STUDIES ON GENETIC MUTATIONS IN *PLASMODIUM FALCIPARUM* STRAINS ASSOCIATED WITH 4-AMINOQUINOLINES (CHLOROQUINE) AND PYRIMETHAMINE-SULPHADOXINE (FANSIDAR) RESISTANCE IN GHANAIAN MALARIA PATIENTS.

A THESIS PRESENTED TO THE DEPARTMENT OF ZOOLOGY,

UNIVERSITY OF GHANA

BY

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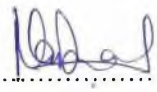
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY IN ZOOLOGY (PARASITOLOGY)

SEPTEMBER, 2001

DECLARATION

This thesis is the result of research work undertaken by Nancy O. Duah in the Department of Zoology, University of Ghana, under the supervisions of Dr Michael D. Wilson, Dr. Kwadwo A. Koram and Dr. Dominic Edoh.



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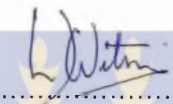

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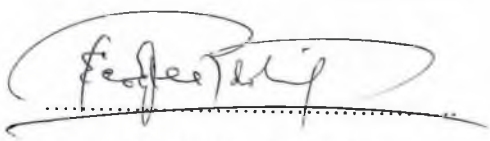

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DEDICATION

TO THE MEMBERS OF THE DUAH FAMILY

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my supervisors Dr. M. D. Wilson, Dr. K. A. Koram and Dr. D. Edoh for the technical advice and guidance, which contributed towards the success of this project.

I would like to acknowledge the tremendous help and goodwill received from Noguchi Memorial Institute for Medical Research (N.M.I.M.R.). In particular, Prof. D. Ofori-Adjei, Director, for allowing the work to be carried out in the Institute. My sincere gratitude also goes to the staff of Epidemiology Unit (Mr. Abuaku, Mr. Fenteng, Mr. Attiogbe, Mr. Osei, Mr Boafo and Miss Ampomah) for their assistance throughout the fieldwork. Thanks are due the following people for their valuable assistance in the laboratory; Dr. D. A. Boakye, Mr. Charles Brown, Mr. Jim Brandful, Mrs. Anita Ghansah, Mr Neils Quashie, Mrs. Bridget-Marian Ogoe, Mr. Boafo Afrim, Miss Janet Midega, Miss Helena Baidoo, Miss Naiki Puplampu, Miss Olivia Tetteh, Miss Marian Opoku-Agyakwah, Mr. Bismark Sarfo, Mr. Evans Glah and all others who variously helped me.

My heartfelt thanks and gratitude go to all the members of my family, especially to my Mum and Dad for their support and encouragement, my sisters, Louisa, Julia, Joana, Naa Merley and my brother Sammie; my cousins, Mavis, Cynthia, Abigail, and Susana; my auntie Paulina; my grannies; my brother-in-law, Steve; and my two sweet nieces, Stephanie and Shawna, for their prayers, support and love. To my friends, Sarah, Cynthia, Akosua, Neils, Derek, Martin, Michael, Andrew, Charles, Emmanuel and Benard, I extend my sincere appreciation for their prayers and love. To my mates, Godfred Futagbe and Samuel Kwapong, thanks for the encouragement.

An outstanding appreciation goes to Almighty God for all the grace that has sustained me till now and all that will keep me growing stronger in the future.

The Multilateral Initiative on Malaria (MIM) grant from WHO/TDR to Dr. Kwadwo A. Koram (N.M.I.M.R) funded this research work.

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LIST OF ABBREVIATIONS

bp	base pair
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
ddw	double-distilled water
DNA	deoxyribonucleic acid
EDTA	Disodium ethylene diamine tetraacetate. $2H_2O$
EtBr	Ethidium bromide
EtOH	Ethanol
M	Molar
Mw	molecular weight
PCR	Polymerase chain reaction
pfcrt	*Plasmodium falciparum* transporter gene
PfCRT	*Plasmodium falciparum* transmembrane protein
*pfmdr*1	*Plasmodium falciparum* multi-drug resistant gene
dhfr	dihydrofolate reductase gene
dhps	dihydropteroate synthase gene
rpm	revolution per minute
sddw	sterile double distilled water
TAE	Tris-acetate-EDTA
Tm	melting temperature
µl	microlitre
µM	micromolar

ABSTRACT

The malaria drug policy for Ghana is chloroquine, Fansidar and quinine as the first line, second line and third line drugs respectively. However, the burden of malaria has been complicated by the emergence of resistance especially to chloroquine, which is a cheap and effective drug. It has therefore become imperative that the levels of resistance of *Plasmodium falciparum* to anti-malarial drugs (chloroquine and Fansidar) in Ghana be established and the information used to develop an appropriate drug policy for effective case management. This present study therefore used molecular techniques, mostly, polymerase chain reaction (PCR) to detect and characterise mutations in the putative *P. falciparum* transporter gene (*pfcrt*) and *P. falciparum* multi-drug resistance gene *(pfmdr1)* that are known from previous studies to be associated with chloroquine resistance. Mutations in the dihydrofolate reductase gene (*dhfr*) and dihydropteroate synthase gene (*dhps*) that are associated with pyrimethamine-sulphadoxine (Fansidar) resistance were also studied. Children aged 5 years and below diagnosed as having uncomplicated malaria were recruited at two sentinel hospitals in Ghana (Hohoe and Navrongo) for the study with an informed consent from parents or guardians. Blood films obtained from the patients were examined for the presence of malaria parasites before treatment (Day 0) and then on Days 7 and 14 after treatment. Filter paper blood blots were also obtained at the same time, for use in PCR to detect the mutations. In addition to these, *in-vitro* chloroquine sensitivity test was performed on *P. falciparum* isolates from 26 patients. The *in vivo* studies revealed that 62% and 31% of the patients from Hohoe and Navrongo respectively were resistant to chloroquine. The classification of resistance according to parasitological clearance at Hohoe was 55%, 33% and 13% for RI, RII and RIII levels respectively; it was 43%, 33% and 23% respectively at

Navrongo. The baseline prevalence of the *pfcrt* 76 and *pfmdr1* 86 mutations were 82.5% and 82.0% in Hohoe and 43.8% and 61.5% in Navrongo. An association between *pfcrt* and *pfmdr*1 mutations and clinical outcome was observed at Hohoe (odds ratio = 12.40, p = 0.0001) but not at Navrongo (odds ratio = 1.16, p = 0.75). The baseline prevalence of the quintuple mutations of *dhfr* and *dhps* were 31.1% and 1.04% for Hohoe and Navrongo respectively. The *in-vitro* results showed that 7 of the 26 isolates were resistant to chloroquine with an IC_{50} value of $1.5x10^{-6}$ mol/litre. The results from this study suggest that mutations in *pfcrt* and *pfmdr1* can be used to predict the outcome of chloroquine treatment at Hohoe but not at Navrongo. The observed differences in the *pfcrt* and *pfmdr1* prevalence rates and in the association between genetic mutations and treatment outcome, is thought to be due to differences in drug pressure at the two areas. The relatively high prevalence of the quintuple mutations of *dhfr* and *dhps* observed at Hohoe gives an idea of the use of Fansidar whilst is the contrary for Navrongo.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Malaria is a serious and a major public health problem facing humanity in most tropical countries particularly sub-Saharan Africa. An estimated 300 to 500 million individuals are infected each year worldwide and between 1.5 and 2.7 million people die from it in Africa annually. Over 90% of the deaths occur in children under 5 years of age (WHO, 1999). According to the WHO (1998), malaria represents 2.3% of the overall global disease burden and 9% in Africa, ranking third among major infectious disease threats after pneumococcal acute respiratory infections (3.5%) and tuberculosis (2.8%).

The disease is caused by protozoan parasites belonging to the family Plasmodiidae and genus *Plasmodium*. There are four species of *Plasmodium* known to cause the disease in its various forms in man, namely; *P. falciparum, P. malariae, P. vivax* and *P. ovale*. Of these four *Plasmodium* species, *P. falciparum* is the most widespread and causes by far the most morbidity and mortality as well as presenting therapeutic challenge of drug resistance (WHO, 1999). In sub-Saharan Africa, the disease is transmitted from person to person by the female anopheline mosquito, mostly members of the *An. gambiae* species complex (Lindsay *et al.*, 1991).

The disease symptoms appear 10-16 days after an infectious bite with the bursting of infected red blood cells that releases merozoites into blood circulation. It is characterised by recurrent attacks or paroxysms with three stages involving chills, followed by fever and then sweating.

