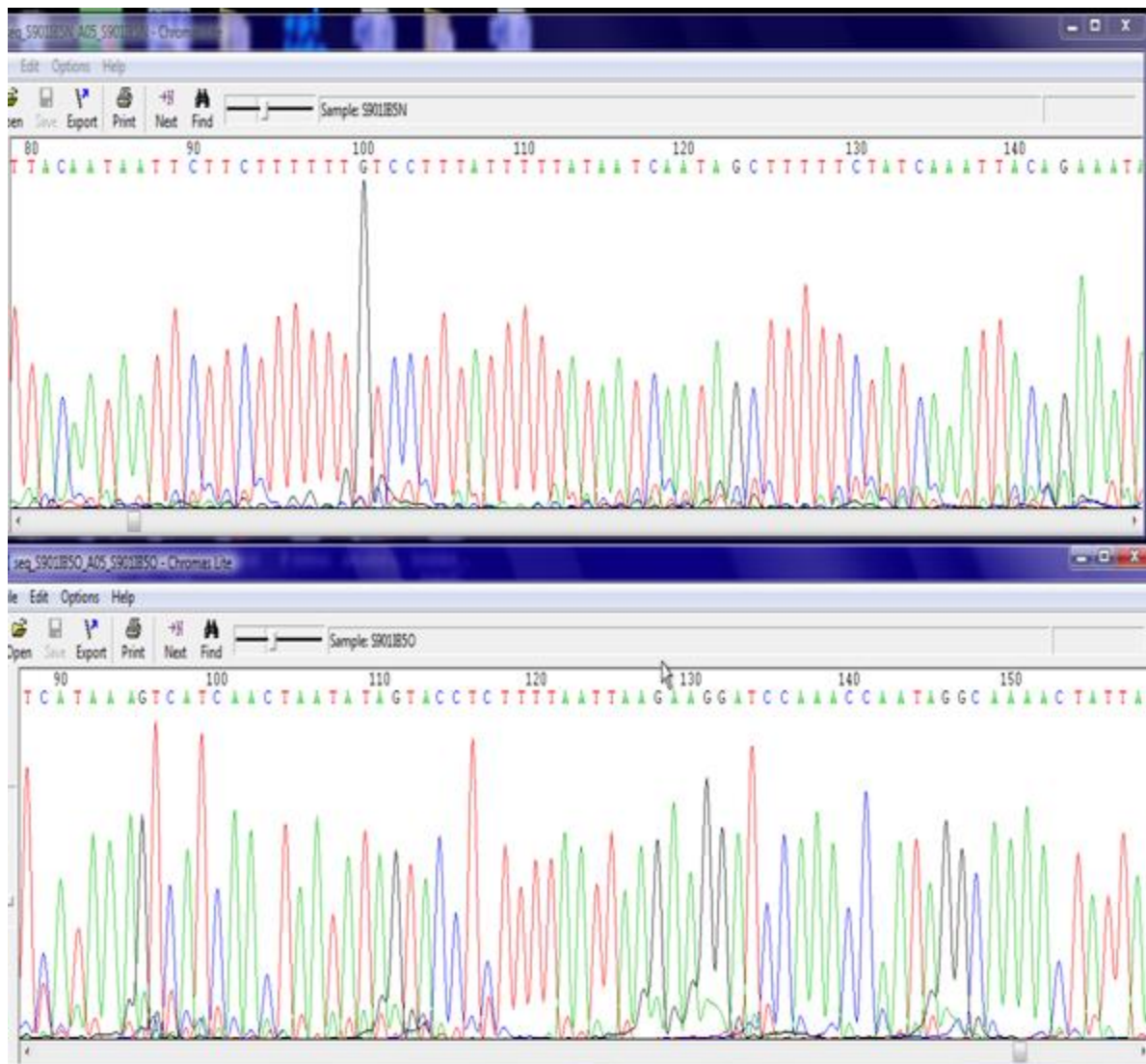
FIGURE 13. Sequenced *Pfmdr1* genes showing Chromatogram

Legend:
The different colours showed forward and reverse reaction showing the possible change in amino acid sequence that determines mutations in different codons.

Table 4. Prevalence of Point Mutations in *Pfmdr1* (FR1, FR3 and FR4) in a cohort of Children Studied

Gene	Fragments	Codon	Number	Prevalence (%) of Point Mutation in Children
Pfmdr1	FR1	86		
		(Wild-type) N86	14	18.9
		(Mutant-type) Y86	46	62.2
		N86 + Y86	14	18.9
Total			74	
		184		
		(Wild-type) Y184	11	15.5
		(Mutant-type) F184	49	69.0
		F184 + Y184	11	15.5
Total			71	
	FR3	1034		
		(Wild-type) S1034	81	100.0
		(Mutant-type) C1034	0	0.0
Total			81	
		1042		
		(Wild-type) N1042	81	100.0
		(Mutant-type) D1042	0	0.0
Total			81	
	FR4	1246		
		(Wild-type) D1246	29	100.0
		(Mutant-type) S1246	0	0.0
Total			29	

Legend:
Y86-	*Pfmdr1* FR1 mutant codon
N86-	*Pfmdr1* FR1 wild codon
F184-	*Pfmdr1* FR1 mutant codon
Y184-	*Pfmdr1* FR1 wild codon
C1034-	*Pfmdr1* FR3 mutant codon
S1034-	*Pfmdr1* FR3 wild codon
D1042-	*Pfmdr1* FR3 mutant codon
N1042-	*Pfmdr1* FR3 wild codon
S1246-	*Pfmdr1* FR4 mutant codon
D1246-	*Pfmdr1* FR4 wild codon

Table 5. Combined analysis of 21 isolates in *Pfcrt* and *Pfmdr1* (FR1, FR3 and FR4) haplotypes

Grouped Alleles	Haplotypes	Frequency (%)
Pfcrt/*Pfmdr1*(FR1,FR3 and FR4)	CVIET + YFSND	13 (61.9)
	CVIET + YYSND	1(4.8)
	CVIET + NFSND	2 (9.5)
	CVIET + NYSND	1 (4.8)
	CVIET + NY/YFSND	2 (9.5)
	CVIET + NY/FSND	1 (4.8)
	CVIET + Y/YFSND	1 (4.8)

Legend:

Y= Mutant Codon 86- *Pfmdr1* FR1
N= Wild Codon 86- *Pfmdr1* FR1
F= Mutant Codon 184 *Pfmdr1* FR1
Y= Wild Codon 184 *Pfmdr1* FR1
S= Wild Codon 1034 *Pfmdr1* FR3
N= Wild Codon 1042 *Pfmdr1* FR3
D= Wild Codon 1246 *Pfmdr1* FR4

4.3.3 Parasitaemia and *Pfcrt* Haplotypes

The relationship between parasite density and the *Pfcrt* haplotypes showed that, of all the positive samples that amplified for *Pfcrt* gene, 100% of the children that had parasite density >250,000p/μl had CVIET (mutant type) haplotype, while CVMNK and CVIET/CVMNK were not seen in the group of children with parasitaemia of >250,000p/μl (P=0.6510) (Table 6).

4.3.4 Parasitaemia and *Pfmdr*1 (FR1, FR3 and FR4) Haplotypes

In the *Pfmdr* 1 FR1, a prevalence of 58.6% was recorded for malaria positive children that had Y/F haplotype in the parasitaemia range 1-500 p/μl and there was no record of Y/F haplotype in the parasitaemia range >250,000 p/μl (Table 7) (P=0.2414). Though, there was no mutation seen in *Pfmdr1* FR3 and FR4, a prevalence of 38.0% of the positive children in parasitaemia group 10001-250000p/μl had the amplified FR3 (P=0.2414), while in FR4, a prevalence of 41.4% was recorded in the parasitaemia group 1-500p/μl (Table 7) (P=0.2414).

Table 6. Parasitaemia and *Pfcrt* haplotypes

Parasitaemia (p/µl)	CVIET %	CVMNK %	CVIET/CVMNK %
1-500	93.9	0.0	6.1
501-1000	80.0	20.0	0.0
1001-10000	95.2	0.0	4.8
10001-250000	89.1	6.5	4.3
>250000	100	0.0	0.0

Legend:

CVIET-   *Pfcrt* resistant haplotype (Asian and African Origin)
CVMNK-   *Pfcrt* wild haplotype
SVMNT-   *Pfcrt* resistant haplotype (Asian and American Origin)

Table 7. Parasitaemia and *Pfmdr*1 FR1, FR3 and FR4 Haplotypes in a Cohort of Malaria Positive Children

*Pfmdr*1 Haplotypes	Parasitaemia p/µl (%)				
	1-500	5001-1000	1001-10000	10001-250000	>250000
Fragment 1 (n=74)					
N/0	3.4	0.0	0.0	0.0	0.0
N/F	3.4	0.0	0.0	13.8	33.3
N/Y	6.9	33.3	0.0	13.8	0.0
NY/F	3.4	33.3	11.1	13.8	0.0
NY/Y	3.4	0.0	0.0	0.0	0.0
NY/YF	3.4	33.3	11.1	6.9	33.3
Y/0	3.4	0.0	0.0	0.0	33.3
Y/F	58.6	0.0	55.6	44.8	0.0
Y/Y	6.9	0.0	0.0	3.4	0.0
Y/YF	6.9	0.0	22.2	3.4	0.0
Fragment 3 (n=81)					
SN	34.2	6.3	15.2	38.0	6.3
Fragment 4 (n=21)					
D	41.4	3.4	24.1	27.6	3.4

N/0-	N86/-
N/F-	N86/F184
N/Y-	N86/Y184
NY/F-	N86 and Y86/F184
NY/Y-	N86 and Y86/Y184
NY/YF-	N86 and Y86/ Y184 and F184
Y/0-	Y86/-
Y/F	Y86/F184
Y/Y	Y86/Y184
Y/YF-	Y86/ Y184 and F184
SN-	S1034 and N1042
D-	D1246

4.4 Amplified *Dhfr and Dhps* Alleles

The *Dhfr* and the *Dhps* genes were amplified using nested PCR. An expected band of 594bp and 711bp were used for the succesful amplification of *Dhfr* and *Dhps* genes respectively. A total of one hundred and fifiteen (115) DNA samples extracted using the chelex method were ran for the two genes amplification. Figure 14 shows the *Dhfr* gel result with the PCR product size (bp) of about 594-bp for the *Dhfr* amplified genes, while figure 15 shows the *Dhps* gel result with the PCR product size (bp) of about 711-bp for the *Dhps* genes that amplified.

4.4.1 Prevalence of *Pfdhfr* and *Pfdhps* genotypes

A total of one hundred and thirteen (113) *Dhfr* and one hundred and fifteen (115) *Dhps* genes were sequenced for SNP. A prevalence of 96.5% *P. falciparum* isolates were found to carry the mutant type Serine 108 to Asparagine (N108) and 107(94.7%) of them carried the Asparagine 51 to Isoleucine (I51) mutation, while the Cysteine 59 to Arginine (R59) was present in 105(92.9%) of the isolated *P. falciparum* (Table 8). A total of four and ten haplotypes were reported for *Dhfr* and *Dhps* respectively. An example of the amplified sequenced *Dhfr* gene showing chromatogram is shown figure 16.

Out of the children *P. falciparum* DNA samples sequenced, 105(92.9%) were found to carry the *Dhfr* haplotype ACIRNVI with mutations in I51, R59 and N108, while 3.5% of the children had the haplotype ACNCSVI (wild type) with no mutations at all. In all the successful isolates, no *Pfdhfr* L164 mutation was seen. T108 and V16 variants which are linked with cycloguanil resistance were not present as well (Table 8).
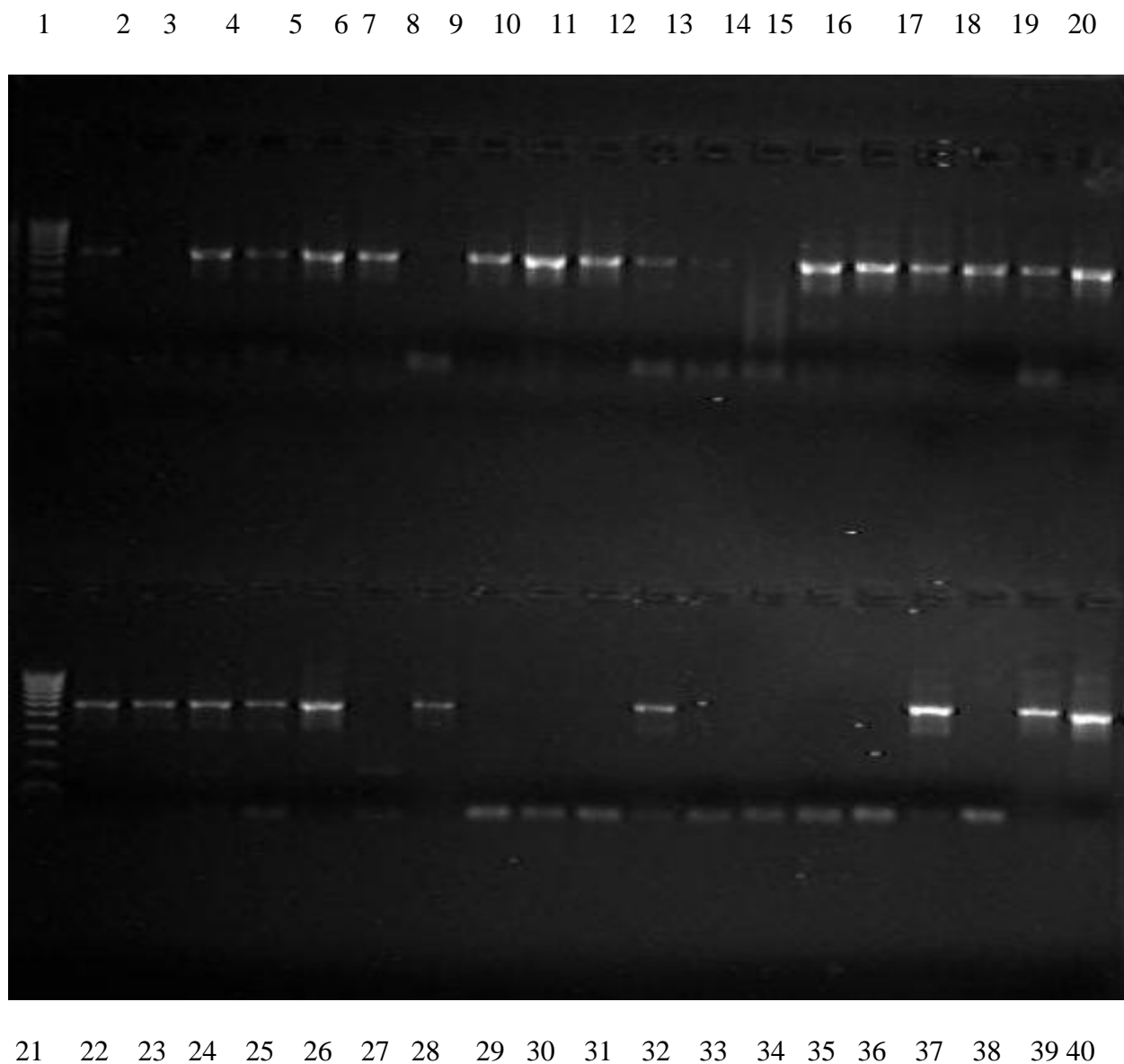
FIGURE 14. PCR products for *Dhfr* gene.