There are principally two approaches to the control of malaria, vector control and chemotherapy. Vector control involves the use of insecticides and environmental management whilst chemotherapy involves mainly the use of antimalarial drugs. Both approaches have limitations, which have hindered the effective control of the disease. The development of insecticide resistance in the vectors and of drug resistance in the parasite are the two major limitations.

The use of chemotherapy to control malaria was known in the 15th century with the use of quinine extracted from the bark of cinchona tree by the Peruvian Indians (WHO, 1999). Chloroquine was introduced in the 1940s and since then chloroquine has been a drug of choice for most control programmes because it is cheap and effective in curing the disease. Other types of drugs, which are basically antifolates, pyrimethamine and sulphones, were also developed later and used.

However, the effective use of the different groups of drugs in disease management has been hampered by the development of resistance to one or more in different parts of the world. Chloroquine-resistant *P. falciparum* was first reported in Thailand in 1961 (Kain *et al.*, 1994) and this rapidly spread worldwide such that there is virtually no endemic area that has not reported it. The problem of drug resistance can be attributed primarily to increased selection pressures on *P. falciparum* in particular, due to indiscriminate and incomplete drug use for self-treatment (WHO, 1995).

The *Plasmodium* parasites are known to have complex genomes and *P. falciparum* resistance to antimalarials has been attributed to mutations in the genome of the

parasite (Wellems *et al.,* 1997). The authors discovered the *cg2* gene on chromosome 7 and provided evidence that chloroquine resistance in Indochina arose with a particular set of mutations in the *cg2* allele, which spread across Asia and Africa. Polymorphisms in this gene then were thought to be associated with chloroquine resistance but allelic modification experiments have ruled out a role for this gene in chloroquine resistance (Su *et al.,* 1997; Basco and Ringwald, 1999; Adagu and Warhurst, 1999; Fidock *et al.,* 2000).

Another genetic mechanism of chloroquine resistance that has been suggested is the single base substitutions in the P. *falciparum* multi-drug-resistance gene (*pfmdr*1) on chromosome 5 which is associated with enhance efflux of the drug from resistant parasites (Foote *et al.*, 1990). The mutation results in the substitution of asparagine for tyrosine at position 86. Association of chloroquine resistance with *pfmdr*1 has been reported in genetic studies (Wellems *et al.*, 1990). Work done in Sudan, Tanzania and Kenya revealed an association between the mutation and chloroquine resistance (IAEA, 2001; Duraisingh *et al.*, 1997). However, in some field studies done in Mali, Cameroon and Southern Africa, no association was found between the presence of the mutation and chloroquine resistance (Basco and Ringwald, 1998; Djimde *et al.*, 2001, McCutcheon *et al*, 1999).

Chloroquine resistance has also been linked to mutant alleles of the *P. falciparum* transporter gene (*pfcrt*). This gene has 13 exons and was identified near *cg2* on chromosome 7 (Fidock *et al.*, 2000). It encodes for the digestive-vacuole transmembrane protein known as PfCRT. Sets of point mutations in *pfcrt* were associated with chloroquine resistance in laboratory lines of *P. falciparum* from

Southeast Asia, Africa and South America (Plowe and Wellems, 1999; Djimde *et al.*, 2001). One mutation that results in the substitution of threonine for lysine at position 76 (K76T) of the gene's DNA sequence was present in all resistant isolates and absent from all sensitive isolates tested *in vitro* (Djimde *et al.*, 2001). Also, genetic transformation experiments with plasmids expressing mutant forms of *pfcrt* conferred chloroquine resistance on three different chloroquine sensitive clones (Djimde *et al.*, 2001).

Fansidar which is a combination of folate antagonists and sulphonamides, has also become important because it is almost as cheap as chloroquine (Sudre *et al.*, 1992) and because it is often effective against chloroquine resistant *P. falciparum* (Mharakurwa and Mugochi, 1994; Soto *et al.*, 1995). However, treatment failure has become so common that, it is no longer a reliable choice for the treatment of *P. falciparum* infection in many parts of the world (Peters, 1987). The resistance of *P. falciparum* to pyrimethamine-sulphadoxine has been attributed to point mutations in the dihydrofolate reductase-thymidylate synthase gene (*dhfr-ts*) as well as dihydropteroate synthase gene (*dhps*) [Peterson *et al.*, 1990; Wang *et al.*, 1997]. The genotypes defined by the *dhfr* mutations are the Asn-108, lleu-51, Arg-59 and Leu-164 whilst those for *dhps* are Gly-437, Glu-540 and Gly-581.

Isolates from areas where *in vivo* Fansidar resistance is common often have one or more of the *dhfr* point mutations associated with pyrimethamine resistance in-vitro (Peterson *et al.*, 1988). Fansidar contains two drugs therefore it is possible that combination of point mutations, each of which has minimal or undetectable effect but may have profound effect together (synergistic). Both sets of mutations tend to

occur in a progressive, step-wise fashion, with higher levels of in vitro resistance occurring in the presence of multiple mutations, leading to the suggestion that different levels of in vivo resistance may be determined by specific sets of *dhfr* and *dhps* mutations (Plowe *et al.*, 1997; Wang *et al.*, 1997). Recently, *in vitro* pharmacokinetic studies of the synergistic action of pyrimethamine and sulphadoxine carried out under physiologic folate and para-aminobenzoic acid conditions suggest that the in vivo response to Fansidar may be determined primarily by parasite sensitivity to pyrimethamine (Watkins *et al.*, 1997). This therefore suggests that mutations in the *dhfr* are responsible for Fansidar resistance in *P. falciparum*.

The fact that there is no third antimalarial drug suitable for widespread use to replace chloroquine and Fansidar, the ability to map resistant malaria quickly and accurately on an epidemiological scale will be important in efforts to control the spread of resistance (Plowe *et al.*, 1999). Mapping of the mutations associated with resistance is one such means for monitoring drug resistance. Even though there are new drugs such as Malarone, Halfan, Mefloquine and others, these are very expensive and therefore cannot be used by the poor malaria endemic countries.

Methods for detection of such mutations using the polymerase chain reaction (PCR), which is an *in vitro* method for DNA amplification already exist (Plowe *et al.*, 1995). The nested PCR, which involves two rounds of amplification, primary and secondary (Snounou, 1993a) increases the sensitivity of detecting even point mutations in DNA sequences.

1.2 Rationale

Ghana is a malaria endemic country and distribution of the disease follows distinct climatic and ecological zones, with more cases occurring in the middle forest ecological zone, followed by coastal zone and then the northern savannah area (Ahmed, 1989). The disease accounts for about 40-42% of all outpatient visits in the country (MOH, 1987). The transmission of the disease occurs throughout the year but more especially just before and after rains (Ahmed, 1989). The commonest malaria parasite in the country is *P. falciparum.*

Chloroquine is the most widely used antimalarial and it is also the first line drug for disease management (Ofori-Adjei, 1989). Fansidar is used as a second line drug with quinine as the third line drug. The occurrence of chloroquine resistant parasite was first reported in 1986 in Accra (Neequaye, 1986) and since then other reports of *.P. falciparum* resistance in patients have also been made (Ofori-Adjei *et al.*, 1988; Neequaye *et al.*, 1988). Afari *et al.* (1992) in a field study assessed sensitivity to chloroquine in three ecological zones in Ghana and confirmed the occurrence of chloroquine resistant *P. falciparum* parasites. Recent studies carried out in some parts of the country revealed that about 40% of patients treated with chloroquine did not respond to treatment in some parts of the country (Ofori-Adjei, 2001).

A study of schoolchildren at Madina, a suburb of Accra revealed a high incidence of Fansidar treatment failure (Landgraf *et al.*, 1994). It is however likely that this may be the situation in the other parts of the country because increasing drug treatment failures have been observed with chloroquine and Fansidar at most hospitals (Koram, 2001).

Probably because of it's effectiveness, low cost and low rate of side effects, chloroquine still remains the drug of choice in most malaria endemic countries but appearance and spread of chloroquine resistant parasites in Africa poses a major challenge to malaria control. With the spread of drug-resistant parasites the efficacy of chloroquine may deteriorate beyond a level at which it will cease to be effective as first line drug for the treatment of malaria.

However, when there is little or no evidence to support a switch in first line drugs, the use of chloroquine may be retained long beyond the point at which it retains its comparative advantage with adverse effects on malaria morbidity and mortality (Koram, 2001). The experience from Kenya and Malawi suggests that this might have been the case and chloroquine was retained long after its efficacy had fallen below desirable levels (Bloland *et al.*, 1993). Although translation of results from simplified *in vivo* tests into treatment policy may be problematic, the absence of reliable data to base such decisions on do not augur well for a control strategy based on chemotherapy. Therefore the following questions need to be answered

1. Is the use of chloroquine as a first line drug in Ghana still justified?
2. What is the level of *P. falciparum* resistance to registered antimalaria drugs in the country?