Legend:
Lane 8 and 30 represent chelex negative control samples without any DNA template. Lanes 39 and 40 represent DNA positive control samples, Lanes 1 and 21 represent DNA marker (100-bp) and the rest of the lanes are the test nested DNA (594-bp).
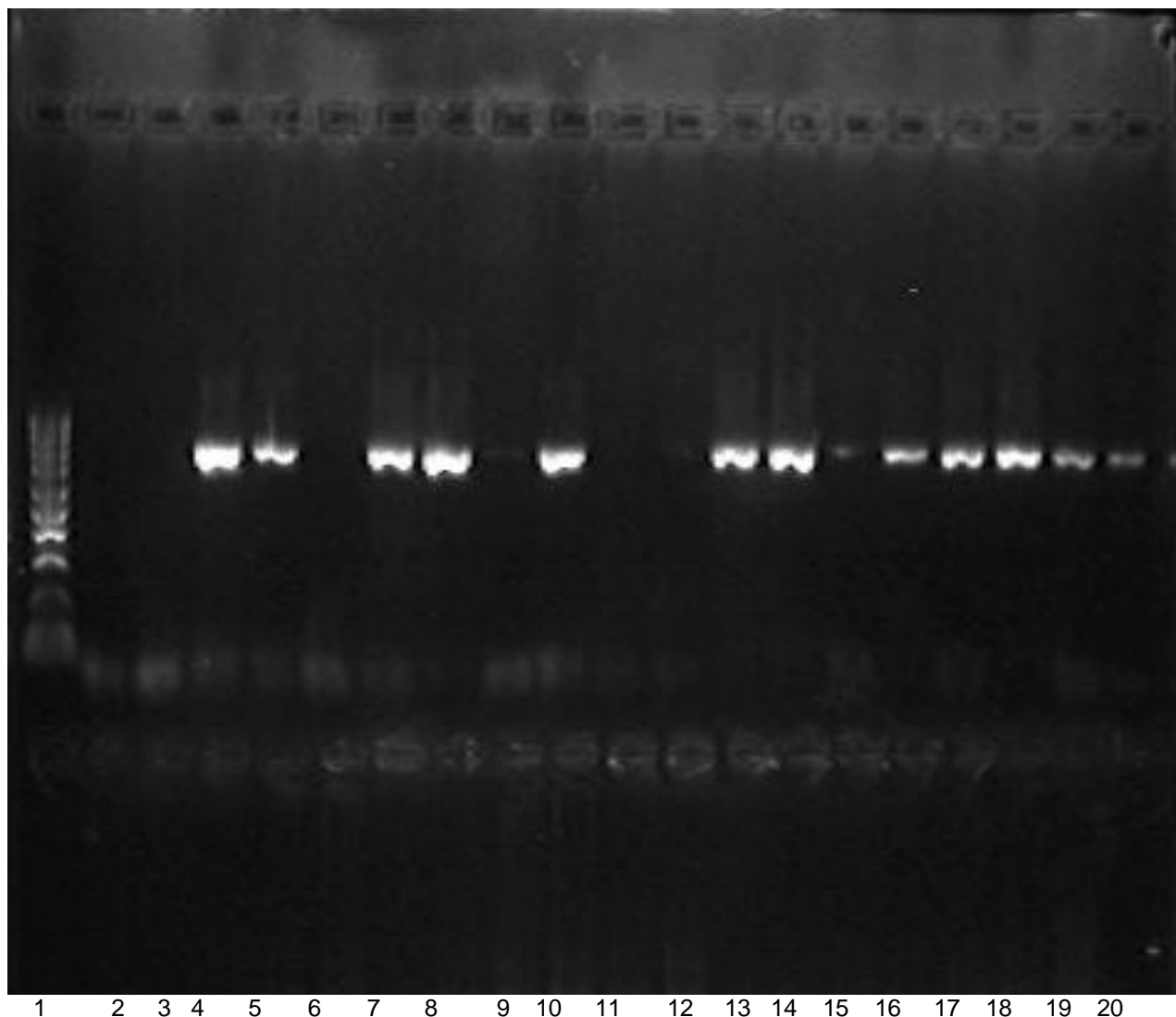
FIGURE 15. PCR products for *Dhps* gene.

Legend:
 Lane 2 and 12 represent chelex negative control samples without any DNA template. Lanes
18, 19 and 20 represent DNA positive control samples, Lanes 1 represents DNA ladder (100-
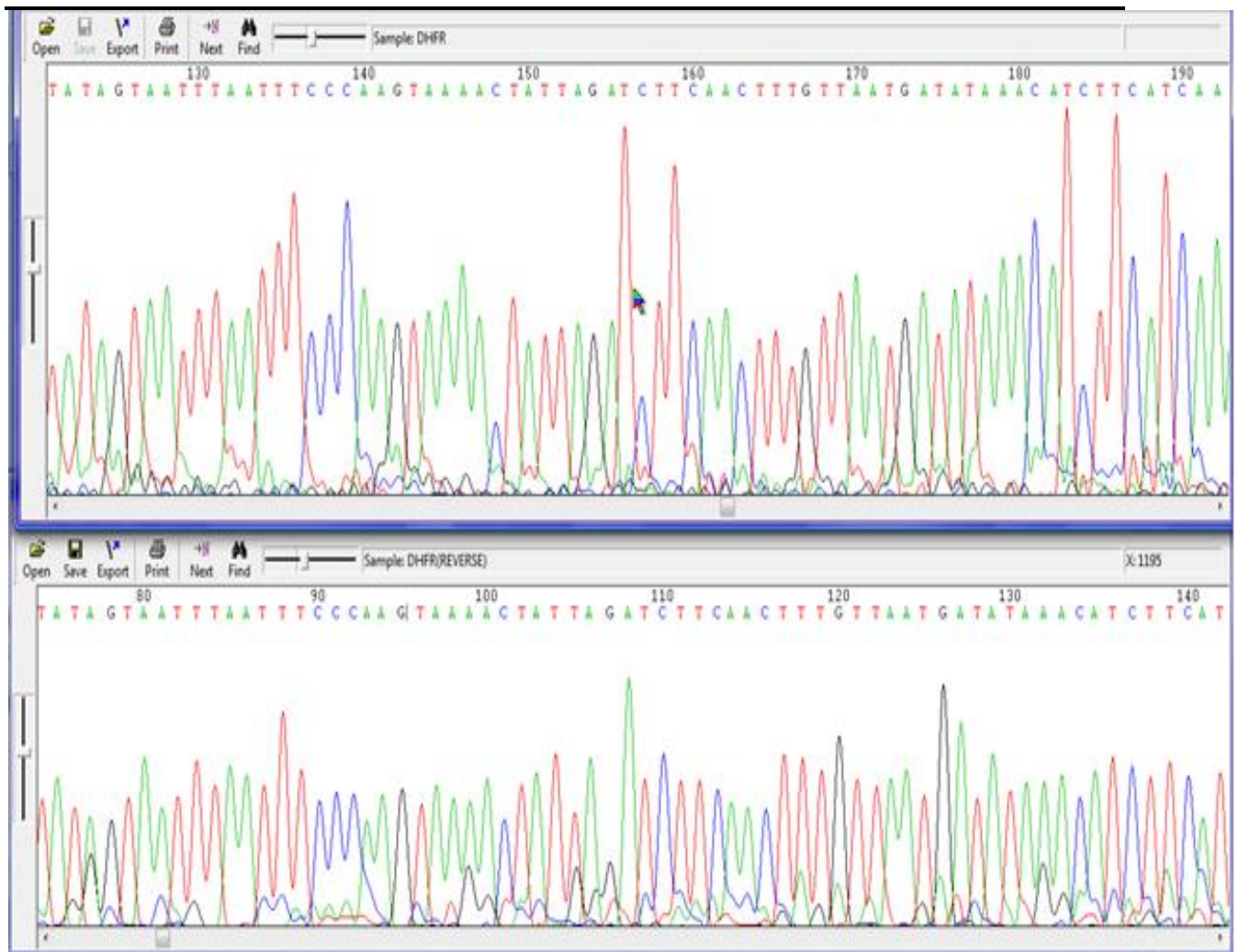bp) and the rest of the lanes are the test nested DNA (711-bp).

FIGURE 16. Sequenced *Dhfr* gene showing chromatogram

Legend:
The different colours showed forward and reverse reaction of a sequenced *Dhfr* genes. The change in amino acid sequence determines mutations in the different codons

Table 8. Prevalence of SP mutations in *Plasmodium falciparum* isolates from children in Lagos, Nigeria

Gene	Mutations	Number	Mutations (%)
Dhfr	I51	113	107 (94.7)
	R59	113	105 (92.9)
	N108	113	109 (96.5)
	L164	113	0 (0)
	R50	113	0 (0)
	V16	113	0 (0)
Dhps	V431	115	22 (19)
	A/F436	115	39/1 (33.6/0.9)
	G437	115	112 (96.6)
	G581	115	22 (19)
	S613	115	30 (25.9)

Legend:
I51-   Mutant *Dhfr* codon 51
R59-   Mutant *Dhfr* codon 59
N108-   Mutant *Dhfr* codon 108
L164-   Mutant *Dhfr* codon 164
R50-   Mutant *Dhfr* codon 50
V16-   Mutant *Dhfr* codon 16
V431-   Mutant *Dhps* codon 431
A/F436-   Mutant *Dhps* codon 436
G437-   Mutant *Dhps* codon 437
G581-   Mutant *Dhps* codon 581
S613-   Mutant *Dhps* codon 613

The *Pfdhfr* gene analysis showed four haplotypes: three associated with drug resistance profiles (ACIRNVI, ACICNVI and ACNCNVI), while one was associated with SP sensitivity (ACNCSVI). The *Pfdhps* gene analysis however showed existence of eleven haplotypes with ten of them associated with drug resistant profiles (ISGKAA, VAGKGS, VAGKAA, IAGKAS, IAAKAA, ISGKGA, IFGKAS, IAGKAA, VSGKGS and ISGKAS) and one (ISAKAA) associated with SP sensitivity (Table 9).

The *Dhps* gene polymorphisms in different loci showed that *Dhps* G437 had a prevalence of 96.6% and A581G had a prevalence of 19%, while the *Dhps* I431V had a prevalence of 19% (Table 8). The *Dhps* haplotype ISGKAA had a prevalence of 60.0%. The VAGKGS and IAGKAS haplotypes have prevalence of 13.9% and 9.6% respectively (Table 9). There was no mutation recorded at codons K540E.

The matched *Dhfr* and *Dhps* haplotypes showed quadruple mutation [triple *dhfr* (51I, 59R and 108N) + *dhps* 437G] representing the haplotype ACIRNVI + ISGKAA with a prevalence of 56.7% in the studied children (Table 10). Six of the isolates (10%) have the haplotype ACIRNVI + VAGKGS representing triple mutant at the *Dhfr* gene and five mutants at the *Dhps* gene.

Table 9: Frequency of haplotypes in *Dhfr* and *Dhps* genes in *Plasmodium falciparum* isolates of children in Lagos, Nigeria

Genes	Haplotypes	Frequency	%
Dhfr	ACIRNVI	105/113	92.9
	ACICNVI	2/113	1.8
	ACNCNVI	2/113	1.8
	ACNCSVI	4/113	3.5
Dhps	ISGKAA	69/115	60.0
	VAGKGS	16/115	13.9
	VAGKAA	5/115	4.3
	IAGKAS	11/115	9.6
	ISAKAA	2/115	1.7
	IAAKAA	2/115	1.7
	ISGKGA	2/115	1.7
	IFGKAS	1/115	0.9
	IAGKAA	5/115	4.3
	VSGKGS	1/115	0.9
	ISGKAS	1/115	1.7

Legend:
ACIRNVI-   *Dhfr* gene haplotype in codons A40/C50/I51/R59/N108/V140/I164
ACICNVI -   *Dhfr* gene haplotype in codons A40/C50/I51/C59/N108/V140/I164
ACNCNVI-   *Dhfr* gene haplotype in codons A40/C50/N51/C59/N108/V140/I164
ACNCSVI-   *Dhfr* gene haplotype in codons A40/C50/N51/C59/S108/V140/I164
ISGKAA-   *Dhps* gene haplotype in codons I431/S436/G437/K540/A581/A613
VAGKGS-   *Dhps* gene haplotype in codons V431/A436/G437/K540/G581/S613
VAGKAA-   *Dhps* gene haplotype in codons V431/A436/G437/K540/A581/A613
IAGKAS-   *Dhps* gene haplotype in codons I431/A436/G437/K540/A581/S613
ISAKAA-   *Dhps* gene haplotype in codons I431/S436/A437/K540/A581/A613
IAAKAA-   *Dhps* gene haplotype in codons I431/A436/A437/K540/A581/A613
ISGKGA-   *Dhps* gene haplotype in codons I431/S436/G437/K540/G581/A613
IFGKAS-   *Dhps* gene haplotype in codons I431/F436/G437/K540/A581/S613
IAGKAA-   *Dhps* gene haplotype in codons I431/A436/G437/K540/A581/A613
VSGKGS-   *Dhps* gene haplotype in codons V431/S436/G437/K540/G581/S613
ISGKAS-   *Dhps* gene haplotype in codons I431/S436/G437/K540/A581/S613

Table 10: Haplotypes reported in *Dhfr* and *Dhps* genes

Grouped alleles	Haplotypes	Number (%)
Triple *Dhfr*/Five *Dhps*	ACIRNVI + VAGKGS	6(10.0)
Triple *Dhfr*/ Four *Dhps*	ACIRNVI + VSGKGS	1(1.7)
Triple *Dhfr*/Three *Dhps*	ACIRNVI + VAGKAA	4(6.6)
Triple *Dhfr*/Three *Dhps*	ACIRNVI + IAGKAS	5(8.3)
Triple *Dhfr*/Three *Dhps*	ACIRNVI + IFGKAS	1(1.7)
Triple *Dhfr*/Two *Dhps*	ACIRNVI + ISGKGA	4(6.6)
Triple *Dhfr*/Two *Dhps*	ACIRNVI + IAGKGS	2(3.3)
Triple *Dhfr*/Two *Dhps*	ACIRNVI + IAGKAA	1(1.7)
Triple *Dhfr*/One *Dhps*	ACIRNVI + ISGKAA	34(56.7)
Two *Dhfr*/Two *Dhps*	ACICNVI + IAGKAA	1(1.7)
One *Dhfr*/One *Dhps*	ACNCNVI+ ISGKAA	1(1.7)

Legend:
ACIRNVI-   *Dhfr* gene haplotype in codons A40/C50/I51/R59/N108/V140/I164
ACICNVI -   *Dhfr* gene haplotype in codons A40/C50/I51/C59/N108/V140/I164
ACNCNVI-   *Dhfr* gene haplotype in codons A40/C50/N51/C59/N108/V140/I164
ACNCSVI-   *Dhfr* gene haplotype in codons A40/C50/N51/C59/S108/V140/I164
ISGKAA-   *Dhps* gene haplotype in codons I431/S436/G437/K540/A581/A613
VAGKGS-   *Dhps* gene haplotype in codons V431/A436/G437/K540/G581/S613
VAGKAA-   *Dhps* gene haplotype in codons V431/A436/G437/K540/A581/A613
IAGKAS-   *Dhps* gene haplotype in codons I431/A436/G437/K540/A581/S613
ISAKAA-   *Dhps* gene haplotype in codons I431/S436/A437/K540/A581/A613
IAAKAA-   *Dhps* gene haplotype in codons I431/A436/A437/K540/A581/A613
ISGKGA-   *Dhps* gene haplotype in codons I431/S436/G437/K540/G581/A613
IFGKAS-   *Dhps* gene haplotype in codons I431/F436/G437/K540/A581/S613
IAGKAA-   *Dhps* gene haplotype in codons I431/A436/G437/K540/A581/A613
VSGKGS-   *Dhps* gene haplotype in codons V431/S436/G437/K540/G581/S613
ISGKAS-   *Dhps* gene haplotype in codons I431/S436/G437/K540/A581/S613

4.4.2   Parasitaemia and *Dhfr* Haplotypes

The relationship between parasitaemia and the *Dhfr* haplotypes showed that out of the amplified isolates, 40(40.8%) carried the haplotype ACIRNVI in the parasitaemia group 10,000 p/µl -250,000p/µl (P=0.8224) (Table 11).

4.4.3   Parasitaemia and *Dhps* Haplotypes

In the *Dhps* haplotypes, out of the amplified isolates, 24(35.8%) carried the haplotype ISGKAA in the parasitaemia group 10,000 - 250,000p/µl and 1(1.5) carried the haplotype ISGKAA in >250,000p/µl (P=0.0465) (Table 12).

Table 11: Parasitaemia and *Dhfr* haplotypes

Parasitaemia (p/µl)	ACICNVI %	ACIRNVI %	ACNCNVI %	ACNCSVI %
1-500	0.0	29.6	0.0	25.0
501-1000	0.0	7.1	0.0	25.0
1001-10000	0.0	18.4	100.0	0.0
10001-250000	100.0	40.8	0.0	50.0
>250000	0.0	3.1	0.0	0.0

Legend:
ACIRNVI-   *Dhfr* gene haplotype in codons A40/C50/I51/R59/N108/V140/I164
ACICNVI -   *Dhfr* gene haplotype in codons A40/C50/I51/C59/N108/V140/I164
ACNCNVI-   *Dhfr* gene haplotype in codons A40/C50/N51/C59/N108/V140/I164
ACNCSVI-   *Dhfr* gene haplotype in codons A40/C50/N51/C59/S108/V140/I164

Table 12: Parasitaemia and *Dhps* haplotypes

Dhps Haplotypes	Parasitaemia p/µl (%)				
	1-500 %	501-1000 %	1001-10000 %	10001-250000 %	>250000 %
IAAKAA	0.0	0.0	0.0	100.0	0.0
IAGKAA	25.0	0.0	25.0	25.0	25.0
IAGKAS	36.4	0.0	0.0	54.5	9.1
IFGKAS	0.0	0.0	100.0	0.0	0.0
ISAKAA	0.0	0.0	0.0	100.0	0.0
ISGKAA	31.3	7.5	23.9	35.8	1.5
ISGKAS	0.0	0.0	100.0	0.0	0.0
ISGKGA	0.0	33.3	66.7	0.0	0.0
VAGKAA	20.0	20.0	20.0	40.0	0.0
VAGKGS	40.0	0.0	6.7	46.7	6.7
VSGKGS	0.0	0.0	0.0	0.0	100.0

Legend:

ISGKAA-	*Dhps* gene haplotype in codons I431/S436/G437/K540/A581/A613
VAGKGS-	*Dhps* gene haplotype in codons V431/A436/G437/K540/G581/S613
VAGKAA-	*Dhps* gene haplotype in codons V431/A436/G437/K540/A581/A613
IAGKAS-	*Dhps* gene haplotype in codons I431/A436/G437/K540/A581/S613
ISAKAA-	*Dhps* gene haplotype in codons I431/S436/A437/K540/A581/A613
IAAKAA-	*Dhps* gene haplotype in codons I431/A436/A437/K540/A581/A613
ISGKGA-	*Dhps* gene haplotype in codons I431/S436/G437/K540/G581/A613
IFGKAS-	*Dhps* gene haplotype in codons I431/F436/G437/K540/A581/S613
IAGKAA-	*Dhps* gene haplotype in codons I431/A436/G437/K540/A581/A613
VSGKGS-	*Dhps* gene haplotype in codons V431/S436/G437/K540/G581/S613
ISGKAS-	*Dhps* gene haplotype in codons I431/S436/G437/K540/A581/S613

CHAPTER FIVE

5.0   DISCUSSION

Malaria is an important cause of febrile illness in children in Africa especially when caused by *Plasmodium falciparum* that infects most people. It is more harzadous in children less than five years old. Malaria as a febrile illness is included in the Integrated Management of Childhood Illness (IMCI) strategy which aims to improve family and community practices in the management of childhood illness (Goves, 1997). Malaria prevalence is reported to be on the high side in young children and the cause of most deaths in this age (WHO, 2009).

In this study, 20.7% of the children (age 0-12years) had malaria. Similar studies to this malaria prevalence reported in this study but not in febrile children; prevalence of 20.8% was reported in Enugu, South-East Nigeria among 1,296 afebrile school children (Ekpenyong and Eyo, 2008), 27.2% in severely ill children (aged,0-8years) in Maiduguri, North-East, Nigeria (Samdi *et al.*, 2005). In contrast to this report, malaria prevalence of 32.9% was reported in children between 1-14years old in Kano State (Adeleke, 2007) and Ejezie and Ezedinachi, (1992) reported a prevalence of 44.8% in children less than 0-12years in Calabar, South-South, Nigeria. As there are divergent reports of malaria prevalence and infectivity in children in Africa, a comparative analysis of the results obtained in this study will empirically bring to bear the current trends. The WHO Malaria report (WHO, 2008a) put the estimated malaria cases at 247 millions, down from the usual estimates of 300-500 million cases (WHO, 2000). The recent WHO Malaria report (WHO, 2009) currently estimates the malaria cases to be 243 million. These reported reductions in malaria cases were attributed to large-scale implementation of malaria interventions such as the use of LLINs, ACTs, and IRS (WHO, 2009).

Among the 0-≤5years old children in this study, malaria prevalence of 16.9% was reported in contrast to a similar study in Jos, North-Central, were a prevalence of 56.9% was reported for febrile children 0-≤5years old in a Primary Health Care Centre (Ikeh and Teclaire, 2008), 67.5% among 0-5 years old in Ogun State, (Ibidapo, 2005) and a prevalence of 87.2% in 0-≤5years old children in another study in Uyo, South-South Nigeria (Opara *et al.*, 2006). Guinovart *et al.*, (2008) also reported a malaria prevalence of 30.5% in children 0-≤15 years of age in Mozambique, in which children aged 0-≥ 5 years represented 36.4% of the cases. In two neighbouring communities in Ghana in 0-12years old children, a malaria prevalence of 37.8% and 12.8% was reported (Ronald *et al.*, 2006).

Consistent with this study, a decline in malaria prevalence has been reported in some parts of sub-Saharan Africa (Bouyou-Akotet *et al.*, 2009; Delacollette *et al.*, 2009). Adherence to the IMCI guildlines on presumptive treatment approach is likely to increase the degree of overtreatment and inevitably a failure to treat alternative causes of fever. Indeed, studies in critically ill patients treated for "malaria" showed significantly higher mortality in those with a negative slide compared to those with a positive slide (Reyburn *et al.*, 2004). With the almost universal introduction of ACT, there have been increasing calls for improving the way diagnosis and case management for malaria is conducted. In order to treat patients appropriately and to ensure the sustainability of ACT and to protect the "useful lifespan" of ACTs, the new guidelines from WHO recommend that a laboratory test should be performed before treating (WHO, 2010). Therefore, efforts should be made to improve access to parasitological diagnostics. Presently, the options available are to increase capacity to carry out microscopy or the widespread use of RDTs which requires no special skill and can be perform without electricity unlike microscopy.

This study reported a prevalence of 11.0% for children 0-≤1year old in contrast to the study among infants by Afolabi *et al*., (2001) in Lagos where they reported a prevalence of 27.1%; and Ikeh and Teclaire (2008) reported prevalence of 52.2% in Jos, in children 0-≤1year old. Malaria prevalence in children >5-12years old was more (42.1%) than the occurrence in the 0-≤5years children (16.9%) in this study. A similar prevalence was reported by Bouyou-Akotet *et al*., (2009) in Gabon where more than 40% of the febrile children aged 5-10 years had malaria parasite. Highest malaria prevalence was also seen in wet and dry season in 5-15years old children in Gambia (Okebe *et al*., 2010), and in a study of healthy Gambians, where peak parasite prevalence was seen in 10-15years old (Satoguina *et al*., 2009). The similarity in the prevalence obtained in Lagos, Gabon and Gambia is interesting as it underscores the importance of this age group to be targeted in interventional programmes though universal target of all age groups is the current thinking. If the 5-10/12 years age group had higher prevalence, it meant that there was an over emphasis on the 0-<5 years in the era of targeted malaria control. Therefore, with the new universal approach, all groups are likely to benefit and receive equal attention. This study therefore identified this age-group, in addition to existing groups to be carefully followed up in future studies.

Among the infants, the prevalence in the 10-12 month-old (14.9%) was high when compared with the 1-3month-old infants (5.8%). Though, there was no significant difference in the malaria prevalence between these age-groups, transplancetally acquired maternal antibody (IgG) is known to protect infants in the first six months of life (White, 2005). It is note worthy to emphasize here that the malaria epidemiology in the Northern part of Nigeria is different from the Southern part as the North is characterized by short rains of about 3 months while in the South, the rains are on for a longer time and thus malaria transmission is more stable in the South than in the North.

We reported higher parasite density in the 0-≤5years old than in the >5-12 years old children in this study in contrast with the study done by Adeleke, (2007) among febrile children in Kano. In Ogun State, South-West Nigeria, parasite density range of 71,500p/µl to 140,024p/µl and a mean parasiteamia of 95,122.63 p/µl was reported in the 1-12 years old children (Osonuga and Oduyemi, 2009). These mean parasite density is higher than than what was obtained in a similar group in this study (43,097.6p/µl).

Despite the change in antimalarial drug policy in the country, the continuous use of CQ and SP is still on the increase because it is cheap and it is readily available in the country. Self treatment with CQ and SP is still on the high side in the population because of the cost and accesibility of ACTs. The poverty level in the country has contributed to the increase in the usage of CQ and SP because the recommended drug (ACTs) is expensive and where it is available through government programme, there are issue on access and stock out. In the presence of already existing resistance to SP, there is likelihood for the SP resistance to increase and therefore jeopardized the efficacy of SP currently used as IPTp. The access to, and high usage of these drugs in the treatment of malaria in the clinical and general population setting makes monitoring for resistance imperative.

Djimde *et al.*, (2001) reported that resistance to CQ is largely determined by K76T mutation in the *Pfcrt* gene (this is enhanced by mutations at other sites of this gene) and mutations in the *Pfmdr1* gene. In this study, the molecular genotyping for the prevalence of mutations in 5 codons of *Pfcrt* (72-76) and in the *Pfmdr*1 genes among natural malaria parasite populations in the febrile children showed that among the three haplotypes (CVMNK, SVMNT, and CVIET) found in the *Pfcrt* gene, the CVIET (mutant type) had a prevalence of 91.6%, CVMNK (wild type) had prevalence of 4.2% and CVMNK/CVIET (mixed infection) had a

prevalence of 4.2%. SVMNT (mutant type) was not found in this study. CVMNK haplotype are known to be clearly associated with CQ-sensitive isolates, whereas the SVMNT and CVIET haplotypes were associated with CQ resistant isolates (Keen *et al.*, 2007; Sutherland *et al.*, 2007). As it has been reported in most Africa countries except in Tanzania and Angola (Alifrangis *et al.*, 2006; Gama *et al.*, 2010), the resistant haplotype, SVMNT was absent in the 119 samples genotyped. This study confirms that the SVMNT (the Asian Haplotypes) do not occur in Lagos. While the resistant haplotypes (CVIET) that have been reported in Africa had a high prevalence of 91.6%, which is similar with a study in Sudan where 93% of people treated with CQ had CVIET for *Pfcrt* (codon 72-76) and in Uganda where 98% of isolates carried the CQ resistant CVIET haplotype (Keen *et al.*, 2007). Four out of the eleven malaria positive children who admitted using CQ before coming to the clinic had the mutant haplotype CVIET.

A prevalence of 62.2% was recorded in this study for *Pfmdr1* Y86 (mutant-type) which have been reported to be responsible for CQ resistance in combination with *Pfcrt* 76T (Nagesha *et al.*, 2001; Duraisingh and Cowman, 2005; Happi *et al.*, 2006). *Pfmdr1* polymorphisms may result in reduction in the therapeutic efficacy of the newly adopted combination treatment for uncomplicated falciparum malaria in Sub-Saharan countries of Africa. Polymorphisms in the *Pfmdr*1 gene was said to be under artemether lumenfantrine selection pressure (Happi *et al.*, 2009). The *Pfmdr1* haplotype 86N-184F-1246D was significant in pre-treatment group and was selected for among post-treatment group treated with artemether lumenfantrine obtained from patients in Ibadan, Nigeria. The reports from East Africa (Dokomajilar *et al.*, 2006; Humphreys *et al.*, 2007) and West Africa (Zongo *et al.*, 2007) show some evidence of clinical and parasitological failure after treatment with artemether lumenfantrine. *In-vivo* selection of *Pfmdr1* 86N allele by artemether-lumefantrine was also reported in Tanzania (Sisowath *et al.*,

2005). Thus, the success of treatment with ACTs may largely depend on the parasite's existing level of tolerance to the partner drugs. Selection of *Pfmdr1* Y86 by amodiaquine and CQ were recorded previously in the Gambia (Duraisingh *et al.*, 1997), and in Kenya (Holmgren *et al.*, 2006). A prevalence of 69.0% was reported for mutations in *Pfmdr1* F184 in this study, though, studies are ongoing on the effect of this codon on CQ resistance.

A study reported a decrease in the frequency of *Pfcrt* 76T mutation with the abolition of CQ in the treatment of *P. falciparum* malaria in the People's Republic of China (Wang *et al.*, 2005), unlike in Nigeria where CQ is not recommended but is highly used. Since resistant phenotypes often have fitness costs (Yang *et al.*, 2007), their prevalence is expected to decline after removal of the selective pressure.

The prevalence of mutant alleles of *Pfcrt* 76T decreased from 64.5% in 2002 to 16% in 2004 and that of the mutant *Pfmdr1* 86Y alleles decreased from 46.6% to 2.7% two and half year after successful withdrawal of CQ in coastal Tanzania (Temu *et al.*, 2006). Kublin *et al.*, (2003) also reported that the prevalence of the CQ-resistant *Pfcrt* 76T genotype decreased from 85% in 1992 to 13% in 2000 in Malawi. In 2001, CQ cleared 100% of 63 asymptomatic *P. falciparum* infections, no isolates were resistant to CQ *invitro*, and no infections with the CQ resistant *Pfcrt* 76T genotype were detected (Kublin *et al.*, 2003). Similarly, it has been demonstrated that CQ was again an efficacious treatment for malaria, 12 years after it was succesfully withdrawn from use in Malawi (Laufer *et al.*, 2006). Similar result was also recorded in Kenya where the frequency of the *Pfcrt*-76 mutant significantly decreased from around 95% to 60%, though, the frequency of *Pfmdr1*-86 did not decline, remaining around 75% (Mwai *et al.*, 2009). In Tanzania, where CQ is no more used, the frequency of the wild type CVMNK haplotype increased from 6% in 2003 to 30% in 2007. This finding may reflect

decreasing drug pressure of 4-aminoquinolines on the parasite populations in the area. The selection pressure of AQ on *Pfcrt* mutants is said to be likely of less importance compared with that of CQ (Alifrangis *et al.*, 2009).

In Asia, especially in Thia-Cambodia border where resistance to CQ first occured, high antimalarial drug resistant genes has been reported in both clinical settings and in the general population. Drug pressure in this area is high for a number of reasons and unfortunately, it is in this area that resistance to ACTs was reported (Rogers *et al.*, 2009). Regular monitoring for drug resistance is recommended in Nigeria and behavioural changes in communication (BCC) should be reinforced to stem the continued use of CQ and indeed resistant CQ genes in the population. It is regretable that the resistant CQ genes were isolated in children who did not use CQ. The explanation for the persistence of K76T mutation in the studied children with *P. falciparum* populations may entail several factors even after it was changed as drug of policy in the country. First, despite the change in policy of CQ to ACTS, the drug is still prescribed and used. Second, CQ and other 4-aminoquinoline drugs are still widely available commercially in Nigerian and most people still use these drugs despite its reported failure. The use of quinine and amodiaquine may also impose positive selection, maintaining CQ resistance, which has been associated to a certain extent with *Pfcrt* 76T and *Pfmdr1* 86Y mutations (Holmgren *et al.*, 2006; Happi *et al.*, 2006).

The results of this study showed that there is an increase in the occurence of key mutations in the *Pfcrt* and *Pfmdr1* genes in the population of the children studied even after CQ was changed. The level *Pfcrt* 72C/73V/74I/75E/76T (CVIET) mutant haplotype (91.6%), *Pfmdr1* Y86(62.2%) and F184 (69%) were higher compared with the lower levels of *Pfcrt* T76 (73.3%) in rural and T76 (74.6%) in urban area of Lagos, South- West Nigeria (Olukosi *et al.*,

2005) and T76 (62%) and Y86 (29%) reported by Happi *et al.*, (2006) in Ibadan, South-West Nigeria and *Pfcrt* T76 (74%), Y86 (29%) and F184 (64%) reported in pre-treatment children in Osogbo, South-West Nigeria (Ojurongbe *et al.*, 2007) and the presence of mutant *Pfcrt*T76, *Pfmdr1*Y86 and *Pfmdr1*F184 alleles in 60%, 33% and 14% respectively in 6 months-12 years children in Ibadan (Folarin *et al.*, 2008). This clearly demonstrates a high frequency of drug resistant mutants circulating in the studied children even after the change in policy to ACTs and by extension the population showed that CQ resistant is increasing in children in Nigeria.