This study will seek to partially answer these questions by determining the level of infection with resistant parasites at two sentinel sites so that drug resistance can be monitored to enable the effective use of the front line drugs in disease management.

This will be achieved by establishing the levels of resistance and the resistance-associated mutations in the parasite to the drugs used to manage malaria in the country.

1.3 General Objective

The overall aim of this study is to determine chloroquine and Fansidar resistance levels and resistance-associated genetic mutations in *P. falciparum* populations at two district hospitals in Ghana. The following mutations in *P. falciparum*; putative transporter gene (*pfcrt*) and *P. falciparum* multi-drug resistance gene (*pfmdr1*) both associated with chloroquine resistance, and dihydrofolate reductase gene (*dhfr*) and dihydropteroate synthetase gene (*dhps*) which are associated with Fansidar (pyrimethamine-sulphadoxine) resistance in patients will be investigated.

1.3.1 Specific objectives

1. To recruit and obtain demographic data on age and sex of a cohort of children aged 5 years and below.
2. To collect filter paper blood blot samples from malaria patients on days 0, 3, 7, 14 and 21 and also venous blood from all recruited patients on day 0 for molecular analysis and parasite culture respectively.
3. To conduct *in vitro* chloroquine sensitivity tests on parasites in venous blood samples from patients.
4. To detect and identify the species of *Plasmodium* present in the blood blot samples using PCR
5. To amplify target *P. falciparum* DNA sequences which have the mutations of *pfcrt*, *pfmdr*, *dhfr* and *dhps* genes using the polymerase chain reaction (PCR).

6. To determine the distribution and frequencies of both resistant and wildtype alleles of parasite in the sampled populations.
7. To analyse the data to reveal any associations between Chloroquine and Fansidar *in vivo* resistance and the mutations.
8. To confirm the association between these genetic mutations and chloroquine resistance using the results from *in-vitro* drug sensitivity test.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria: The Disease

Malaria and its symptoms have been known since time immemorial in man's recorded history with the occurrence of mosquito in amber suggesting its prevalence in pre-historic times (Smith, 1996). The symptoms of the disease were first described by Hippocrates, who related them to the time of the year and to where the patients lived (Bradley, 1996). A variety of names were given in describing the disease such as shakes, intermittent fever, ague and chills. It was realised then that there was an aetiological relationship between the disease and swamps. This led the Romans to begin drainage programmes because of the bad air that was associated with fever-producing areas and hence the term *mala aria*, written *mal'aria* (Smith, 1996). With time the apostrophe was removed to get the present day term *malaria.*

The causal agent was discovered by Alphonse Laveran in 1880. Ronald Ross later worked out the transmission of parasite from one person to another by *Anopheles* mosquitoes in 1898. The first known intervention against the disease was by native Peruvian Indians (before the discovery of the parasite and vector) in 1600, who used the bitter bark of cinchona tree (Bradley, 1996). By 1649, the bark was available in England as "Jesuits powder" (Bruce-Chwatt, 1980) to cure agues as it was called then. Vector control through insectides began in 1942 with the discovery of DDT, which was very effective but there was rapid insectide resistance. Other methods were implemented such as coating marshes with paraffin, draining stagnant water and the use of nets. Chemotherapy has been the main method for parasite control

with the use of drugs like quinine, chloroquine, pyrimethamine-sulphadoxine and mefloquine.

2.1.1 Disease symptoms

Clinical manifestations of the disease are dependent on the immune status of the host and also the species of *Plasmodium* an individual is harbouring. The first symptoms of malaria are non-specific and resemble influenza. These symptoms are similar for all four species of *Plasmodium*. They include headache, muscular ache, vague abdominal discomfort, lethargy, lassitude and dysphoria. These precede fever up to 2 days. Then there is fever with temperature rising intermittently (>37.5^{O}C), shivering, mild chills, worsening headache, malaise and loss of appetite. The periodicity of fever depends on the type of parasite species a patient harbours. If the infection is left untreated, the fever in *P. vivax* and *P. ovale* infections regularises to a 2-day cycle (tertian) and *P. malariae* fever occurs every 3 days. For *P. falciparum*, fever remains erratic and may not regularise to tertian pattern (White, 1996). There is paroxysm characterised by teeth-chattering rigors, cold intense headache and muscular ache, which can last up to 30 minutes, and then followed by profuse sweats. As the infection continues there is splenomegaly and hepatomegaly and development of anaemia.

Severe malaria, which is the acute form of falciparum malaria, as defined by WHO (1990) includes features such as severe anaemia, renal failure, pulmonary oedema, hypoglycaemia, circulatory collapse, bleeding, convulsions, haemoglobinuria, coma hyperparasitaemia, jaundice and hyperpyrexia. Cerebral malaria is the most prominent feature of severe malaria and is defined strictly as unarousable coma. This

is caused by the sequestration of infected erythrocytes in the microvasculature of the brain. Most deaths due to malaria are caused by severe malaria.

2.2 The Global Distribution of Malaria

The distribution of the disease was previously widespread but over the past 50 years, the geographical area affected by malaria has shrunk considerably and the disease is mainly confined to poorer tropical areas of Africa, Latin America, and Asia (Fig 2.1) where there are problems of controlling the disease because of inadequate health structures and poor socio-economic conditions (WHO, 1998). Malaria is endemic in a total of 101 countries and territories which have been divided into WHO's malaria regions. There are 45 countries in WHO's African Region, 21 in WHO's Americas Region, 4 in WHO's European region, 14 in WHO's Eastern Mediterranean Region, 8 in WHO's South-East Asia Region, and 9 in WHO's Western Pacific Region. The disease is now a public health problem in these countries, which are inhabited by an estimated total of 2400 million people representing 40% of the world's population (WHO, 1998). It was further estimated that worldwide prevalence of the disease is 300-500 million clinical cases each year of which more than 90% are in sub-Saharan Africa. Furthermore, an estimated mortality due to malaria is over 1 million deaths each year with the vast majority of deaths occurring among young children in Africa, especially those in remote rural areas with poor health services. Pregnant women, non-immune travellers, refugees, displaced persons and labourers entering endemic areas are also at risk of death due to the disease. According to WHO, distribution of the disease varies from one country to another and even within countries because of the flight range of the vector, which is about 2 miles and is irrespective of the prevailing wind.

Fig. 2.1 Global distribution of malaria (after Bradley, 1996). Shaded areas represent malaria endemic regions

2.3 The Socio-economic Impact of Malaria

Malaria has been a major obstacle to development in disease endemic areas especially in Africa, which is the worst affected malaria region. More than any other disease, malaria hits the poor and the disease endemic countries are some of the world's poorest (WHO, 1998). The disease causes 10.6% of lost disability adjusted life years (DALYs) being second only to HIV/AIDS (WHO, 1999).

In Africa, malaria accounts for up to a third of all hospital admissions and up to a quarter of all deaths of children under the age of 5 (WHO, 1998). There are up to 800,000 infantile mortalities and a substantial number of miscarriages and very low birth weight (VLBW) babies per year due to the disease (Bradley, 1996). The cost of malaria in economic terms is also high; treatment ranges in cost between $US 0.80 and $US 5.30 depending on local drug resistance, and the total cost in Africa is estimated at about $US1.8 billion per year (NIAID, 2000). A bout of malaria typically costs 10 working days, adding to the economic burden. It is also estimated that, costs to countries include costs for control and lost workdays to be 1-5% of GPD in Africa. For the individual, costs include the price of treatment and prevention and lost income (WHO, 1998).

The rural communities are the most affected because the rainy season is often a time of intense agricultural activity and time for poor families to earn most of their annual income. The disease can make these families even poorer due to the lost of labour. In children, malaria leads to chronic school absenteeism and there can be impairment of learning ability. Urban malaria is increasing due to unplanned development around large cities, particularly in Africa and South Asia.

Malaria epidemics related to political/civil upheavals, economic difficulties and environmental problems also contribute in the most dramatic way to death tolls and human suffering (WHO, 1998). Increased risk of the disease is linked with changes in land use activities like road construction, mining, logging and agricultural and irrigation projects, particularly in "frontier" areas like the Amazon and in South-East Asia. Other causes of its spread include global climatic change, disintegration of health services, armed conflicts and mass movements of refugees. The emergence of multi-drug resistant strains of parasite is also exacerbating the situation. New drugs developed are expensive, further straining the economies of the rural communities and endemic countries.