It took the malaria stake holders good diplomacy to get artemisinin monotherapies out of the market. Countries should use the same diplomacy in ensuring that in-effective antimalarial drugs are withdrawn completely from the public. It is clear that when a drug is no more recommended, it should be withdrawn and not accesible for use. This accounts for the reduction in CQ resistant genes in other countries. In Nigeria however, CQ is still available, sold and used in large quantities because it is cheaper than the current ACTs and there is restricted access to it when it is available. Patients having malaria still rely on CQ because that is what they can afford especially in the declining economic fortunes and poverty and it is accessible. The lower the frequency of mutant genes to CQ (which is one of the 4-aminoquinolines), the higher the chances that ACTs using amodiaquine and lumefantrine will be more efficacious as the drug pressure will be relieved. The continous use of CQ and SP as monotherapy could also pose a threat to Artemisinin partner drugs such as (amodiaquine, mefloquine, lumenfantrine and SP).

The results of the association between the different parasitaemia groups and the *Pfcrt* and *Pfmdr1* haplotypes showed that there was no significant difference in the parasite density and the amplified genes. In contrast to what has been reported where higher parasitaemia was

related to decreased ability to clear infections with *Pfcrt*-76 and *Pfmdr1*-86 mutations after chloroquine treatment (Khalil *et al.,*. 2005). There was also no significant relationship between the parasite density groups and the *Pfcrt* and *Pfmdr1* mutant and wild type.

The result of the SP resistant *P falciparum* in this study indicated a prevalence of 92.9% for triple mutation in the *Pfdhfr* genes sequenced; 51I, 59R and 108N (51I+59R+108N). Consistent with this study, similar prevalence in triple mutations in codon I51, R59 and N108 were reported in children and adults samples genotyped (Zhong *et al.*, 2008; De Almeida *et al.*, 2009). In addition, an *in-vivo* work in children reported a prevalence of 89% for triple mutations at *Dhfr*-108, *Dhfr*-51 and *Dhfr*-59 in Congo (Alker *et al.*, 2008). In contast to this result, low triple mutants prevalence were reported both in children and adults (Biswas *et al.*, 2000; Ahmed *et al.*, 2004; Hapuarachchi *et al.*, 2006; Zakeri *et al.*, 2007). Triple mutations at the *Pfdhfr* gene (51I+59R+108N) have been reported to be associated with high levels of pyrimethamine resistance (Plowe, 2003).

Out of the triple mutants that was reported in this study, the mutation at codon 108N of the *Pfdhfr* gene, a major determinant of pyrimethamine resistance (Peterson *et al.*, 1991; Zhong *et al.*, 2008), has a prevalence of 96.5% which is has been reported in some studies in children and adults (Schönfeld *et al.*, 2007; De Almeida *et al.*, 2009). A prevalence of 92.9% in R59 and a prevalence of 94.7% in I51 were seen in this study. In contrast to R59 prevalence in this study, some studies have reported low prevalence in codon R59 in children and adults compared to the mutation in codon I51 and N108 (Schönfeld *et al.*, 2007; Elbasit *et al.*, 2008). The low prevalence of I51 was also reported in children and adults in the Papua New Guinea. Pyrimethamine sensitivity which is associated with ACNCSVI haplotype was seen in 4(3.5%) of the samples genotyped. There was no ACNRNVI haplotype which was reported in

Tanzania, Indian and Brazil (Pearce *et al.*, 2003; Ahmed *et al.*, 2004; Gama *et al.*, 2009). The L164, R50, and V16 mutations were absent from this study as it was reported in the study carried out in children in Nigeria by Happi *et al.*, (2005). The 164L that was formerly reported to be absent within Africa (Plowe, 2003) was reported in Kenya (McCollum *et al.*, 2006). In a similar study in children in Nigeria, by Happi *et al.*, (2005), they reported mutations in *dhfr* Asn-108 (79%), *dhfr* Ile-51 (36%) and *dhfr* Arg-59 (32%) lower than what was reported in this study.

In the *Dhps* gene sequenced, A437G have a prevalence of 96.6% in the *Plasmodium falciparum* isolated. Reports have showed that *Pfdhps* 437G was the first to emerge as a result of sulpha drugs pressure (Nzila *et al.*, 2000; Ngo *et al.*, 2003), which confers resistance to sulphadoxine in *P. falciparum* (Snounou *et al.*, 1993; Jelinek *et al.*, 1999; Syarfruddin *et al.*, 2005). In contrast to the 437G prevalence seen in this study, a prevalence of 34% was seen for G437 in children in Ibadan South-West Nigeria (Happi *et al.*, 2005). Alker *et al.*, (2008), have reported that mutations at either *Dhps*-437 or *Dhps*-540 were strongly associated with molecular determinants of SP treatment failure. The *Dhps* A/F436 had prevalence of 33.6% for A436 and 0.9% for F436 in the *Plasmodium falciparum* isolated. In Kenya, studies showed that mutant F436 allele was rare (< 5%), and mutant A436 allele was not detected (Zhong *et al.*, 2008). Mutation at codon 613 was also detected in this study which was not seen in some studies done earlier in Congo, Sudan and Kenya (Alker *et al.*, 2008; Elbasit *et al.*, 2008; Zhong *et al.*, 2008).

Nineteen percent (19%) of the sample had mutation at *Dhps* G581. Mutation in this codon G581 has earlier been reported in some studies (Schönfeld *et al.*, 2007; Sutherland *et al.*, 2009). Of interest is the presence of mutations in a new codon V431 (19%) in the samples

genotyped, this was reported in a recent work in the Nigerians in UK (Sutherland *et al.*, 2009). There was no mutation in *Dhps* E540, similar to what have been reported in Africa (Zakeri *et al.*, 2007) and in Nigerians attending traveler's clinic in the UK (Sutherland *et al.*, 2009), but many studies have reported the occurrence of *Dhps* E540 in children and in adults (Happi *et al.*, 2005; Alker *et al.*, 2008; Zhong *et al.*, 2008). Linkage was observed among codon 431, 581 and 613. Out of the 22 children that had mutation at codon V431, seventeen of them also had mutation at both codon G581 and S613. This was also reported in a study done by Sutherland *et al.*, (2009), where they discovered rare occurrence together of mutations at positions 581 and 613, in 5 Nigerian isolates and all these carried the novel I431V substitution and so were of the quintuple mutant haplotype VSGKGS, suggesting there may be linkage of the 431, 581 and 613 codons in some Nigerian parasite populations. More studies are needed to evaluate the relationship among these three codons.

This study grouped *Dhps* and *Dhfr* genes into different haplotypes as reported in some studies (Gama *et al.*, 2009; Sutherland *et al.*, 2009). *Pfdhfr* haplotypes such as ACIRNVI, ACICNVI, ACNCNVI and ACNCSVI were described in this study. While for *Pfdhps* haplotypes, ISGKAA, VAGKGS, VAGKAA, IAGKAS, ISAKAA, IAAKAA, ISGKGA, IFGKAS, IAGKAA, VSGKGS and ISGKAS were reported. This study also reported a haplotype that was revealed recently (ISGKAS) (Sutherland *et al.*, 2009) and another haplotype (IFGKAS) was reported in the isolates genotyped in this study.

The combination of the *Dhps* and the *Dhfr* haplotypes showed that most of the combined haplotypes were seen in ACIRNVI + ISGKAA (56.7%), which is synonymous to the quadruple mutation [triple *dhfr* (51I, 59R and 108N) + *dhps* 437G). This has been reported in study done by De Almeida *et al.*, (2009), where 82.3% of the population studied had the

quadruple mutation. Quintuple mutation (51I/59R/108N of *Pfdhfr* and 437G/540E of *Pfdhps*) that was seen in some studies was not seen in samples genotyped (Happi *et al*., 2005; Alker *et al*., 2008; Zhong *et al*., 2008). The quadruple and quintuple mutations have been implicated to be responsible for high level of SP resistance in most studies (Happi *et al*., 2005; Ndounga *et al*., 2007).

In this study, there was no mutation in E540, but mutations are seen in other *Dhps* mutant codons. Six of the isolates in the present study have combined haplotypes of the ACIRNVI + VAGKGS, which correlate with mutations in triple *Dhfr* (51,59 and 108) and five *Dhps* genes (431, 436, 437, 581 and 613). The role of mutations at *Dhps*-431, *Dhps*-436, *Dhps*-581 and *Dhps*-613 in treatment failure is less clear, and more studies are needed on these mutations as they have received less attention due to their low prevalence in Africa. Aubouy *et al*., (2003), have concluded in their study that, failure of SP treatment in Gabon is related to the combination of at least two *Dhfr* (C59R and S108N) and one *Dhps* mutations (S436A or A437G).

The results of the association between the parasitaemia groups and the *Dhfr* haplotypes showed that that there was no significant difference in the level of parasitaemia and mutations in *Dhfr* genes. In the *Dhps* genes, there is significant relationship between parasitaemia and *Dhps* haplotypes. Similar result was reported in children with parasiteamia above 45,000 p/µl by Alker *et al*., (2008), where high parasitaemia was reported to be associated with SP treatment failure and mutations in *Dhps* 437 and 540 codons. High parasitaemia may be a sign of low partial immunity. The larger effect of these mutations at high parasitaemia might be caused by the inability of the immune system to clear resistant parasites and resistance might have a greater impact in less immune individuals (Alker *et al*., 2008). However, further

studies are needed to determine the association between resistant parasites and level of parasitaemia.

The total removal of SP from the population has been reported to bring about reduction in resistant parasites. Studies on molecular surveillance for drug resistance have reported the sensitivity of SP after its total removal from the population in Amazon region of Peru (Zhou *et al.*, 2008; Bacon *et al.*, 2009).

5.1   CONTRIBUTIONS TO KNOWLEDGE

This study contributed to knowledge in the following ways;

- This study reports the shift in the malaria prevalence and intensity from the less than 5 years old children to the 5-12 years old children and this shows the need for malaria prevention in children above 5 years, which justifies the universal target in the current malaria control programme.

- This study reports an increased in SP resistance using sequencing for SNPs to detect point mutations in *Dhfr* and *Dhps* genes and haplotypes in *Dhfr* and *Dhps* (4 *Dhfr* and 11 *Dhps* haplotypes) in Nigerian children with uncomplicated malaria.

- High frequency of *Pfcrt* mutant haplotypes and *Pfmdr1* point mutations reported in this study provides evidence of threat to partner drug(s) of Artemisinin if CQ use is not curtailed.

CHAPTER SIX

6.0   CONCLUSION/RECOMMENDATION

The trend in malaria prevalence of 20.7% in 0-12years and 16.9% in 0-≤5years old children showed that there is a decline in incidence of malaria compared to the documented prevalence. Shift in malaria prevalence occured in >5-12 years (42.1%) compared to 0-≤5years old (16.9%) that is well reported. There was also a shift in malaria prevalence from the well reported high prevalence in the 0-≤5years to the greater than 5 years old age group of children (>5-12years) and justifies the universal target in the current malaria control programme.

The persistence and increase in prevalence of CQ resistant haplotypes (CVIET) with a prevalence of 91.6% *Pfcrt* mutant haplotype (CVIET) and the persistence of the mutant *Pfmdr1* genes also in both Y86 (62.2%) and F184 (69.0%) even after the supposed change in antimalarial indicates increase in the resistant malaria parasites in the general population. The rise in these resistant malaria parasites could pose threat to Artemisinin partner drugs (such as mefloquine, amodiaquine, lumenfantrine etc) if CQ use is not curtailed.

High frequency of mutation in the *Dhfr* genes at codon N108 (96.5%), R59 (92.9%) and I51 (94.7%) and in *Dhps* genes at codons G437 (96.6%) suggest that the continuous use of SP may contribute to higher patterns of inefficacy in the treatment of falciparum malaria and with more availability of SP there is possibility of increasing resistance to SP from moderate to high and with the rapid spread of SP resistance.

7.0.   REFERENCES

Abdallam, S., Weatherall. D.J., Wickramasinghe, S.N. and Hughes, M. (1980). The anaemia of *P. falciparum* malaria. *Br. J. Haematol*. **46**: 171-183

Adeleke, S.I. (2007). Malaria Parasitaemia and its correlation with age in children diagnosed at Aminu Kano Teaching Hospital, Kano, Nigeria. *Int. Jor. P. App. Scs*. **1**(2): 39-42

A-Elbasit, I.E., Khalil, I.F., Elbashir, M.I., Masuadi, E.M., Bygbjerg, I.C., Alifrangis, M. and Giha, H.A. (2008). High frequency of *Plasmodium falciparum* CICNI/SGEAA and CVIET haplotypes without association with resistance to sulfadoxine/pyrimethamine and chloroquine combination in the Daraweesh area, in Sudan. *Eur.J.Clin. Microbiol. Infect. Dis*. **27**: 725–732

Adagu, I.S., Warhurst, D.C., Carucci, D.J. and Duraisingh, M.T. (1995). *pfmdr1* mutations and chloroquine-resistance in *Plasmodium falciparum* isolates from Zaria, Nigeria. *Trans. R. Soc. Trop. Med. Hyg*. **89**: 132

Adagu, I.S., Dias, F., Pinheiro, L., Rombo, L., doRosario, V. and Warhurst, D.C. (1996). Guinea-Bissau: association of chloroquine resistance of *Plasmodium falciparum* with the Tyr 86 allele of the multiple drug resistance gene *pfmdr1*. *Trans. R. Soc. Trop. Med. Hyg*. **90**: 90-91

Ademowo, O.G., Falusi, A.G. and Mewoyeka, O.O. (1995). Prevalence of asymptomatic parasitaemia in an urban and rural community in south Western Nigeria. Cent. *Afr. J. Med*. **40**(1): 18-21

Afolabi, B.M., Salako, L.A., Mafe, A.G., Oviwigho, U.B., Rabiu, K.A., Sanyaolu, N.O. and Ibrahim, M.M. (2001). Malaria in the first 6 months of life in urban African infants with anaemia. *Am. J. Trop. Med. Hyg*. **65**(6): 822–827

Agbenyega, T., Angus, B., Bedu-Addo, G., Baffoe-Bonnie, B., Griffin, G., Valance, P. and Krishna, S. (2000). Glucose and lactate kinetics in children with severe malaria. *J. Clin. Endocrinol. Metab*. **85**: 1569-1576

Ahmed, A., Bararia, D., Vinayak, S., Yameen, M., Biswas, S., Dev, V., Kumar, A., Ansari, M.A. and Sharma, Y.D. (2004). *Plasmodium falciparum* isolates in India exhibit a progressive increase in mutations associated with sulfadoxine-pyrimethamine resistance. *Antimicrob. Agents. Chemother.* **48**: 879-889

Alifrangis, M., Dalgaard, M.B., Lusingu, J.P., Vestergaard, L.S., Staalsoe, T., Jensen, A.T., Enevold, A., Ronn, A.M., Khalil, I.F., Warhurst, D.C., Lemnge, M.M., Theander, T.G. and Bygbjerg, I.C. (2006). Occurrence of the Southeast Asian/South American SVMNT haplotype of the chloroquine resistance transporter gene in *Plasmodium falciparum* in Tanzania. *J. Infect. Dis.* **193**: 1738 1741

Alifrangis, M., Lusingu, J.P., Mmbando, B., Dalgaard, M.B., Vestergaard, L.S., Ishengoma, D., Khalil, I.F., Theander, T.G., Lemnge, M.M. and Bygbjerg, I.C. (2009). Short Report: Five-Year Surveillance of Molecular Markers of *Plasmodium falciparum* Antimalarial Drug Resistance in Korogwe District, Tanzania: Accumulation of the 581G Mutation in the *P. falciparum* Dihydropteroate Synthase Gene. *Am. J. Trop. Med. Hyg.* **80**(4): 523–527

Alker, A.P., Kazadi, W.M., Kutelemeni, A.K., Bloland, P.B., Tshefu, A.K. and Meshnick, S.R. (2008). *Dhfr* and *Dhps* genotype and sulfadoxine-pyrimethamine treatment failure in children with falciparum malaria in the Democratic Republic of Congo. *Trop. Med. Int. Health.* **13**(11): 1384–1391

Angus, B.J., Chotivanich, K., Udomsangpetch, R. and White, N.J. (1997). Invivo removal of malaria parasites from red blood cells without their destruction in acute falciparum malaria. *Blood.* **90**: 2037

Aubouy, A., Jafari, S., Huart, V., Migot-Nabias, F., Mayombo, J., Durand, R., Bakary, M., Le Bras, J. and Deloron, P. (2003). *DHFR* and *DHPS* genotypes of *Plasmodium falciparum* isolates from Gabon correlate with *in vitro* activity of pyrimethamine and cycloguanil, but not with sulfadoxine–pyrimethamine treatment efficacy. *J. Antimicrob. Chemo.* **52**: 43–49

Awad-el-Kariem, F.M., Miles, M.A. and Warhurst, D.C. (1992). Chloroquine-resistant *Plasmodium falciparum* isolates from the Sudan lack two mutations in the *pfmdr1* gene thought to be associated with chloroquine resistance. *Trans. R. Soc. Trop. Med. Hyg.* **86**: 587-589.