2.4 The Life Cycle and Transmission of Human *Plasmodium*

The life cycle of the parasite is split between a vertebrate host and an insect vector. Parasites are transmitted from one human to another by female Anopheline mosquito (Figure 2.2). There are three phases of development in the life cycle of the parasite namely, exo-erythrocytic stages in liver, erythrocytic schizogony in erythrocytes and sexual process in the mosquito (Smith, 1996). A human infection begins with a bite from an infected *Anopheles* mosquito when it takes a blood meal. During the process numerous threadlike sporozoites, the infective stage of the parasite, may be injected as the mosquito feels about with its proboscis before it strikes a small capillary. The sporozoites are directly injected into the blood stream. Some die but the survivors after 30 minutes migrate to the liver (Smith, 1996). All sporozoites leave peripheral blood circulation within 45 minutes of injection into the host (WHO, 1987).

In the hepatocytes, the sporozoites multiply asexually (schizogony) to become a schizont that contains tiny rounded merozoites. When schizogony is completed usually after 9-16 days, merozoites are released and invade the erythrocytes. The invasion into the erythrocytes is achieved through endocytosis (Aikawa, 1980). Within the erthrocytes they fuel their activities by consuming haemoglobin and develop into ring-shaped trophozoites. There is another round of asexual reproduction within the erythrocytes, which takes 48 hours and this time when the merozoites are released some invade other erythrocytes whilst others change into sexual forms (micro and macrogametocytes) within 10-12 days (Mons, 1985). Normally, a variable number of asexual cycles occur before any gametocytes are produced (Carter & Gwadz, 1980).

The gametocytes have no further activity in the human host. These circulate in the blood stream until they are picked up by another mosquito as it takes blood from the infected human. In the gut of the mosquito, there is exflagellation of the microgametocytes and subsequent fertilization of the macrogametocytes. The zygote, which is called ookinete, penetrates the wall of the midgut and develops into an oocyst. Sporogony within the oocyst produces many threadlike sporozoites, which are released as the oocyst ruptures. The sporozoites develop and become up to 1000 times more infective than when in the oocyst (WHO, 1987). They then migrate to the salivary glands where they reside until injection into another human host.

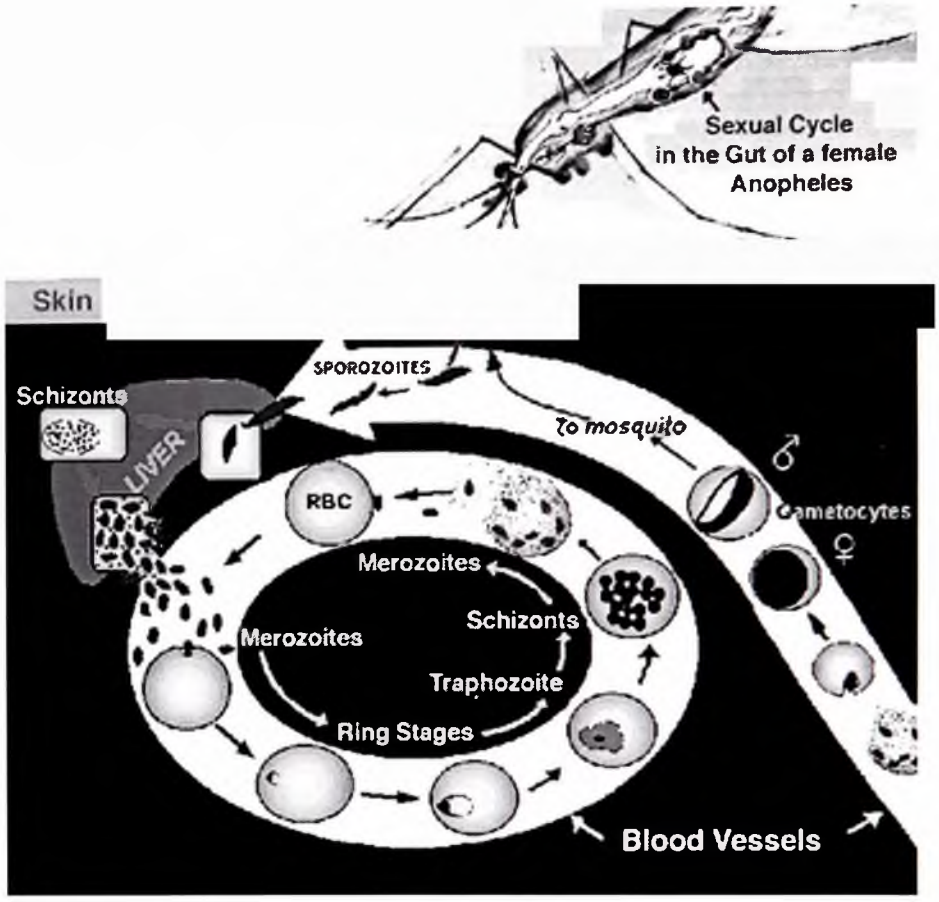

Fig. 2.2 Schematic illustration of the life cycle of *P. falciparum* (from Malaria Foundation International Website, after Shobhona Sharma, 2001)

2.5 The Life Cycle of the Anopheline Vectors of Malaria

There are four distinct stages in the life cycle of the mosquito, namely, egg, larva, pupa, and adult stages (Fig. 2.3). The immature stages are aquatic whilst the adult is terrestrial.

After mating, the female mosquito feeds on blood, which helps in the development of the eggs. Each blood meal provides enough nutrition for a female mosquito to lay a batch of eggs. Anophelines show the most regular cycle of blood feeding and egg laying. The females will also feed on nectar between blood meals. Blood feeding can take place before or after mating; this depends on the species and local circumstances (White, 1998).

Depending on the type of mosquito species, there are specific breeding sites where the female mosquito lays her eggs. Eggs are deposited on damp soil or vegetation, in moist tree-holes or containers, and sometimes on to water. It is the choice of specific oviposition sites by female mosquitoes that determines the breeding places of each species (White, 1998). The female mosquito lays 30-500 or more eggs at one oviposition. The number of eggs deposited depends much on the species although all of them are not deposited at one site (Service, 1993). The number of eggs laid depends also on nutrition and size of adults. Mosquitoes of the genus *Anopheles* lay single eggs that float on the water's surface. *Aedes* mosquitoes for example, lay their eggs singly on damp soil and the eggs hatch only when flooded that is, they undergo diapause. In the case of *Culex* and *Culiseta* species, the eggs are stuck together in groups or rafts of 200 or more on the surface of the water. Eggs hatch soon after completion of the embryonic

development, in as little as two or three days in tropical species but a week or more in temperate and subartic species. Temperature plays a role in the success of eggs hatching into larvae. Eggs of tropical *Anopheles. stephensi* and *An. culicifacies* die when the temperatures fall below about 10°C (Service, 1993).

All mosquito larvae are aquatic and metapneustic and pass through four larval instars (Service, 1993). They breathe at the water's surface through a specialized siphon tube. Mosquito larvae are very active and move almost continuously as they shuttle to the surface to obtain oxygen and dive to the bottom to find food. There are five functional feeding types among larvae namely, scavengers that ingest mainly dead food by scraping and nibbling at animal carcasses and detritus; bottom-feeders that browse over bottom debris, feeding on living and nonliving material; filter-feeders that strain phytoplankton and zooplankton from the water; filter-feeders that feed from the surface film and predators that consume other living organisms.

Rates of larval growth are influenced by environmental factors such as water temperature, photoperiodicity, food supply, degree of overcrowding and the species (Service, 1993; White, 1998). No known mosquito has larvae that can withstand desiccation although larvae may be able to survive short periods among wet mud etc. Larvae shed (moult) their skins four times, growing larger after each moulting.

Within a few days to several months, depending on the species and environmental

factors, the larvae develop into pupae. Pupation occurs at the water surface and the whole process takes about three to five minutes. Pupae are non-feeding and normally spend most of their time at the water surface breathing through the paired trumpets. When pupae are disturbed by shadows or vibrations, they descend in a zigzagging fashion. Mosquito pupae are the most active of any insect pupae (White, 1998).

Pupal duration is mainly determined by temperature. In hot tropical countries it is usually two or three days, but can be as short as 26 hours at temperature of about 30°C (Service, 1993). Duration also varies according to species. When this process is complete, the fully formed adult emerges from the pupal case. Emergence usually takes 12-15 minutes and within minutes afterwards the newly emerged adult can make very short flights. Male larvae generally pupate before females and in most species male pupal life is shorter than female pupal life. Males usually emerge a day or so before females (Service, 1993). The entire life cycle from egg to adult can be completed in less than 10 days during periods of favourable temperatures.

After emergence from the pupa the teneral adult usually seeks a shelter amongst vegetation until ready for mating, which in the case of the female usually occurs a few hours after emergence, but sometimes much sooner (Service, 1993). In some species one or two days may elapse before emergence and mating. Males do not mate until their genitalia have rotated through 180°C, a process that takes 20-24 hours but in some species as little as 6-12 hours (Service, 1963; White, 1998). Mating usually occurs in flight and commonly around dusk. Adult mosquitoes can live for several weeks to several months, depending upon numerous environmental conditions.

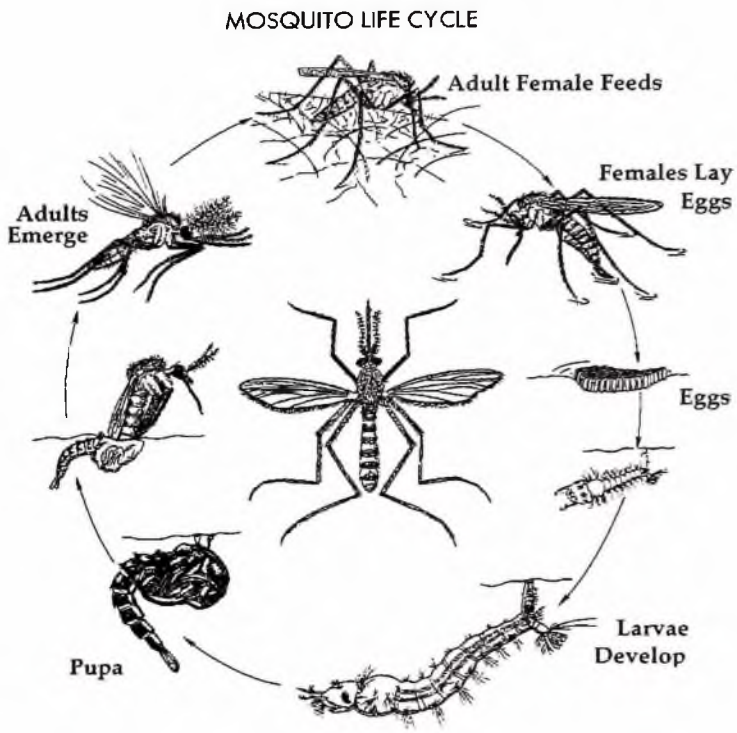

Figure 2.3: Schematic illustration of the life cycle of *Anopheles* vectors of malaria (After Service, 1980).

2.6. Control of Malaria

2.6.1 Historical overview

Systematic control of malaria could only begin after the discovery of the causative parasite by Alphonse Laveran in 1880 and the demonstration by Ronald Ross in 1897 that mosquito was the vector.

The first control mounted against the disease was in the 1950s and 1960 (NIAID, 2000). With the discovery of dichlorodiphenyltrichloroethane (DDT) during World War II, it was thought eradication of malaria was possible (WHO, 1998). The WHO Global Malaria Eradication Programme was then created with the aim of spraying homes with residual insecticide in this case DDT and manipulation of the environment to reduce mosquito breeding. This strategy proved successful in large areas of North America, Southern Europe, the former Soviet Union and some territories of Asia and South America by halting transmission and thereby eradicating malaria. The disease however persisted in most countries in Latin America, Asia and Africa. Large-scale eradication was never attempted in Africa because of the problems and logistics associated with malaria control, which were beyond the scope of the vast majority of the countries then.

Whilst malaria has remained endemic in most African countries, the disease is re-emerging in areas where it was previously under control or eradicated, for example, in the Central Asian Republics of Tajikistan and Azerbaijan, and in Korea (WHO, 1999). Easy international travel has enhanced the importation of cases of malaria and therefore it is now more frequently registered in developed countries

Malaria has been a priority for WHO since its inception in 1948. The WHO's Programme on Communicable Diseases (CDS) coordinated control activities worldwide. The four basic technical elements of the current WHO's Global Malaria Control Strategy are the provision of early diagnosis and prompt treatment for the disease, the planning and implementation of selective and sustainable preventive measures including vector control, early detection for the prevention or containment of epidemics and the strengthening of local research capacities to promote regular assessment of countries' malaria situations, in particular the ecological, social and economic determinants of the disease (WHO, 1998).

The UNDP, UNICEF, WHO and World Bank in 1998 launched the Roll Back Malaria programme (RBM) with the aim of obtaining deeper commitment to win the fight against malaria (WHO, 1998). This was going to require not only the commitment of the health sector, but also other governmental sectors, the private sector where activities may directly or indirectly affect the malaria situation, nongovernmental organizations, and affected communities themselves. The achievements of the RBM so far, have been political commitment to malaria control, a progressive strengthening of national and local capacities for assessing malaria situations and selecting appropriate measures aimed at reducing or preventing the disease (WHO, 1998). National plans of action have also been developed in more than 80% of malaria endemic countries.

2.6.2 Vector control

The main approach to controlling adult mosquitoes has been the spraying of inside surfaces of walls and ceiling or roof of homes with residual insecticide. The rationale being that mosquitoes will rest on the walls before or after biting and remain long enough to pick up lethal dose of the insecticide.

The main insecticide used during the 1950s and 1960s malaria control programmes was DDT. It was widely used because of its cheapness per unit weight and its durability, which enabled spraying to be carried out twice a year, or only once in areas with a short annual malaria mosquito season (Curtis, 1996). Earlier studies demonstrated that DDT was also a repellent, irritant and has toxic effects on malaria vectors (WHO, 1998). When DDT was sprayed on house walls at doses of 2 g/m^2, it exerted powerful control over indoor transmission of malaria (Cavalie and Mouchet, 1962). There were claims in the 1960s and 70s about supposed effects of DDT on human health such as DDT residues in human breast milk which was usually attributed to contaminated food (Curtis, 1994). Also resistance to DDT developed in the insects, which rendered it ineffective after a while. DDT was then replaced with organophosphate or carbamate insecticides such as malathion or bendiocarb in Sri Lanka, parts of India, Pakistan, Turkey and Central America. However, these insecticides proved to be considerably more expensive to use than DDT and malathion does not persist well on mud walls.

Pyrethroids such as deltamethrin and lambdacyhalothrin are used presently and are effective at far lower doses than DDT (0.25 mg/sq metre compared with 2 gm/sq metre). Although more expensive per unit weight, these pyrethroids are not much more expensive per house protected per year (Curtis, 1994). They are also much more acceptable to householders because they leave no visible deposit on walls and also because they kill nuisance insects such as cockroaches. Like DDT, pyrethroids tend to irritate mosquitoes so that they do not rest on deposits for long. However, the pyrethroids paralyse the nervous system so fast that contact for a few minutes is enough to kill, whereas much longer contact is required with DDT and mosquitoes may escape it without picking up a lethal dose. For these reasons, better malaria control has generally been achieved with pyrethroids rather than with DDT and other chemical insecticides.

Large scale use of insecticide impregnated treated bednets (IIBNS) in endemic countries is being promoted as a means to control malaria. Studies conducted in rural northern Ghana showed a reduction in mortality in users of the IIBNS and it also revealed that protection extends about 100m to non-user neighbours (Binka *et al.*, 1998). Other studies conducted in rural Tanzania, Ifakara, over a three-year period reported of reduction in mortality such that IIBNS prevented 1 in 20 child deaths in the community (Schellenberg *et al*, 2001). To assess the impact of distributing IIBNS on malaria parasitaemia and anaemia in young children following the Ifakara studies, it was revealed that IIBNS had a protective efficacy against parasitaemia and anaemia in children and therefore a substantial effect on morbidity (Abdulla *et al*, 2001). Nets have long been appreciated as a protection against night biting mosquitoes including malaria

vectors. However, nets are often torn or hung in such a way that mosquitoes can enter or bite through them. The initial motive for impregnating them with an insecticide, which was safe for close human contact, was to add a chemical barrier to the imperfect physical barrier presented by the net. This apparently arises because a treated net either kills or irritates and drives away mosquitoes before they have found a hole in the net to enter. An additional argument for treating bednets is that they are a rational place in which to deploy a residual insecticide because mosquitoes are attracted to them by the carbon dioxide and body odour emitted by the sleeper. Thus the net acts like a baited trap. In comparison with spraying a family's house, the amount of insecticide needed to treat their nets is much less and synthetic netting is a more favourable substrate for a residual insecticide than is a mud wall. The concern has been raised that large scale use of pyrethroid impregnated nets may select for pyrethroid resistance of a physiological kind or may change mosquito behaviour so that they bite out of doors instead (WHO, 1999).

Anopheles breeding is sufficiently limited in extent and definable therefore, larval control also makes a significant contribution to malaria control. Thus draining or filling breeding sites, screening of tanks, spraying of larvicide such as Temephos even into potable water, stocking breeding sites with larvivorous fish such as *Ctenopharyngodon idella*, spraying breeding sites with bacterial toxin from *Bacillus thuringiensis israelensis* have all been used to control larval populations (Curtis, 1991).