Bacon, D.J., McCollum, A.M., Griffing, S.M., Salas, C., Soberon, V., Santolalla, M., Haley, R., Tsukayama, P., Lucas, C., Escalante, A.A. and Udhayakumar, V. (2009). Dynamics of malaria drug resistance patterns in the Amazon basin region following changes in Peruvian national treatment policy for uncomplicated malaria. *Antimicrob. Agents. Chemother.* **53**: 2042-2051

Basco, L.K., LeBras, J., Rhoades, Z. and Wilson, C.M. (1995). Analysis of *Pfmdr1* and drug susceptibility in fresh isolates of *Plasmodium falciparum* from SubSaharan Africa. *Mol. Biochem. Parasitol.* **74**: 157-166.

Basco, L.K. and Ringwald, P. (2001). Analysis of the key *pfcrt* point mutation and *in vitro* and *in vivo* response to chloroquine in Yaounde, Cameroon. *J. Infect. Dis.* **183**: 1828-1831.

Basco, L.K. (2003). Molecular epidemiology of malaria in Cameroon. XVI. Longitudinal surveillance of in vitro pyrimethamine resistance in *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **69**: 174–178

Bhattarai, A. and Ali, S. (2007). 'Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar'. *Plos. Med.* **4**(11): e309

Biemba, G., Dolmans, D., Thuma, P.E., Weiss, G. and Gordeuk, V.R. (2000). Severe anaemia in Zambia children with *Plasmodium falciparum*.malaria. *Trop. Med. Int. Health.* **5**: 9 -16

Biswas, S., Escalante, A., Chaiyaroj, S., Angkasekwinai, P. and Lal, A.A. (2000). Prevalence of point mutations in the dihydrofolate reductase and dihydropteroate synthetase genes of *Plasmodium falciparum* isolates from India and Thailand: a molecular epidemiologic study. *Trop. Med. Int. Health.* **5**: 737-743

Bjorkman, A. (2002). Malaria associated anaemia, drug resistance and antimalarial combination therapy. *Int. J. Parasitol.* **32**: 1637-1643

Bjorkman, A. and Bhattarai, A. (2005). Public health impact of drug resistant Plasmodium falciparum malaria. *Acta. Tropica.* **94**: 163–169

Bloland, P.B., Boriga, D.A. and Ruebush, T.K. (1999). Longitudinal cohort study of the epidemiology of malaria infections in area of intense transmission II. Descriptive epidemiology of malaria infection and disease among children. *Am. J. Trop. Med. Hyg.* **60**: 641-648

Bonner, P.C., Zhon, Z., Mirel, L.B., Ayisi, J.G., Shi, Y.P., Eijk, A.M., Otieno, J.A., Nahlen, B.L., Steketee, R.W. and Udhayakumar, V. (2005). Placental malaria diminishes development of antibody responses to *Plasmodium falciparum* epitopes in infants residing in area of Western Kenya where *P falciparum* is endemic. *Clin. Diag. Lab. Immunology.* **12**(I3): 375-379

Bouyou-Akotet, M.K., Dzeing-Ella, A., Kendjo, E., Etoughe, D., Ngoungou, E.B., Planche, T., Koko, J. and Kombila, M. (2009). Impact of *Plasmodium falciparum* infection on the frequency of moderate to severe anaemia in children below 10 years of age in Gabon. *Malaria. J.* **8**: 166

Branch, O.H., Udhayakumar, V., Hightower, A.H., Oloo, A.J., Hawley, W.A., Nahlen, B.L., Bloland, P.B. and Lal, A.A. (1998). A longitudinal investigation of IgG and IgM antibody responses to the merozoite surface protein-1 19-kilodalton domain of *Plasmodium falciparum* in pregnant women and infants: associations with febrile illness, parasitemia, and anemia. *Am. J. Trop. Med. Hyg.* **58**: 211–219.

Bray, P.G., Martin, R.E., Tilley, L., Ward, S.A., Kirk, K. and Fidock, D.A. (2005). Defining the role of PfCRT in *Plasmodium falciparum* chloroquine resistance. *Mol. Microbiol.* **56**: 323–333

Brinkmann, U. and Brinkmann, A. (1991). Malaria and Health in Africa: The present situation and epidemiological trends. *Trop. Med. Parasitol.* **42**: 204-213

Brooks, D., Wang, P., Read, M., Watkin, W., Sims, P. and Hyde, J. (1994). Sequence variation in the hydroxylmethyldihdropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, *Plasmodium falciparum,* with differing resistance to sulfadoxine. *Eur. J. Biochem.* **224**: 397–405

Canfield, C.J., Pudney, M. and Gutteridge, W.E. (1995). Interaction of atovaquone with other antimalarial drugs against *Plasmodium falciparum. Exp. Parasitol.* **80**: 373-381

Casals-Pascual, C. and Robert, D.J. (2002). Clinical and haematological features of malaria. *Heamatology.* p 41 -47

Charlwood, J.D., Smith, T., Lyimo, E., Kitua, A.Y., Masanja, H., Booth, M., Alonso, P.L. and Tanner. (1998). Incidence of *Plasmodium falciparum* Infection In infants an relation to exposure to sporozoite-infected anophelines. *Am. J. Trop. Med. Hyg.* **59**: 243-251

Chen, N., Russell, B., Staley, J., Kotecka, B., Nasveld, P. and Cheng, Q. (2001). Sequence polymorphisms in *pfcrt* are strongly associated with chloroquine resistance in *Plasmodium falciparum. J. Infect. Dis.* **183**: 1543-1555

Chen, N., Kyle, D.E., Pasay, C., Fowler, E.V., Baker, J., Peters, J.M. and Cheng, Q. (2003). *Pfcrt* allelic types with two novel amino acid mutations in chloroquine-resistant *Plasmodium falciparum* isolates from the Philippines. *Antimicrob. Agents. Chemother.* **47**: 3500–3505

Conway, D.J., Cavanagh, D.R., Tanabe, K., Roper, C., Mikes, Z.S., Sakihama, N., Bojang, K.A., Oduola, A.M.J., Kremsner, P.G., Arnot, D.E., Greenwod, B.M. and McBride, J.S. (2000). A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. *Nat. Med.* **6**: 689–692

Conway, D.J. (2007). Molecular epidemiology of malaria. *Clin. Mic. Rev.* **20**(1): 188–204

Cortese, J.F. and Plowe, C.V. (1998). Antifolate resistance due to newand known *Plasmodium falciparum* dihydrofolate reductase mutations expressed in yeast. *Mol. Biochem. Parasitol.* **94**: 205–214

Cowman, A.F., Morry, M.J., Biggs, B.A., Cross, G.A. and Foote, S.J. (1988). Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum. Proc. Natl. Acad. Sci. USA.* **85**: 9109-9113

Cox-Singh, J., Singh, B., Alias, A. and Abdullah, M.S. (1995). Assessment of the association between three *pfmdr1* point mutations and chloroquine resistance *in vitro* of Malaysian *Plasmodium falciparum* isolates. *Trans. R. Soc. Trop. Med. Hyg.* **89**: 436-437

Cox-Singh, J., Davis, T.M.E., Lee, K.S., Shamsul, S.S.G., Matusop, A., Ratnam, S., Rahman, H.A., Conway, D.J. and Singh, B. (2008). *Plasmodium knowlesi* Malaria in Humans Is Widely Distributed and Potentially Life Threatening. *Clin. Infect. Dis.* **46**: 165–171

Crawley, J., Smith, S., Kirkham, F., Muthinji, P., Waruiru, C. and Marsh, K. (1996) Seizures and status epilepticus in childhood cerebral malaria. *Q. J. Med.* **89**: 591

Crawley, J.W.C., Mithwani, S., Mwangi, I., Watkins, W., Ouma, D., Winstanley, P., Peto, T. and Marsh, K. (2000). Effect of Phenobarbital on seizure frequency and mortality in childhood cerebral malaria. *Lancet.* **355**: 701-707

Das, A. and Dash, A.P. (2007). Evolutionary paradigm of chloroquine-resistant malaria in India. *Trends. Parasitol.* **23**: 132–135

Delacollette, C.D., Souza, C., Christophel, E., Thimassan, K., Abdur, R., Bell, D., Dai, T.C., Gopinath, D., Lu, S., Mendoza, R., Ortega, L., Rastogi, R., Tantinimitkul, C. and Ehrenberg, J. (2009). Malaria trends and challenges in the greater Mekong Subregion. *Southeast. Asian. J. Trop. Med. Pub. Health.* **40**(4): 674-791

De Almeida, A., Arez, A.P., Cravo, P.V.L. and do Rosário, V.E. (2009). Analysis of genetic mutations associated with anti-malarial drug resistance in *Plasmodium falciparum* from the Democratic Republic of East Timor. *Malaria. J.* **8**: 59

Djimde, A., Doumbo, O.K., Cortese, J.F., Kayentao, K., Doumbo, S., Diourte, Y., Dicko, A., Su, X.Z., Nomura, T., Fidock, D.A., Wellems, T.E., Plowe, C.V. and Coulibaly, D. (2001). A molecular marker for chloroquine-resistant falciparum malaria. *N. Engl. J. Med.* **344**: 257–263

Djimde, A.A., Doumbo, O.K., Traore, O., Guindo, A.B., Kayentao, K., Diourte, Y., Niare-Doumbo, S., Coulibaly, D., Kone, A.K., Cissoko, Y., Tekete, M., Fofana, B., Dicko, A., Diallo, D.A., Wellems, T.E., Kwiatkowski, D. and Plowe, C.V. (2003). Clearance of drug-resistant parasites as a model for protective immunity in *Plasmodium falciparum* malaria. *Am. J. Trop. Med. Hyg.* **69**: 558-563

Dlamini, S.V., Beshir, K. and Sutherland, C.J. (2010). Markers of anti-malarial drug resistance in *Plasmodium falciparum* isolates from Swaziland: identification of *pfmdr1*-86F in natural parasite isolates. *Malaria. J.* **9**: 68

Dokomajilar, C., Nsobya, S.L., Greenhouse, B., Rosenthal, P.J. and Dorsey, G. (2006). Selection of *Plasmodium falciparum Pfmdr1* alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. *Antimicrob. Agents. Chemother.* **50**: 1893–1895

Dorsey, G., Kamya, M.R., Singh, A. and Rosenthal, P.J. (2001). Polymorphisms in the *Plasmodium falciparum pfcrt* and *pfmdr-1*genes and clinical response to chloroquine in Kampala, Uganda. *J. Infect. Dis.* **183**: 1417-1420

Duraisingh, M.I., Drakeley, C.I., Muller, O., Bailey, R., Snounou, G., Targett, G.A., Greenwood, B. and Warhurst, D. (1997). Evidence for selection for the tyrosine 86 allele of *pfmdr1* gene of *Plasmodium falciparum* by chloroquine and amodiaquine. *Parasitology.* **114**: 205–211

Duraisingh, M.T., Jones, P., Sambou, I., von Seidlein, L., Pinder, M. and Warhurst, D.C. (2000). The tyrosine-86 allele of the pfmdr1 gene of *Plasmodium falciparum* is associated with increased sensitivity to the antimalarials mefloquine and artemisinin. *Mol. Biochem. Parasitol*. **108**: 13 23

Duraisingh, M.T. and Cowman, A.F. (2005). Contribution of the *pfmdr1* gene to antimalarial drug resistance. *Acta. Trop*. **94**: 181–190

Durrand, V., Berry, A., Sem, R., Glaziou, P., Beaudou, J. and Fandeur, T. (2004). Variations in the sequence and expression of the *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) and their relationship to chloroquine resistance in vitro. *Mol. Biochem. Parasitol*. **136**: 273–285

Dzeing–Ella, A., Obiang, P.C., Tchoua, R., Planche, T., Mboza, B., Mbounja, M., Muller-Roemer, U., Jarvis, J., Kendjo, E., Ngou-Milama, E., Kremsner, P.G., Krishna, S. and Kombila, M. (2005). Severe falciparum malaria in Gabonese children. *Clinical. Laboratory. Features*. **4**: 1

East African Network for Monitoring Antimalarial Treatment (EANMAT). (2003). The efficacy of antimalarial monotherapies, sulphadoxine-pyrimethamine and amodiaquine in East Africa: implications for sub-regional policy. *Trop. Med. Int. Health*. **8**(10): 860-867

Ejezie, G.C. and Ezedinachi, E.N. (1992). Malaria parasite density and body temperature in children under 10 years of age in Calabar, Nigeria. *Trop. Geographical. Med*. **44**(1-2): 97-101

Ekanem, O.J., Ezedinachi, E.N.U., Molta, N.B., Watila, I.M., Chukwuani, C.M., Meremikwu, M.M., Akpede, G. and Ojar, E.A. (2000). Treatment of malaria in North-Eastern and South-Eastern Nigeria: a population study of mefloquine, sulphadoxine, pyrimethamine combination vs chloroquine. *West Afr J Med*; **19**: 293-297.

Ekpenyong, E.A. and Eyo, J.E. (2008). Malaria control and treatment strategies among school children in semi-urban tropical communities. *West. Indian. Med. J.* **57**(5): 456-461

Ekvall, H. (2003). Malaria and anemia. *Curr. Opin. Hematol.* **10**: 108–114

Elbasit, I.E.A., Khalil, I.F., Elbashir, M.I., Masuadi, E.M., Bygbjerg, I.C., Alifrangis, M. and Giha, H.A. (2008). High frequency of *Plasmodium falciparum* CICNI/SGEAA and CVIET haplotypes without association with resistance to sulfadoxine/pyrimethamine and chloroquine combination in the Daraweesh area, in Sudan. *Eur. J. Clin. Microbiol. Infect. Dis.* **27**: 725–732

English, M., Waruiru, C., Amukoye, E., Murphy, S., Crawley, J., Mwangi, I., Peshu, N. and Marsh, K. (1996). Deep breathing in children with severe malaria: indicator of metabolic acidosis and poor outcome. *Am. J. Trop. Med. Hyg.* **55**: 521-52

English, M. (2000). Life-threatening severe malarial anaemia. *Trans. R. Soc. Trop. Med. Hyg.* **94**: 585-588

Etuk, E.U. and Umoh, U.F. (2001). The evaluation of some commercially available oral preparations of chloroquine in Sokoto urban. *J. Life. Environ. Sci.* **2**(3): 103–107

Falade, C.O., Salako, L.A., Sowunmi, A., Oduola, A.M.J. and Larcier, P. (2007). Comparative efficacy of halofantrine, chloroquine and sulphadoxine-pyrimethamine for treatment of acute uncomplicated malaria in Nigeria children. *Trans. R. Soc. Trop. Med. Hyg.* **91**: 58-62

Fidock, D.A., Nomura, T., Talley, A.K., Cooper, R.A., Dzekunov, S.M., Ferdig, M.T., Ursos, L.M., Sidhu, A.B., Naude, B., Deitsch, K.W., Su, X.Z., Wootton, J.C., Roepe, P.D. and Wellems, T.E. (2000). Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol. Cell.* **6**: 861–871

Federal Ministry of Health (FMOH). (2005). National Antimalarial Treatment Policy. National Malaria and Vector Control Division Abuja, Nigeria

Folarin, O.A., Gbotosho, G.O., Sowunmi, A., Olorunsogo, O.O., Oduola, A.M.J. and Happi, T.C. (2008). Chloroquine Resistant *Plasmodium falciparum* in Nigeria: Relationship between *pfcrt* and *pfmdr1* Polymorphisms, *In-Vitro* Resistance and Treatment Outcome. *Open. Trop. Med. J.* **1**: 74–82

Foote, S.J., Thompson, J.K., Cowman, A.F. and Kemp, D.J. (1989). Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. *Cell.* **57**: 921–930

Foote, S.J., Kyle, D.E., Martin, R.K., Oduola, A.M., Forsyth, K. and Kemp, D.J. (1990). Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature*. **345**: 255-258