There is considerable interest in the idea of rendering mosquito populations genetically harmless by introducing genes, which make them non-susceptible to *Plasmodium* (Collins and Paskewitz, 1995) and also to divert them from being strongly attracted to biting humans and animals. Thus, for the desirable genes to be spread in *Anopheles* populations, genetic systems such as transposable elements or the intracellular symbiont *Wolbachia* have been used (Curtis, 1996). However, this approach is still being researched and may be a long time yet before it becomes operational.

2.6.3. Chemotherapy

Chemotherapy has been the main method for parasite control and clinical management with the use of antimalarial drugs such as quinine, chloroquine and others.

Antimalarials can be grouped under four categories according to the stage in the life cycle of the parasite that they attack. The four categories are tissue schizonticides, blood schizonticides, gametocytocidals and sporonticidals. The causal prophylactics appear to kill the early but not the late liver stages and prevent erythrocytic infections. Drugs that destroy all exo-erythrocytic forms are referred to as tissue schizonticides. Blood schizonticides act on the asexual erythrocytic parasites. The gametocytocidal drugs act on gametocytes, particularly the immature forms. The antimalarials which when taken up in a blood meal inhibit the development of oocyst and thereby prevent the production of sporozoites in the mosquito are referred to as sporonticidals.

Artemisinin

The infusion of qinghao *Artemisia annua* has been used in China for at least the last 2000 years (WHO, 1999) and its active ingredient "qinghaosu" also known as artemisinin have recently been identified. The two most widely used derivatives of artemisinin are artesunate and artemether and both are blood schizonticides. Artesunate is given orally in the form of tablets at a dose of 4mg/kg for 3 days and sodium artesunate is in the form of powder that is reconstituted for intravenous injection. Artemether is given through intramuscular injection (anterior thigh) at a dose of 3.2mg/kg and then 1.6kmg/kg (Fernandez, 2001). While they are widely used in Southeast Asia, they are not licensed in much of the "Western World" including Australia (Davis, 2001). A high rate of treatment failures has been reported and it is now being combined with mefloquine for the treatment of *P. falciparum* malaria (WHO, 1998).

Halofantrin

Halofantrin belongs to a class of compound called the phenanthrene-methanols and it is not related to quinine. It was first introduced in the 1980s as a blood schizonticidal against the erthrocytic stages of chloroquine resistant *P. falciparum* and also the erythrocytic stages of *P.vivax* but not the hypnozoites. The drug is used to treat acute forms o f uncomplicated and multi-resistant *P. falciparum* malaria and as a 'stand by' drug if chemoprophylaxis fails and there is no medical aid available. It is given at 8mg/kg in 3 doses. It is slowly and irregularly absorbed if taken orally (but aided by a

fatty meal), with a peak plasma concentration 4-6 hours later. Its half-life is 1-2 days and it is eliminated through the faeces. But due to its short half life of 1 to 2 days, it is therefore not suitable as a prophylactic (Davis, 2001). Halofantrin has been associated with neuropsychiatric disturbances and also it is contra-indicated during pregnancy and not advised for women who are breastfeeding (WHO, 1998). Abdominal pain, diarrhoea, prurities and skin rash have also been reported as side effects by WHO.

Malarone

Malarone was first introduced in 1998 in Australia. It is a combination of proguanil and atovaquone. Atovaquone was introduced in 1992 and it was used with success for the treatment of *Pneumocystis carrinii*. It is a synthetic derivative of hydroxynaphthoquinone, and may exert its effect by selectively inhibiting electron transport in the mitochondria. When combined with proguanil there is a synergistic effect and the combination is at the present time a very effective antimalarial treatment (Davis, 2001). It is given at 1000mg/day for 3 days. Malarone has undergone several large scale clinical trials and has been found to be 95% effective in drug resistant *P. falciparum* malaria (WHO, 1998). It has been claimed to be largely free from undesirable side effects even though proguanil is an antifolate. The common side effects listed in order of occurrence are rash, nausea, diarrhoea, headache, fever and vomiting. However, it is a very expensive drug.

Mefloquine

Mefloquine was first introduced in 1971. It is a quinoline methanol derivative that is related structurally to quinine. Mefloquine is a blood schizonticide and it is also effective in killing hypnozoites if given as a combination treatment with primaquine. The compound was found to be effective against malaria that was resistant to other forms of treatment and because of its long half life is also a good prophylactic.

The drug interferes with transportation of haemoglobin products and other substances from the host cell to the parasite's food vacuole. (Hellgren *et al.,* 1997; Weidekamm *et al.,* 1998). Because of its relationship to quinine, the two drugs are not recommended to be used together. Mefloquine is taken orally at 25mg/kg once and is rapidly absorbed. Though it has a slow onset of action, it is very long acting with a plasma half-life of 30 days. There have been reports of various undesirable side effects including several cases of acute brain syndrome, which is estimated to occur in 1 in 10,000 to 1 in 20,000 of the people taking this drug (WHO, 1998). This side effect usually develops about two weeks after starting mefloquine and generally resolves after a few days. Widespread resistance has now developed to mefloquine and this together with undesirable side effects has resulted in a decline in its use (Fernandez, 2001).

Mepacrine

Mepacrine was introduced in 1935 but is now considered obsolete. This drug, which is a 9-amino-acridine, is an effective blood schizonticide but among its disadvantages is the fact that it is laid down in the skin and the recipient turns bright yellow. It was used as a prophylactic on a large scale during the World War II (WHO, 1998). It had a major

influence in reducing the incidence of malaria in troops serving in South East Asia (Davis, 2001). It is given intravenously at 20mg/kg over 4 hours followed by 10mg/kg over 8 hours (Fernandez, 2001) It is now considered to have too many undesirable side effects and is no longer used.

Primaquine

Primaquine was first used in 1951, replacing the related pamaquine, which was one of the first synthetic antimalarials. Both are 8-aminiquinolines which destroy pre-erythrocytic stages (sporontocides). The important role of primaquine is in the treatment of *P. vivax* infection which has persistent liver stages. It is taken orally and is rapidly absorbed and metabolised. Primaquine is given as its base, 15 mg/day for 14 days. The half-life of the drug is 3-6 hours. Its main unwanted effect is due to an inherited genetic metabolic condition, a deficiency of glucose-6-phosphate dehydrogenase in the red blood cell. Large doses of primaquine usually result in methaemaglobinaemia with cyanosis haemolysis (Fernandez, 2001).

Proguanil

Proguanil was first synthesised in 1946 and introduced in 1948 as a Biguanide (Davis, 2001). It has a biguanide chain attached at one end to a chlorophenyl ring and its structure is very close to pyrimethamine. It destroys the early tissue stages, especially in *P. falciparum* and hence is used as causal prophylactics. It also acts as a blood schizonticide working more slowly than chloroquine and as a sporontocide. It is given at 200mg for 3 to 7 days (Navy Medical Dept, 2001). It is free from unpleasant side effects and because of its mode of action, it is among the compounds known as folate

antagonists or antifolates and destroys the malarial parasite by binding to the enzyme dihydrofolate reductase. It is still used as a prophylactic in some countries.

Pyrimethamine

Pyrimethamine was introduced in 1952 as a diaminopyrimidine with similar activities as proguanil. It is a drug that affects the synthesis and utilisation of folate. Pyrimethamine (2,4, diaminopyrimidine) acts by inhibiting the dihydrofolate reductase necessary for synthesis of tetrahydrofolate, a precursor in the parasite DNA synthesis. Pyrimethamine is given at 25mg weekly has a half life of 4 days (Navy Medical Dept, 2001). Some of the side effects are skin rashes and higher doses will affect human dihydrofolate releases leading to megaloblastic anaemia. Folate supplement is usually given in pregnancy, but this reduces the efficacy of the drug (Nwanynwu *et al.*, 1996; Basco and Ringwald, 1998).