Gadalla, N.B., Elzaki, S.E., Mukhtar, E., Warhurst, D.C., El-Sayed, B. and Sutherland, C.J. (2010). Dynamics of pfcrt alleles CVMNK and CVIET in chloroquine-treated Sudanese patients infected with Plasmodium falciparum. *Malaria. J.* **9**: 74

Gay-Andrieu, F., Adhossi, E., Veronique, L., Gagara, M., Laminou, I., Hama, K. and Hamadou, B. (2005). Epidemiological, clinical and biological features of malaria among children in Niamey, Niger. *Malaria. J.* **4**: 10

Gama, B.E., Almeida de Oliveira, N.K., Zalis, M.G., de Souza, J.M., Santos, F., Daniel-Ribeiro, C.T. and Ferreira-da-Cruz, M.F. (2009). Chloroquine and sulphadoxine-pyrimethamine sensitivity of *Plasmodium falciparum* parasites in a Brazilian endemic area. *Malaria. J.* **8**: 156

Gama, B.E., Pereira-Carvalho, G.A.L., Lutucuta Kosi, F.J.I., Almeida de Oliveira, N.K., Fortes, F., Rosenthal, P.J., Daniel-Ribeiro, C.T. and Ferreira-da-Cruz, M.F. (2010). *Plasmodium falciparum* isolates from Angola show the StctVMNT haplotype in the *pfcrt* gene. *Malaria. J.* **9**: 174

Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., Carlton, J.M., Pain, A., Nelson, K.E., Bowman, S., Paulsen, I.T., James, K., Eisen, J.A., Rutherford, K., Salzberg, S.L., Craig, A., Kyes, S., Chan, M.S., Nene, V., Shallom, S.J., Suh, B., Peterson, J., Angiuoli, S., Pertea, M., Allen, J., Selengut, J., Haft, D., Mather, M.W., Vaidya, A.B., Martin, D.M., Fairlamb, A.H., Fraunholz, M.J., Roos, D.S., Ralph, S.A., McFadden, G.I., Cummings, L.M., Subramanian, G.M., Mungall, C., Venter, J.C., Carucci, D.J., HoVman, S.L., Newbold, C., Davis, R.W., Fraser, C.M. and Barrell, B. (2002). Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature*. **419**: 498–511

Gellert, S., Hassan, B.Y., Meleh, S. and Hiesqen, G. (1998). Malaria prevalence and outcome in the in-patients of the paediatric. Department of the state specialist Hospital (SSH) Maiduriguri, Nigeria. *J. Trop. Peadiatr*. **44**(2): 109-113

Gilles, H.M. (1991). Management of severe and complicated malaria, Geneva, World Health Organisation

Gimenez, F. and Barraud de Lageric, S. (2003). Tumour necrosis factor alpha in the pathogenesis of cerebral malaria. *Cellular. Mol. Life. Sci*. **60**: 1623-1635

Ginsburg, H. (2005). Should chloroquine be laid to rest? *Acta. Trop*. **96**: 16-23

Goves, S. (1997). Integrated management of childhood illness by out-patient health workers: technical basis and overview. *Bulletin of the World Health Organization*. **75**: 7-24

Greenwood, B.M. and Armstrong, J.R.M. (1991). Comparison of two simple methods for determining malaria parasite density. *Trans. R. Soc. Trop. Med. Hyg*. **85**: 186–188

Gregson, A. and Plowe, C.V. (2005). Mechanisms of resistance of malaria parasites to antifolates. *Pharmacol. Rev*. **57**: 117–145

Guinovart, C., Bassat, Q., Sigaúque, B., Aide, P., Sacarlal, J., Nhampossa, T., Bardají, A., Nhacolo, A., Macete, E., Mandomando, I., Aponte, J.J., Menéndez, C. and Alonso,

P.L. (2008). Malaria in rural Mozambique. Part I: Children attending the outpatient clinic. *Malaria. J.* **7**: 36

Happi, T.C., Thomas, S.M., Gbotosho, G.O., Falade, C.O., Akinboye, D.O., Gerena, L., Hudson, L.T., Sowunmi, A., Kyle, A.D.E., Milhous, W., Wirth, D.F. and Oduola, A.M.J. (2003). Point mutations in the *pfcrt* and *pfmdr-1* genes of *Plasmodium falciparum* and clinical response to chloroquine, among malaria patients from Nigeria. *Annals. Tropical. Med. Paras*. **97** (5): 439–451

Happi, C.T., Gbotosho, G.O., Folarin, O.A., Akinboye, D.O., Yusuf, B.O., Ebong, O.O., Sowunmi, A., Kyle, D.E., Milhous, W., Wirth, D.F. and Oduola, A.M.J. (2005). Polymorphisms in Plasmodium falciparum dhfr and dhps genes and age related in vivo sulfadoxine-pyrimethamine resistance in malaria-infected patients from Nigeria. *Acta. Tropica*. **95**(3): 183-193

Happi, C.T., Gbotosho, G.O., Folarin, O.A., Bolaji, O.M., Sowunmi, A., Kyle, D.E., Milhous, W., Wirth, D.F. and Oduola, A.M.J. (2006). Association between mutations in *plasmodium falciparum* chloroquine resistance transporter and *p. falciparum* multidrug resistance 1 genes and *in vivo* amodiaquine resistance in *p. falciparum* malaria–infected children in Nigeria. *Am. J. Trop. Med. Hyg*. **75**(1): 155–161

Happi, C.T., Gbotosho, G.O., Folarin, O.A., Sowunmi, A., Hudson, T., O'Neil, M., Milhous, W., Wirth, D.F. and Oduola, A.M.J. (2009). Selection of Plasmodium falciparum multidrug resistance gene 1 alleles in asexual stages and gametocytes by artemether-lumefantrine in Nigerian children with uncomplicated falciparum malaria. *Antimic. Agents. Chemoth*. **53**(3): 888-895

Hapuarachchi, H.C., Dayanath, M.Y.D., Bandara, K.B.A.T., Abeysundara, S., Abeyewickreme, W., De Silva, N.R., Hunt, S.Y. and Sibley, C.H. (2006). Point mutations in the dihydrofolate reductase and dihydropteroate synthase genes of *Plasmodium falciparum* and resistance to sulfadoxine-pyrimethamine in Sri Lanka. *Am. J. Trop. Med. Hyg*. **74**: 198-204

Hastings, I.M. and Watkins, W.M. (2006). Tolerance is the key to understanding antimalarial drug resistance. *Trends. Parasitol.* **22**(2): 71-7

Hatton, C., Peto, T., Bunch, C., Pasvol, G., Russel, S., Singer, C., Edward, G. and Winstanley, P.A. (1986). Frequency of severe neutropenia associated with amodiaquine prophylaxis against malaria. *Lancet.* **1**: 411-414

Haruki, K., Bray, P.G., Ward, S.A., Hommel, M. and Ritchie, G.Y. (1994). Chloroquine resistance of *Plasmodium falciparum*: further evidence for a lack of association with mutations of the *pfmdr1* gene. *Trans. R. Soc. Trop. Med. Hyg.* **88**: 694

Helleberg M, Goka BQ, Akanmori BD, Obeng-Adjei O, Rodriques O and Kurtzhals JAL (2005). Bone marrow suppression and severe anaemia associated with persistent *Plasmodium falciparum* infection in African children with microscopically undetectable parasitaemia. *Malaria J.* **4**: 56

Holding, P.A. and Snow, R.W. (2001), Impact of *Plasmodium falciparum* malaria on performance and learning: review of the evidence. *Am. J. Trop. Med. Hyg.* **64**: 68

Holmgren, G., Gil, G.P., Ferreira, P.M., Veiga, M.I., Obonyo, C.O. and Bjorkman, A. (2006). Amodiaquine resistant *Plasmodium falciparum* malaria invivo is associated with selection of *Pfcrt* 76T and *Pfmdr*1 86Y. *Infect. Genet. Evol.* **6**: 309-314

Huaman, M.C., Roncal, N., Nakazawa, S., Long, T.T. Gerena, L. and Garcia, C. (2004). Polymorphism of the *Plasmodium falciparum* multidrug resistance and chloroquine resistance transporter genes and *in vitro* susceptibility to aminoquinolines in isolates from the Peruvian Amazon. *Am. J. Trop. Med. Hyg.* **70**: 461-466

Humphreys, G.S., Merinopoulos, I., Ahmed, J., Whitty, C.J., Mutabingwa, T.K., Sutherland, C.J. and Hallett, R.L. (2007). Amodiaquine and artemether-lumefantrine select distinct alleles of the *Plasmodium falciparum mdr1* gene in Tanzanian children treated for uncomplicated malaria. *Antimicrob. Agents. Chemother.* **51**: 991–997

Ibidapo, C.A. (2005). Perception of causes of malaria and treatment-seeking behaviour of nursing mothers in a rural community. *Aust. J. Rural. Health*. **13** (4): 214-218

Idro, R., Aloyo, J., Mayende, L., Bitarakwate, E., John, C. and Kivumbi, G.W. (2006). Severe malaria in children in areas with low, moderate and high transmission intensity in Uganda. *Trop. Med. Int. Health*. **11**(1): 115-124

Ikeh, E., Peletiri, I.C. and Angyo, I.A. (2002). The prevalence and Intensity of malaria parasite in children at Jos University Teaching Hospital, Nigeria. *Highland. Med. Res. J*. **1**(1): 9-10

Ikeh, E.I. and Teclaire, N.N. (2008). Prevalence of malaria parasitaemia and associated factors in febrile under-5 children seen in Primary Health Care Centres in Jos, North Central Nigeria *Niger. Postgrad. Med. J*. **15** (2): 65-69

Jelinek, T., Kilian, A.H.D., Kabagambe, G. and Sonnenberg, F.V. (1999). *Plasmodium falciparum* resistance to sulfadoxyne/pyrimethamine in Uganda: correlation with polymorphisms in the dehidrofolate reductase and dihydropterate synthetase genes. *Am. J. Trop. Med. Hyg*. **61**: 463-466

Kahigwa, E., Schellenberg, D., Sanz, S., Aponte, J.J., Wigayi, J., Mshinda, H., Alonso, P. and Menendez, C. (2002). Risk factors for presentation to hospital with severe anaemia in Tanzanian children: a case-control study. *Trop. Med. Int. Health*. **7**: 823-830

Kanchana, R., Wanna, C., Aree, S., Kesara, N. and Sodsri, T. (2009). Association between chloroquine resistance phenotypes and point mutations in *pfcrt* and *pfmdr1* in *Plasmodium falciparum* isolates from Thailand. *Acta. Tropica*. **109**: 37–40

Keen, J., Farcas, G.A., Zhong, K., Yohanna, S., Dunne, M.W. and Kain, K.C. (2007). Real-time PCR assay for rapid detection and analysis of PfCRT haplotypes of chloroquineresistant *Plasmodium falciparum* isolates from India. *J. Clin.Microbiol*. **45**: 2889–2893

Khalil, I.F., Alifrangis, M., Tarimo, D.S., Staalso, T., Satti, G.M. and Theander, T.G. (2005). The roles of the pfcrt 76T and pfmdr1 86Y mutations, immunity and the initial level of parasitaemia, in predicting the outcome of chloroquine treatment in two areas with different transmission intensities. *Ann. Trop. Med. Parasitol.* **99**: 441–448

Koram, K.A., Abuaku, B., Duah, N. and Quashie, N. (2005). Comparative efficacy of antimalarial drugs including ACTs in the treatment of uncomplicated malaria among children under 5 years in Ghana. *Acta. Trop.* **95**(3): 194-203

Krishna, S., Waller, D.W., ter Kuile, F., Kwiatkowski, D., Holloway, P.A. and White, N.J. (1994). Lactic acidosis and hypoglycaemia in children with severe malaria: pathophysiological and prognostic significance. *Trans. R. Soc. Trop. Med. Hyg.* **88**: 67-73

Krishna, S. and White, N.J. (1996). Pharmacokinetics of quinine, cholorquine and amodiaquine clinical implication. *Clin. Pharmacokinetics.* **30**: 263-299

Krishna, S., Planche, T., Agbenyega, T., Woodrow, C., Agranoff, D., Bedu-Addo, G., Owusu-Ofori, A.K., Appiah, J.A., Ramanathan, S., Mansor, S.M. and Navaratnam, V. (2001). Bioavailability and preliminary clinical efficacy of intrarectal artesunate in Ghanaian children with moderate malaria. *Antimicrob. Agent. Chemother.* **45**: 509-516

Kublin, J.G., Dzinjalamala, F.K., Kamwendo, D.D., Malkin, E.M., Cortese, J.F., Martino, L.M., Mukadam, R.A., Rogerson, S.J., Lescano, A.G., Molyneux, M.E., Winstanley, P.A., Chimpeni, P., Taylor, T.E. and Plowe, C.V. (2002). Molecular markers for failure of sulfadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *J. Infect. Dis.* **185**: 380-388

Kublin, J.G., Cortese, J.F., Njunju, E.M., Mukadam, R.A., Wirima, J.J., Kazembe, P.N., Djimde, A.A., Kouriba, B., Taylor, T.E. and Plowe, C.V. (2003). Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *J. Infect. Dis.* **187**: 1870–1875

Kyabayinze, D., Cattamanchi, A., Kamya, M.R., Rosenthal, P.J. and Dorsey, G. (2003). Validation of a simplified method for using molecular markers to predict sulfadoxine-pyrimethamine treatment failure in African children with falciparum malaria. *Am. J. Trop. Med. Hyg*. **69**: 247-252

Laufer, M.K., Thesing, P.C., Eddington, N.D., Masonga, R., Dzinjalamala, F.K., Takala, S.L., Taylor, T.E. and Plowe, C.V. (2006). Return of chloroquine antimalarial efficacy in Malawi. *N. Engl. J. Med*. **355:** 1959-1966

Le Bras, J. and Durand, R. (2003). The mechanisms of resistance to antimalarial drugs in *Plasmodium falciparum. Fundam. Clin. Pharmacol*. **17**: 147-153

Le Hesran, J.Y., Cot, M., Personne, P., Frevet, N., Dubois, B., Beyeme, M., Boudin, C. and Deloron, P. (1997). Maternal placental infection with *Plasmodium falciparum* and malarial morbidity during the first 2years of life. *Am. J. Epidemiol*. **146**: 826-831

Lokeshwar, M.R. (2000). Malaria in children – An Overview

Malisa, A.L., Pearce, R.J., Abdulla, S., Mshinda, H., Kachur, P.S., Bloland, P. and Roper, C. (2010). Drug coverage in treatment of malaria and the consequences for resistance evolution--evidence from the use of sulphadoxine/pyrimethamine. *Malaria. J*. **5**: 9

Marsh, K., Forster, D., Waruiru, C., Mwangi, I., Winstanley, M., Marsh, V., Newton, C., Winstanley, P., Wara, P. and Peshu, N. (1995). Indicator of life threatening malaria in African children. *N. Engl. J. Med*. **332**: 1399-1404

Mbanugo, J.I. and Emenalo, S. (2004). Prevalence of malaria parasitaemia among blood donors in Owerri, Imo State, Nigeria. *Nig. J. Parasitology*. **25**: 75-80

McCollum, A.M., Poe, A.C., Hamel, M., Huber, C., Zhou, Z., Shi, Y.P., Ouma, P., Vulule, J., Bloland, P., Slutsker, L., Barnwell, J.W., Udhayakumar, V. and Escalante, A.A. (2006) Antifolate resistance in Plasmodium falciparum: multiple origins and identification of novel dhfr alleles. *J. Infect. Dis*. **194**: 189–197

McElroy, P.D., ter Kuile, F.O., Lal, A.A., Bloland, P.B., Hawley, W.A., Oloo, A.J., Monto, A.S., Meshnick, S.R., Nahlen, B.L. and McElroy, P.D. (2000). Effect of *Plasmodium falciparum* parasitemia density on hemoglobin concentrations among full-term, normal birth weight children in western Kenya, IV. The Asembo Bay Cohort Project. *Am. J. Trop. Med. Hyg*. **62**: 504-512

Mehlotra, R.K., Fujioka, H., Roepe, P.D., Janneh, O., Ursos, L.M., Jacobs-Lorena, V., McNamara, D.T., Bockarie, M.J., Kazura, J.W., Kyle, D.E., Fidock, D.A. and Zimmerman, P.A. (2001). Evolution of a unique *Plasmodium falciparum* chloroquine-resistance phenotype in association with *pfcrt* polymorphism in Papua New Guinea and South America. *Proc. Natl. Acad. Sci. USA*. **98**: 12689–12694

Meyer, C.G., May, J., Arez, A.P., Gil, J.P. and Do, R.V. (2002). Genetic diversity of *Plasmodium falciparum*: asexual stages. *Trop. Med. Int. Health*. **7**: 395-408

Miller, L.H., Shunichi, U. and Chien, S. (1971). Alteration in the rheologic properties of *Plasmodium Knowlesi* infected red cells. A possible mechanism of cerebral malaria. *J. Clin. Invest*. **50**: 1451-1455

Mita, T., Kaneko, A., Lum, J.K., Bwijo, B., Takechi, M., Zungu, I.L., Tsukahara, T., Tanabe, K., Kobayakawa, T. and Bjorkman, A. (2003). Recovery of chloroquine sensitivity and low prevalence of the *Plasmodium falciparum* chloroquine resistance transporter gene mutation K76T following the discontinuance of chloroquine use in Malawi. *Am. J. Trop. Med. Hyg*. **68**: 413-415

Mnyika, K.S. and Kihamia, C.M. (1991). Chloroquine-induced pruritus: its impact on chloroquine utilization in malaria control in Dar es Salaam. *J. Trop. Med. Hyg.* **94**(1): 27-31

Mockenhaupt, F.P., Ehrhardt, S., Burhardt, J., Bosomtwe, S.Y., Laryea, S., Aneamana, S.D., Otchwemah, R.N., Cramer, J.P., Dietz, E. and Gellert, S.B. (2004). Manifestation and outcome of severe malaria in children in Northern Ghana. *Am. J. Trop. Med. Hyg*. **71**: 167-172

Moody, A. (2002). Rapid diagnostic test for malaria parasites. *Clinc. Micro. Rev*. **15**: 66-72

Molyneux, M.E., Taylor, T.E., Wirima, J.J. and Borgstein, A. (1989). Clinical features and prognostic indicators in paediatrics cerebral malaria: a study of 131 comatose Malawian children *Q.J. Med*. **71**: 441

Mwai, L., Ochong, E., Abdirahman, A., Kiara, S.M., Ward, S., Kokwaro, G., Sasi, P., Marsh, K., Borrmann, S., Mackinnon, M. and Nzila, A. (2009). Chloroquine resistance before and after its withdrawal in Kenya. *Malaria. J*. **8**: 106

Myrick, A., Munasinghe, A., Patankar, S. and Wirth, D.F. (2003). Mapping of the *Plasmodium falciparum* multidrug resistance gene 5'-upstream region, and evidence of induction of transcript levels by antimalarial drugs in chloroquine sensitive parasites. *Mol. Microbiol*. **49**: 671-683

Mu, J., Ferdig, M.T., Feng, X., Joy, D.A., Duan, J., Furuya, T., Subramanian, G., Aravind, L., Cooper, R.A., Wootton, J.C., Xiong, M. and Su, X.Z. (2003). Multiple transporters associated with malaria parasite responses to chloroquine and quinine. *Mol. Microbiol*. **49**: 977-989

Muller, I., Bockarie, M., Alpers, M. and Smith, T. (2003). The epidemiology of malaria in Papua New Guinea. *Trends. Parasitol*. **19**: 253–259

Murphy, S.C. and Breman, J.G. (2001). Gaps in the childhood malaria burden in Africa: Cerebral malaria, neurological sequelae, anaemia, respiratory distress, hypoglycaemia, and complications of pregnancy. *Am. J. Trop. Med. Hyg*. **64** (Suppl 1-2): 57-67

Mutabingwa, T.K., Anthony, D., Heller, A., Hallett, R., Ahmed, J., Drakeley, C., Greenwood, B.M. and Whitty, C.J. (2005). Amodiaquine alone, amodiaquine+sulfadoxine-pyrimethamine, amodiaquine+artesunate, and artemether-lumefantrine for outpatient treatment of malaria in Tanzanian children: a four-arm randomized effectiveness trial. *Lancet*. **365**: 1474-1480

Nagel, R.L. (2002). Malaria anaemia. *Haemoglobin*. **26**: 329 -343

Nagesha, H.S., Din, S., Casey, G.J., Susanti, A.I., Fryauff, D.J., Reeder, J.C. and Cowman, A.F. (2001). Mutations in the *pfmdr1, dhfr* and *dhps* genes of *Plasmodium falciparum* are associated with *in-vivo* drug resistance in west Papua, Indonesia. *Trans. R. Soc. Trop. Med. Hyg*. **95**: 43–49

Nagesha, H.S., Casey, G.J., Rieckmann, K.H., Fryauff, D.J., Laksana, B.S., Reeder, J.C., Maguire, J.D. and Baird, J.K. (2003). New haplotypes of the *Plasmodium falciparum* resistance transporter (*pfcrt*) gene among chloroquine- resistant parasite isolates. *Am. J. Trop. Med. Hyg*. **68**: 398–402

Ndounga, M., Tahar, R., Basco, L.K., Casimiro, P.N., Malonga, D.A. and Ntoumi, F. (2007). Therapeutic efficacy of sulfadoxine-pyrimethamine and the prevalence of molecular markers of resistance in under 5-years olds in Brazzaville, Congo. *Trop. Med. Int. Health*. **12**: 1164-1171

Newton, C.R., Crawley, J. and Sowunmi, A. (1997). Intracranial hypertension in Africans with cerebral malaria. *Arch. Dis. Child*. **76**: 219

Newton, C.R. and Krishna, S. (1998). Severe falciparum malaria in children: current understanding of pathophysiology and supportive treatment. *Pharmacol. Ther*. **79**: 1

Newton, C.R., Taylor, T.E. and Whitten, R.O. (1998). Pathophysiology of fatal falciparum malaria in Africa children. *Am. J. Trop. Med. Hyg*. **58**: 673-683

Newton, C.R. and Warell, D.A. (1998). Neurological manifestation of falciparum malaria. *Ann. Neurol*. **43**: 695

Newton, C.R., Hien, T.T. and White, N. (2000). Cerebral malaria. *J. Neurol. Neurosurg. Psychiatry*. **69**: 433

Ngo, T., Durasingh, M., Reed, M., Hipgrave, D., Biggs, B. and Cowman, A.F. (2003). Analysis *Pfcrt, Pfmdr1, dhfr*, and *dhps* mutations and drugs sensitivies in *P. falciparum* isolates from patients in Vietnam before and after treatment with artemisinin. *Am. Soc. Trop. Med. Hyg*. **68**: 350-356

Nmor, C. and Egwunyenga, O.A. (2004). Survey of blood parasites among blood donors at Eku, Delta State, Nigeria. *Nig. J. Parasitol.* **25**: 87-92

Nosten, F. and White, N.J. (2007). Artemisinin-based combination treatment of falciparum malaria. *Am. J. Trop. Med. Hyg.* **77**: 181–192

Nzila, A.M., Mberu, E.K., Sulo, J., Dayo, H., Winstanley, P.A., Sibley, C.H. and Watkins, W.M. (2000). Towards an understanding of the mechanism of pyrimethamine-sulfadoxine resistance in *P. falciparum*: genotyping of dihydrofolate reductase and dihydropteroate synthase of Kenyan parasites. *Am. Soc. Microbiol.* **44**: 991-996

Ogungbamigbe, T., Ogunro, P., Elemile, P., Egbewale, B., Olowu, O. and Abiodun, O. (2005). Presciption patterns of antimalarial drugs among medical practitioners in Osogbo Metropolis, South-West Nigeria. *Trop. Med. Health.* **33**: 201-208.

Ojurongbe, O., Ogungbamigbe, T.O., Fagbenro-Beyioku, A.F., Fendel, R., Kremsner, P.G. and Kun, J.F. (2007). Rapid detection of Pfcrt and Pfmdr1 mutations in *Plasmodium falciparum* isolates by FRET and in vivo response to chloroquine among children from Osogbo, Nigeria. *Malar. J.* **6**: 41

Okafor, H.U. and Oguonu, T. (2006). Epidemiology of malaria in infancy at Enugu, Nigeria. *Niger. J. Clin. Pract.* **9**(1): 14-17

Okebe, J.U., Walther, B., Bojang, K., Drammeh, S., Schellenberg, D., Conway, D.J. and Walther, M. (2010). Prescribing practice for malaria following introduction of artemether-lumefantrine in an urban area with declining endemicity in West Africa. *Malaria. J.* **9**: 180

Okogun, G.R.A. and Amadi, A.N. (2005). Epidemiology, therapeutic agents and cost of management of paediatric malaria in a Nigerian tertiary hospital. *J. Vect. Borne. Dis.* **42**: 87-94

Olarewaju, W.I. and Johnson, A.W. (2002). Malaria in children in Ilorin, Nigeria. *East. Afr. Med. J.* **78**(3): 131-134

Olukosi, Y.A., Iwalokun, B.A., Magbagbeola, E.O., Akinwande, O., Adewole, T.A. and Agomo, P.U. (2005). Pattern of rural-urban aquisition of Pfcrt T76 allele among Nigerian children with uncomplicated Plasmodium falciparum malaria. *Afr. J. Biotec*. **4** (4): 361-366

Opara, K.N., Atting, I.A., Ukpong, I.G., Nwabueze, A.A. and Inokon, I.I. (2006). Susceptibility of genetic indices to falciparum malaria in infants and young children in southern Nigeria. *Pak. J. Bio. Sci*. **9**(3): 452-456

Osonuga, O.A. and Oduyemi, O.I.O. (2009). Parasitaemia Changes in the Course of Treatment of Severe Malaria Patients with Artemether and Quinine (A Preliminary Study). *Maced. J. Med. Sci*. doi:10.3889/MJMS.1857-5773.2009.0077

Pearce, R.J., Drakeley, C., Chandramohan, D., Mosha, F. and Roper, C. (2003). Molecular determination of point mutation haplotypes in the dihydrofolate reductase and dihydropteroate synthase of *Plasmodium falciparum* in three districts of northern Tanzania. *Antimicrob. Agents. Chemother*. **47**: 1347-1354

Peterson, D.S., Walliker, D. and Wellems, T.E. (1988). Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. *Proc. Natl. Acad. Sci. USA*. **85**: 9114-9118

Peterson, D.S., Di, S.S., Povoa, M., Calvosa, V.S., Do, R.V. and Wellems, T.E. (1991). Prevalence of the dihydrofolate reductase Asn-108 mutation as the basis for pyrimethamine-resistant falciparum malaria in the Brazilian Amazon. *Am. J. Trop. Med. Hyg*. **45**: 492-497

Pillai, D.R., Labbe, A.C., Vanisaveth, V., Hongvangthong, B., Pomphida, S., Inkathone, S., Zhong, K. and Kain, K.C. (2001). *Plasmodium falciparum* malaria in Laos: chloroquine treatment outcome and predictive value of molecular markers. *J. Infect. Dis*. **183**: 789-795

Planche, T., Agbenyega, T., Bedu-Addo, G., Ansong, D., Owusu-Ofori, A., Micah, F., Anakwa, C., Asafo-Agyei, E., Hutson, A., Stacpoole, P.W. and Krishna, S.A. (2003). A prospective comparison of malaria with other severe diseases in African children; prognosis and optimization of management. *Clin. Infect. Dis.* **37**: 890-897

Plowe, C.V. (2003). Monitoring antimalarial drug resistance: making the most of the tools at hand. *J. Exp. Biol.* **206**: 3745–3752

Plowe, C.V., Cortese, J.F., Djimde, A., Nwanyanwu, O., Watkins, W., Winstanley, P., Estrada-Franco, J., Mollinedo, R., Avila, J.C., Cespedes, J.L., Carter, D. and Doumbo, O. (1997). Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. *J. Infect. Dis.* **176**: 1590–1596

Price, R.N., Simpson, J.A., Nosten, F., Luxemburger, C., Hkirjaroen, L., ter Kuile, F., Chongsuphajaisiddhi, T. and White, N.J. (2001). Factors contributing to anaemia after uncomplicated falciparum malaria. *Am. J. Trop. Med. Hyg.* **65**: 614-622

RBM, (1999). Nigeria Supports Roll Black Malaria Movement September Newsletter.Abuja; WHO 1999. P2

Reed, M.B., Saliba, K.J., Caruana, S.R., Kirk, K. and Cowman, A.F. (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum. Nature.* **403**: 906–909

Reyburn, H., Mbatia, R., Drakeley, C., Carneiro, I., Mwakasungula, E., Mwerinde, O., Saganda, K., Shao, J., Kitua, A., Olomi, R., Greenwood, B.M. and Whitty, C.J. (2004). Overdiagnosis of malaria in patients with severe febrile illness in Tanzania: a prospective study**.** *Bmj.* **329**: 1212

Rogers, W.O., Sem, R., Tero, T., Chim, P., Lim, P., Muth, S., Socheat, D., Ariey, F. and Wongsrichanalai, C. (2009). Failure of artesunate-mefloquine combination therapy for uncomplicated *Plasmodium falciparum* malaria in southern Cambodia *Malaria. J.* **8**: 10

Ronald, L.A., Kenny, S.L., Klinkenberg, E., Akoto, A.O., Boakye, I., Barnish, G. and Donnelly, M.J. (2006). Malaria and anaemia among children in two communities of Kumasi, Ghana: a cross-sectional survey *Malaria. J.* **5**: 105

Runsewe – Abiodun, I.T., Ogunfowora, O.B. and Fetuga, B.M. (2006) Neomatal malaria in Nigeria – a 2 year review *BMC. Paediatrics*. **6**: 19

Rwagacondo, C.E., Niyitegeka, F., Sarushi, J., Karema, C., Mugisha, V., Dujardin, J.C., Van Overmeir, C., van Den, E.J. and D Alessandro, U. (2003). Efficacy of amodiaquine alone and combined with sulfadoxine pyrimethamine and of sulfadoxine pyrimethamine combined with artesunate. *Am. J. Trop. Med. Hyg.* **68**: 743-747

Salako, L.A., Ajayi, F.O., Sowunmi, A. and Walker, O. (1990). Malaria in Nigeria: a revisit. *Annals. Trop. Medi. Parasitol.* **84**: 435–445

Samdi, L.M., Oguche, S., Molta, N.B., Watila, I.M., Agomo, P.U. and Ene, A.C. (2005). *Plasmodium* infection in severely ill children Aged 0-8 years in Maiduguri metropolis, North-Eastern Nigeria. *J. Med. Sci.* **5**(4): 294-297

Satoguina, J., Walther, B., Drakeley, C., Nwakanma, D., Oriero, E.C., Correa, S., Corran, P., Conway, D.J. and Walther, M. (2009). Comparison of surveillance methods applied to a situation of low malaria prevalence at rural sites in The Gambia and Guinea Bissau**.** *Malar. J.* **8**: 274

Schellenberg D, Menendez C, Kahigwa E, Font F, Galindo C, Acosta C, Schellenberg JA, Aponte JJ, Kimario J, Urassa H, Mshinda H, Tanner M, Alonso P (1999). African children with malaria in area of intense *Plasmodium falciparum* transmission: features on admission to the hospital and risk factors for death. *Am J Trop Med Hyg*; **61**: 431-438

Schoepflin, S., Marfurt, J., Goroti, M., Baisor, M., Mueller, I. and Felger, I. (2008). Heterogeneous distribution of *Plasmodium falciparum* drug resistance haplotypes in subsets of the host population. *Malaria. J.* **7**: 78

Schönfeld, M., Miranda, I.B., Schunk, M., Maduhu, I., Maboko, L., Hoelscher, M., Berens-Riha, N., Kitua, A. and Löscher, T. (2007). Molecular surveillance of drug-resistance associated mutations of *Plasmodium falciparum* in south-west Tanzania. *Malaria. J.* **6**: 2

Sidhu, A.B., Verdier-Pinard, D. and Fidock, D.A. (2002). Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. *Science*. **298**: 210–213

Sirawaraporn, W., Sathitkul, T., Sirawaraporn, R., Yuthavong, Y. and Santi, D.V. (1997). Antifolate-resistant in mutants of *Plasmodium falciparum* dihydrofolate reductase. *Proc. Natl. Acad. Sci. USA*. **94**: 1124-1129

Sharma, Y.D. (2005). Genetic alteration in drug resistance markers of *Plasmodium falciparum*. *Indian. J. Med. Res*. **121**: 13–22

Sisowath, C., Stromberg, J., Martensson, A., Msellem, M., Obondo, C., Bjorkman, A. and Gil, J.P. (2005). In vivo selection of *Plasmodium falciparum* pfmdr1 86N coding alleles by artemether lumefantrine (Coartem). *J. Infect. Dis*. **191**(6)**:** 1014-1017

Snounou, G., Viriyakosol, S., Zhu, X.P., Jarra, W., Pinheiro, L., Rosário, V.E., Thaithong, S. and Brown, K.N. (1993). High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Mol. Biochem. Parasitol*. **1**: 315-320

Solomon, T., Felix, J.M., Samuel, M., Dengo, G.A. and Saldanha, R.A. (1994). Hypoglycaemia in paediatric admission in Mozambique. *Lancet*. **343**: 149-150.