Quinine

Quinine is an alkaloid originally extracted from the bark of *Cinchona ledgeriana* (Cinchona tree). It is one of the four main alkaloids found in the bark and is the only drug, which over a long period of time has remained largely effective for treating the disease (Davis, 2001). The antifebrile properties of the bitter bark of the cinchona tree were known by local populations well before the 15th century (WHO, 1999). History has it that the Spanish learned about quinine from the Peruvian Indians in the 1600s. Export of quinine to Europe and to United States became a lucrative business until World War II cut off access to the world supply of cinchona bark. Quinine is a bitter

tasting blood schizonticide and in case of *P. vivax* and *P. malariae* also acts as a gametocytocide. It was isolated in 1820 by pharmacist Pelletier and Caventou (Haas, 1994). It is now used only for emergency treatment of *P. falciparum* malaria, when response to chloroquine and most antimalarials have failed. Its mechanism of action is similar to Chloroquine, it causes cytotoxicity of the parasite by inhibiting the plasmodial haem polymerisation with the subsequent built up of toxic haem. It also intercalates with parasite DNA. It is given orally at 10mg.salt/kg as a 7-day course (Fernandez, 2001). It is well absorbed in the gut but 80% of it may be bound to plasma protein. Its half-life is 10 hours and is metabolised in the liver and excreted in urine within 24 hours. Side effects are numerous and could be fatal. In the 1930's and 40's it was common for people to take quinine when they thought they had "a touch of malaria" in Africa. The association of repeated infections with falciparum malaria and inadequate treatment with quinine, resulted in the development in some of acute massive intravascular haemolysis and haemoglobinuria (black water fever). In the 1940s, an intensive research program to find alternatives to quinine resulted in the manufacture of chloroquine and other chemical compounds that are more effective and less toxic (NIAID, 2000).

Sulphones

Sulphones such as Dapsone and Sulphonamides have antimalarial activity as blood schizonticides and are usually used in conjunction with other blood schizonticides usually pyrimethamine. They are also folate antagonists and have antibacterial activity whilst Dapsone is an antileprosy agent. A combination of Dapsone and pyrimethamine is

marketed as Maloprim. Sulphonamide (e.g. sulphadoxine) and sulphones (Dapsone) act by competing for the enzyme dihydropteroate synthetase with para-aminobenzoic acid and therefore inhibit folate synthesis. They act on erythrocytic *P. falciparum*, but not sporozoites or hypnozoites. It is a top up treatment after a 7-day course of quinine in acute attack of chloroquine resistant *falciparum* malaria. It is also a prophylaxis for chloroquine resistant strain *P. falciparum* or *P. vivax*. Proguanil or pyrimethamine-dapsone (Fansidar) is combined with chloroquine to prevent transmission by killing gametocytes. It is orally administered and slow but well absorbed.

Chloroquine

Chloroquine is one of the most successful antimalarial drug ever synthesised, because of its safety, affordability, ease of use and its great efficacy (Ginsburg *et al.*, 1999). Chloroquine and its close relative Amodiaquine are all 4-aminoquinolines. It was introduced in 1945 and is an excellent blood schizonticide. It is also a gametocytocide against *P. vivax, P. ovale* and *P. malariae*. For the rapid control of acute malaria, chloroquine is usually the drug of choice. It is a weak base and concentrates in the parasites' lysosome, possibly by a parasite-specific drug-concentrating mechanism. Its beneficial effects on public health have been very enormous but after nearly a half-century of use, how it works at the molecular level remains unknown (Wellems, 1992).

The drug is administered orally, unless where not feasible or in severe attack, then it is given by continuous intravenous infusion and frequent intramuscular or subcutaneous injection. It is rapidly and completely absorbed and extensively distributed throughout the tissues. There is a slow release from the tissue thus resulting in long term protection. It is metabolised in the liver to be excreted in urine. The half-life of the drug is 50 hours, though 70% comes out as intact drug. It is also used for prophylactic purposes. It is considered safe for pregnant and lactating women and also for children. However, it is a recommendation to discourage pregnant women from non-endemic areas to travel to endemic areas because of the difficulties associated with treatment and the risk to the foetus should they contract malaria.

Chloroquine now no longer offers protection against South East Asian *P. falciparum* and increasingly in other regions because of resistance (Navy Med. Dept, 2001). While effective in suppressing *P. falciparum* in some parts of Africa and most strains of *P. vivax*, resistant forms of *P. vivax* are appearing and have been reported in Papua New Guinea, Indonesia, Thailand and India (WHO, 1998).

Mode of action of chloroquine

Although, the mechanism of action of chloroquine is not known with certainty, many studies are under way to discover the cause of resistance in the parasite. Also, the

biochemical and molecular mechanisms of drug resistance in P. *falciparum* remains unknown (Wellems et. al., 1997; Martiney *et al.*, 1999).

Slater and Cerami (1992) first reported that chloroquine interferes with a haem detoxification enzyme that is essential to the survival of the parasites in red blood cells. The rapid propagation of the parasites in part is met through the digestion of the host cell haemoglobin in the parasites' acid food vacuoles (Wellems, 1992). The digestion of haemoglobin results in the release of large amounts of a toxic haem moiety called ferriprotoporphyrin IX (FPIX), which cannot be degraded by the parasite. As such FPIX is sequestered within the food vacuoles as innocuous dark brown granules called the malaria pigment or haemozoin. Chloroquine and other related quinoline ring groups of antimalarials attack the intra-erythrocytic stages of pigment-producing parasites by inhibiting pigment production. Chloroquine is thought to bind tightly to soluble FPIX to form a complex in the food vacuole (Fitch, 1983). The toxic FPIX-drug complexes thus would poison the acid food vacuole and thereby kill the parasite (Wellems, 1992).

It is thought that chloroquine being a weak base accumulates in the acidic food vacuole of the intra-erythrocytic parasite (Ginsburg et al., 1999). FPIX is toxic to the parasite, it increases membrane permeability, which if not detoxified may end up in lysis of the cell. The polymerisation of the toxic FPIX into harmless haemozoin (HZ) is a mechanism used by the parasite to protect itself from the toxicity. Chloroquine inhibits the polymerisation of the FPIX because of its ability to form a complex which results in an increase of the FPIX concentration in the parasite. Chloroquine also causes fragmentation of the parasite's RNA and intercalates with its DNA (Ward, 1988).

Fansidar

Fansidar, a combination of pyrimethamine and sulphadoxine was pressed into service as the first line of defence against chloroquine-resistant *P. falciparum* malaria in South America and Southeast Asia (Plowe *et al.*, 1997). It is used for uncomplicated malaria that cannot be cured by chloroquine, or as first-line treatment where chloroquine has been dropped from use (Krogstad, 1996). Fansidar is cheap, practically, only one dose is needed and is highly effective in much of Africa and South America. As a combination drug, each tablet contains 500mg of sulphadoxine and 25mg of pyrimethamine. It is given as: Adults: 3 tablets in a single dose; Children: 1 tab in children 4-8 years old, 2 tabs in adolescents 9-14 years old, 3 tabs in those >14 years old.

The effects of Fansidar are confined to late trophozoites. As a result, it is said to have a slow onset of action, and is not generally recommended for severe malaria. The rate of parasite clearance achieved by Fansidar is about the same as that of quinine (Winstanley, 1996). Drug-sensitive *P. falciparum* malaria is generally treated with a single oral dose of Fansidar, which makes the treatment of out-patients very practicable. An intramuscular (IM) formulation is available, and is used for patients with protracted vomiting and its efficacy in severe forms of childhood malaria has been compared favourably with that of quinine (Simao *et al.*, 1991). Oral pyrimethamine and sulphadoxine are extensively and rapidly absorbed at about 12 and 4 hours respectively (Winstanley *et al.*, 1992a). Pyrimethamine, but not Sulphadoxine, is absorbed more slowly after IM injection, possibly because of its poor aqueous solubility (Winstanley, 1996). The elimination half life of Pyrimethamine varies from 40 to 100 hours, and that

of Sulphadoxine is about 200 hours. It is because of this slow elimination rate that Fansidar need only be given once.

In Southeast Asia, where parasites are often resistant to both pyrimethamine and sulphadoxine, Fansidar is clinically useless (Winstanley, 1996). Resistance to pyrimethamine has been reported in Africa (WHO, 1990) and also resistance to Fansidar is now widespread and serious side effects have been reported (WHO, 1998).

Mode of action of Fansidar

Plasmodium parasites synthesise their folic acid, which is needed to make the nucleotide building blocks of DNA *de novo*. Folic acid has three major components namely, glutamic acid, para-aminobenzoic acid and pteridine. Fansidar has two main modes of action. In the presence of para-aminobenzoic acid, folate can be synthesised but sulphadoxine in Fansidar inhibits the incorporation of para-aminobenzoic acid [PABA] into dihydropteroate, a precursor of dihydrofolate, by competitive inhibition of dihydropteroate synthetase (*dhps*).

In the presence of folate, dihydropteroate is reduced to dihydrofolate by an enzyme known as dihydrofolate synthase and then to tetrahydrofolate by dihydrofolate reductase (*dhfr*). Pyrimethamine is a competitive inhibitor of dihydrofolate reductase (*dhfr*) and it binds and inhibits dihydrofolate reductase (*dhfr*). The sulphadoxine and pyrimethamine both act synergistically against the parasite (Chulay *et al.*, 1984).