Snow, R.W. and Marsh, K. (1995). Will reducing *Plasmodium falciparum* transmission alter malaria mortality among African children? *Parasitol. Today*. **11**: 188-190

Snow, R.W., Omumbo, J.A. and Lowe, B. (1997). Relation between severe malaria morbidity in children and level of *Plasmodium falciparum* transmission in Africa. *Lancet*. **349**: 1650-1654

Stauffer, W. and Fischer, P.R. (2003). Diagnosis and Treatment of malaria in children. *Clin. Infect. Dis.* **37**: 1340-1348

Su, X., Kirkman, L.A., Fujioka, H. and Wellems, T.E. (1997). Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant *Plasmodium falciparum* in Southeast Asia and Africa. *Cell.* **91**: 593-603

Suh, K.N., Kain, K.C. and Keystone, J.S. (2004). Malaria. *C.M.A.J.* **170** (11): 1693-1702

Sutherland, C.J., Fifer, H., Pearce, R.J., bin Reza, F., Nicholas, M., Haustein, T., Njimgye-Tekumafor, N.E., Justin, F., Doherty, J.F., Gothard, P., Polley, S.D. and Chiodini, P.L. (2009). Novel *pfdhps* haplotypes among imported cases of *Plasmodium falciparum* malaria in the UK. *Antimicrob. Agents. Chemother.* doi:10.1128/AAC.00024-09

Sutherland, C.J., Haustein, T., Gadalla, N., Armstrong, M., Doherty, J.F. and Chiodini, P.L. (2007). Chloroquine resistant *Plasmodium falciparum* infections among UK travellers returning with malaria after chloroquine prophylaxis. *J. Ant. Chem.* **59**: 1197-1199

Syarfruddin, D., Asih, P.B.S., Casey, G.J., Maguire, J., Baird, J.K., Nagesha, H.S., Cowman, A.F. and Reeder, J.C. (2005). Molecular epidemiology of *Plasmodium falciparum* resistance to antimalarial drugs in Indonesia. *Am. J. Trop. Med. Hyg.* **72**: 174-181

Taylor, T.E. and Strickland, G.T. (2000). Malaria. In Strickland GT editor. *Hunter's tropical medicine and emerging infectious diseases.*8th ed. Philadelphia WB Saunders, p. 614-643

Taylor, T.E., Molyneux, M.E., Wirima, J.J., Fletcher, K.A. and Morris, K. (1998). Blood glucose levels in Malawian children before and during the administration of intravenous quinine for severe falciparum malaraia. *N. Engl. J. Med.* **319**: 1040 – 1047

Talisuna, A.O., Nalunkuma-Kazibwe, A., Langi, P., Mutabingwa, T.K., Watkins, W.W., Van Marck, E., Egwang, T.G. and D'Alessandro, U. (2004). Two mutations in dihydrofolate reductase combined with one in the dihydropteroate synthase gene predict sulphadoxine-pyrimethamine parasitological failure in Ugandan children with uncomplicated falciparum malaria. *Infect. Genet. Evol.* **4**: 321–327

Talisuna, A.O., Langi, P., Mutabingwa, T.K., Watkins, W., Van Marck, E., Egwang, T.G. and D'Alessandro, U. (2003). Population-based validation of dihydrofolate reductase gene mutations for the prediction of sulfadoxine-pyrimethamine resistance in Uganda. *Trans. R. Soc. Trop. Med. Hyg.* **97**: 338–342

Temu, E.A., Kimani, I., Tuno, N., Kawada, H., Minjas, J.N. and Takagi, M. (2006). Monitoring chloroquine resistance using *Plasmodium falciparum* parasites isolated from wild mosquitoes in Tanzania. *Am. J. Trop. Med. Hyg.* **75**: 1182-1187

Theresa, K.N. (2005). Prevalence of falciparum malaria together with acute diarrhoea in children residing in malaria endemic zone. *Afr. Health. Sci.* **12** (1-2): 26-30.

Tinto, H., Guekoun, L., Zongo, I., Guiguemde, R.T., D'Alessandro, U. and Ouedraogo, J.B. (2008). Chloroquine-resistance molecular markers (Pfcrt T76 and Pfmdr-1 Y86) and amodiaquine resistance in Burkina Faso. *Trop. Med. Int. Health.* **13**: 238– 240

Tran, T.H., Day, N.P., Nguyen, H.P., Nguyen, T.H., Tran, T.H., Pham, P.L., Dinh, X.S., Ly, V.C., Ha, V., Waller, D., Peto, T.E. and White, N.J. (1996). A controlled trial of artemether or quinine in Vietnamese adults with severe falciparum malaria. *N. Engl. J. Med.* **335**: 76-83

Trape, J.F. (2001). The public health impact of chloroquine resistance in Africa. *Am. J. Trop. Med. Hyg.* **64**: 12-17

Trape, J.F. and Rogier, C. (1996). Combating malaria morbidity and mortality by reducing transmission. *Parasitol. Today.* **12**: 236-240

Triglia, T., Wang, P., Sims, P.F., Hyde, J.E. and Cowman, A.F. (1998). Allelic exchange at the endogenous genomic locus in Plasmodium falciparum proves the role of dihydropteroate synthase in sulfadoxineresistant malaria. *EMBO. J.* **17**: 3807-3815

Ursing, J., Zakeri, S., Gil, J.P. and Bjorkman, A. (2006). Quinoline resistance associated polymorphisms in the *pfcrt*, *pfmdr1* and *pfmrp* genes of *Plasmodium falciparum* in Iran. *Acta. Trop.* **97**: 352–356

Van den, B., Amsalu, R., Balasegaram, M., Hepple, P., Alemu, E., Hussein, E., Al-faith, M., Montgomery, J. and Checchi, F. (2005). Efficacy of two artemisinin combination therapies for uncomplicated falciparum malaria in children under 5years, Malakal, Upper Nile, Sudan. *Malaria. J.* **4**: 14

Van Eijk, A.M.A., Slutsker, I.J., ter Kuile, F.O., Otieno, J.A., Misore, A.Q., Shi, Y.P., Rosen, D.H., Steketee, R.W. and Nahlen, B.L. (2003). Placental malaria is associated with increased risk of parasitemia during infancy. *Am. J. Trop. Med. Hyg.* **69**(Suppl.): 315–316.

Vathsala, P.G., Pramanik, A., Dhanasekaran, S., Devi, C.U., Pillai, C.R., Subbarao, S.K., Ghosh, S.K., Tiwari, S.N., Sathyanarayan, T.S., Deshpande, P.R., Mishra, G.C., Ranjit, M.R., Dash, A.P., Rangarajan, P.N. and Padmanaban, G. (2004). Widespread occurrence of the *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) gene haplotype SVMNT in *P. falciparum* malaria in India. *Am. J. Trop. Med. Hyg.* **70**: 256-259

Vinayak, S., Biswas, S., Dev, V., Kumar, A., Ansari, M.A. and Sharma, Y.D. (2003). Prevalence of the K76T mutation in the *pfcrt* gene of *Plasmodium falciparum* among chloroquine responders in India. *Acta. Trop.* **87**: 287-293

Walker, O., Salako, L.A., Sowunmi, A., Thomas, J.O., Sodeinde, O. and Bondi, F.S. (1992). Prognostic risk factors and post mortem findings in cerebral malaria in children. *Trans. R. Soc. Trop. Med. Hyg.* **86**: 491-493

Wang, P., Lee, C.S., Bayoumi, R., Djimde, A., Doumbo, O., Swedberg, G., Dao, L.D., Mshinda, H., Tanner, M., Watkins, W.M., Sims, P.F.G. and Hyde, J.E. (1997). Resistance to antiolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. *Mol. Biochem. Parasitol.* **89**: 161-177

Wang, X., Um, J., Li, Q., Chen, P., Guo, X., Fu, L., Chen, L., Su, X. and Wellems, T.E. (2005). Decreased prevalence of the *Plasmodium falciparum* chloroquine resistance transporter 76T marker associated with cessation of choroquine use against *P. falciparum* malaria in Hainan, people's Republic of China. *Am. J. Trop. Med. Hyg.* **72**: 410-414

Watkins WM and Mosobo M (1973). Treatment of *Plasmodium falciparum* malaria with pyrimethamine-sulphadoxine: selective pressure for resistance is a function of long elimination half-life. *Trans R Soc Trop Med Hyg,* **87**: 75–78

Warell, D.A. (1999). Management of severe malaria. *Parasitologi.* **41**; 287-294

Wattagoon, Y., Srivilairit, S., Looareesuwan, (1994). Convulsion in Childhood malaria. *Trans. R. Soc. Trop. Med. Hyg.* **88**: 426-428

Wellems, T.E. and Plowe, C.V. (2001). Chloroquine-resistant malaria. *J. Infect. Dis.* **184**: 770–776

Wellems, T.E. (2002). *Plasmodium* chloroquine resistance and the search for a replacement anti-malarial drug. *Science.* **298**: 124–126

Wellems, T.E. (2004). Transporter of a malaria catastrophe. *Nat. Med.* **10**: 1169–1171

Wellems, T.E., Walker-Jonah, A. and Panton, L.J. (1991). Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. *Proc. Natl. Acad. Sci. USA.* **88**: 3382-3386

White, N.J. (1994). Clinical pharmacokinetics and pharmacodynamics of artemisinin and derivatives. *Tran. R. Soc. Trop. Med. Hyg.* **88**(Suppl 1): 541-543

White, N.J. (1995). Optimal regimes of parenteral quinine. *Trans. R. Soc. Trop. Med. Hyg.* **89**: 462-464

White, N.J. (1999). Antimalarial drug resistance and combination chemotherapy. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **354**: 739-749

White, N.J. (1999). Delaying anti-malarial drug resistance with combination chemotherapy. *Parasitologia.* **41**: 301-308.

White, N.J. (2005). Intermittent presumptive treatment of malaria. *PLos. Med.* **2**: e3

White, N.J. (2008). "How antimalarial drug resistance affect post-treatment prophylaxis." *Malar. J.* **7**: 9

White, N.J., Miller, K.D. and Churchhill, F.C. (1988). Chloroquine treatment of severe malaria in children. Pharmacokinetics toxicity and new dosage recommendations. *N. Eng. J. Med.* **319**: 1493-1500

WHO (1986). ''WHO Expert Committee on Malaria, Eighteen Report.''

WHO (2000). Current global malaria situation. WHO Expert committee on malaria. WHO Technical Report Series; **892**: 3 – 12

WHO (2003a). 'Assesment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated falciparum malaria.' http://www.who.int/malaria/docs/ProtocolWHO.pdf

WHO (2003b). 'The Abuja Declaration and the Plan of Action.' http://www.who.int/malaria/whomalariapublications.htm#2003

WHO (2004). Malaria Epidemics: Forecasting, prevention, early detection and control from policy to practice. Report of an informal consultation, Leyisn 8-10 December 2003. WHO/HLT/MAL/2004.1098

WHO (2008a). Roll Back Malaria. *World Malaria Report*. WHO/HTM/GMP/2008.1

WHO (2008b). 'The Global Malaria Action Plan.' http://www.rbm.who.int/gmap/gmap.pdf

WHO (2009). *World Malaria Report* 2009.

WHO (2010). *Guidelines for the treatment of malaria* 2nd edition. Geneva: World Health Organization

Winstanley, P.A., Watkins, W.M., Newton, C.R.J.C, Nevill, C., Mberu, E., Peshu, N., Warm, P.A., Waruiru, C.M., Mwangi, I.N., Warell, D.A. and Marsh (1992). The disposition of oral and intrasmuscular pyrimethamine-sulfadoxine in Kenya children with high parasitaemia but clinically non-severe falciparum malaria *Br. J. Clin. Pharmacol.* **33**: 143-148

Winstanley, P., Ward, S., Snow, R. and Breckenridge, A. (2004). Therapy of falciparum malaria in sub-Saharan Africa: from molecule to Policy. *Clin. Microbiol. Rev*. 612-637

Winters, R.A. and Murray, H.W. (1992). Malaria-the mime revisited: fifteen more years of experience at a New York teaching hospital. *Am. J. Med*. **93**: 243-246.

Wilson, C.M., Serrano, A.E., Wasley, A., Bogenschutz, M.P., Shanker, A.H. and Wirth, D.F. (1989). Amplification of a gene related to mammalian *mdr* genes in drug-resistant *Plasmodium falciparum*. *Science*. **224**: 1184-1186

Wongrischanalai, C., Pickard, A.L., Wernsdofer, W.H. and Meshnick, S.R. (2002). Epidemiology of drug resistant malaria. *Lancet. Infect. Dis*. **2**: 209-218

Wooden, J., Kyes, S. and Sibley, C.H. (1993). PCR and strain identification in *Plasmodium falciparum*. *Parasitol. Today*. **9**: 303–305

Yang, Z., Zhang, Z., Sun, X., Wan, W., Cui, L., Zhang, X., Zhong, D., Yan, G. and Cui, L. (2007). Molecular analysis of chloroquine resistance in *Plasmodium falciparum* in Yunnan Province, China. *Trop. Med. Intl. Health*. **12**: 1051-1060

Yeung, S., Van Damme, W., Socheat, D., White, N.J. and Mills, A. (2004). Access to artemisinin combination therapy for malaria in remote areas of Cambodia. *Malar. J.* **7**: 96

Zakeri, S., Afsharpad, M., Raeisi, A. and Djadid, N.D. (2007). Prevalence of mutations associated with antimalarial drugs in *Plasmodium falciparum* isolates prior to the introduction of sulphadoxine-pyrimethamine as first-line treatment in Iran. *Malaria. J.* **6**: 148

Zakeri, S., Afsharpad, M., Kazemzadeh, T., Mehdizadeh, K., Shabani, A. and Djadid, N.D. (2008). Association of pfcrt but not pfmdr1 alleles with chloroquine resistance in Iranian isolates of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **78**: 633–640

Zhong, D., Afrane, Y., Githeko, A., Cui, L., Menge, D.M. and Yan, G. (2008). Molecular epidemiology of drug-resistant malaria in western Kenya highlands. *BMC. Infec. Dis.* **8**: 105

Zhou, Z., Griffing, S.M., de Oliveira, A.M., McCollum, A.M., Quezada, W.M., Arrospide, N., Escalante, A.A. and Udhayakumar, V. (2008). Decline in sulfadoxine-pyrimethamine-resistant alleles after change in drug policy in the Amazon region of Peru. *Antimicrob. Agents. Chemother.* **52**: 739-741

Zindrou, S., Dung, N.P., Sy, N.D., Skold, O. and Swedberg, G. (1996). *Plasmodium falciparum*: mutation pattern in dihydrofolate reductase-TS genes Vietnamese isolates, a novel mutation, and coexistence of two clones in Thai patient. *Exp. Parasitol.* **84***: 56–64

Zongo, I., Dorsey, G., Rouamba, N., Tinto, H., Dokomajilar, C., Guiguemde, R.T., Rosenthal, P.J. and Ouedraogo, J.B. (2007). Artemether-lumefantrine versus amodiaquine plus sulfadoxine-pyrimethamine for uncomplicated falciparum malaria in Burkina Faso: a randomised non-inferiority trial. *Lancet*. **369**: 491–498

Zurovac, D., Ndhloru, M., Sipilanyambe, N., Chanda, P., Hamer, D.H., Simon, J.L. and Snow, R.W. (2007). Paediatric malaria case management with artemether-lumefantrune in Zambia: a repeat cross-sectional study *Malaria. J.* **6**: 31