2.7 Genetic Mutations Associated with Chloroquine and Fansidar Resistance in *Plasmodium falciparum*

2.7.1 Chloroquine resistance

2.7.1.1 Mutations in the *Pfcrt* gene

The effectiveness of chloroquine depends on its concentration in the parasite's digestive vacuole and in resistant parasites the accumulation of chloroquine inside the vacuole is diminished (Marsh, 1998). This is attributed to the mutation in the transporter gene such that chloroquine entry into the digestive vacuole is hindered because of mutant transporter proteins.

Most chloroquine resistance studies to find point mutations in the parasite genome have been conducted *in vitro*. Mutations have been reported to occur in *P. falciparum* digestive-vacuole transmembrane proteins referred to as PfCRT. The gene that encodes for the protein is known as the *pfcrt* gene which is located on chromosome 7 with 13 exons (Plowe and Wellems, 1999; Fidock *et al*., 2000; Djimde *et al.,* 2001).

A single substitution of Threonine for Lysine at position 76 (*pfcrt* 76) was found to be present in chloroquine-resistant isolates and absent in chloroquine-sensitive isolates (Plowe and Wellems, 1999). In addition to *pfcrt* 76, multiple mutations have also been identified in various arrangements at positions 72, 74, 75, 97, 220, 271, 326, 356 and 371 (Plowe and Wellems, 1999). The roles of these other mutations are still being investigated. But studies conducted in Mali by Djimde *et al.* (2001) suggest the mutation in *pfcrt* 76 putatively confers chloroquine resistance in *P. falciparum*.

2.7.1.2 Mutation in the *Pfmdr*1 gene

Foote *et al.* (1990) have suggested that single base mutations in the *P. falciparum* multidrug resistance gene (*pfmdr 1*) on chromosome 5 are associated with enhanced efflux of chloroquine from resistant parasites. The *pfmdr 1* gene encodes for P-glycoproteins in the digestive vacuole referred to as Pgh 1 (Foote *et al.*, 1989). The mutation is as a result of a single base substitution of Asparagine to Tyrosine at position 86 (*pfmdr*1 86). Some field works done have found an association between *pfmdr*1 and chloroquine resistance (Foote *et al.*, 1990) whilst others have not (Basco and Ringwald, 1998; McCutcheon *et al*, 1999).

2.7.2 Fansidar resistance

2.7.2.1 Mutations in the *Dhfr* gene

Point mutations in the *dhfr* gene which have been reported to be associated with Fansidar resistance occur in codons 108, 51, 59 and 164 (Zolg *et al.*, 1989). The mutations are as a result of substitution of single bases in the gene sequence, which results in altering the shape of the proteins active site cavity (Plowe *et al.*, 1997). There are two point mutations at codon 108, one is a Serine to Asparagine change and the other is a Serine to Threonine change. Asparagine to Isoleucine change occurs at codon 51 whilst Cysteine to Arginine change occurs at 59. The Isoleucine to Leucine change at 164 together with Asn-108, Ile-51 and Arg-59 have been reported to confer high-level resistance to the drug (Peterson *et al.*, 1988).

2.7.2.2 Mutations in the *Dhps* gene

The mutations in the *dhps* gene that are associated with Fansidar resistance are found at codons 437 and 540. The substitutions result in Alanine to Glycine change and Lysine to Glutamic acid change respectively (Brooks *et al.*, 1994). These mutations have been associated with decreased susceptibility to sulphadoxine (Plowe *et al.*, 1997). In addition to these two mutations, others that have been reported include codons 436-Serine to Alanine and Serine to Phenylalanine, 581-Alanine to Glycine and 613-Alanine to Threonine/ Serine (Wang *et al*, 1997).

2.8 Methods for Detection of *Plasmodium falciparum*

2.8.1 Microscopy

This method of diagnosing malaria is efficient in confirming an infection with *Plasmodium.* It involves the preparation of blood films for malaria parasites from capillary blood or venous blood. There are two types of blood films, thin and thick, which are both made on glass slides. The thin blood film is used for the identification of malarial parasite species and consists of one layer of evenly distributed blood cells whilst the thick blood film is made by concentrating the blood spot on the glass slide. After staining in Giemsa stain (10%), the parasites appear as pinkish rings among white cells against a background of lightly stained red cell debris under the microscope.

In *P. falciparum* infection, schizonts are rarely seen because erythrocytic schizogony takes place in the internal organs. Therefore only trophozoites (rings) and gametocytes are seen in blood films. Absolute numbers of parasites (number/µl) can be estimated in thick blood films by counting the parasite against white cells and using fixed white blood cell (WBC) count value to calculate the number of parasite per µl of blood by the equation:

Parasite number/ul blood = number of parasites x 8000/number of WBC

2.8.2 *In vitro* test of parasite sensitivity to chloroquine

The culturing of malaria parasites was first developed by Trager and Jensen (1976). This involves the culturing of parasites in various concentrations of the drug. The principle

underlying the culture is maintaining human erythrocytes under conditions that support intracellular development of the parasites.

The *in vitro* drug sensitivity test has been developed to include the measurement of extent of growth of parasites using the radio labelled metabolite. It involves the incorporation of the radioactively labelled precursor ^{3}H-hypoxantin as a marker for parasite growth (Desjardins *et al.*, 1979). The principle involved here is that since the malaria parasite cannot synthesise purines de novo, they rely on the host for their supply of bases using a salvage pathway. The parasites have different degrees of preference for obtaining host-derive purines, the preference being hypoxanthin followed by adenosine and adenine. Hence on the addition of ^{3}H-hypoxanthin to a culture media, the parasite rapidly picks up the radioactively labelled hypoxanthin. The use of ^{3}H-hypoxanthin as an index of parasite growth is usually preferred over microscopic methods because it yields results more rapidly and more reliably. However it must be stressed that the microscopic method is still important in studies in which the effect of drugs on the various group stages is being conducted. Its disadvantages however are that it is time consuming, strenuous and has a higher degree of error.

The major problem in assessing drug sensitivity of parasites in *in vitro* culture is the presence of different stages of development of the parasite. With the use of radio labelled hypoxanthin (^{3}H-hypoxantin), the problem seems a bit solved. This nucleic acid precursor appears to be efficient because it is preferentially incorporated into the more matured parasites of *P. falciparum*.

A culture medium consists of RPMI-1640 (composition shown in appendix II) developed by Moore *et al.* (1967), supplemented with Herpes buffer and normal human serum or complex mixtures of protein, lipid and other growth factors. It also contains erythrocyte suspension giving a typical 5% hematocrit and 1% or less initial malaria parasitaemia (Trager and Jensen, 1978). The dish or flask containing the culture is incubated at 37-38^{O}C in an atmosphere of 3-5% CO_2 and 17% or less of O_2 under aseptic conditions. In the simplest set up, this mixture of gases is obtained using a "candle jar". The candle jar is basically a dessicator with a stop cork in the lid with a plain white candle inside. After the dish or flask has been set in, the candle is lit and the cover put on with the stop-cork opened. The moment the candle goes out due to the accumulation of CO_2, the stop cork is closed and the dessicator is set in an incubator at 37^{O}C. At least once a day the dish is removed from the dessicator and provided with fresh medium by following the appropriate procedure. This simple candle jar method is ideal for many kinds of work and lends itself to screening for new antimalarial agents (Trager, 1978), and for drug resistance experiments (Nguyen-Dinh and Trager, 1978).

2.8.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is an *in vitro* method of nucleic acid synthesis by which a target DNA fragment is exponentially replicated (Erlich, 1989). It uses a thermo stable DNA polymerase isolated from *Thermus aquaticus* and two oligonucleotide primers that flank the target DNA to be amplified. The reaction involves repeated cycles of heat denaturing of DNA, annealing of the primers to their complementary sequences at a lower temperature, and extension of the annealed primers with the DNA polymerase. The primers hybridise to the opposite strands of target DNA and are

oriented (3' ends pointing towards each other) so that DNA synthesis by the polymerase enzyme proceeds across the region between the primers. The extension products are complimentary to and capable of binding primers, therefore successive cycles of amplification result in the doubling of the target DNA synthesised in the previous cycle.

The primers are usually designed such that the annealing temperatures in the PCR are as high as possible to ensure specificity during amplification. Under standard conditions the annealing temperature in a reaction should be $5^{O}C$ lower than the melting temperatures (T_m) of the primers, which can be determined using the following formula (after Thein and Wallace, 1986):

$$T_m = 4(G + C) + 2(A + T)$$

Where G, C, A and T are guanine, cytosine, adenine and thymine respectively.

Ideally, primers with sequences containing runs of nucleotides that might promote annealing with strands outside the target sequences and also with significant secondary structures are avoided.

In designing primers to detect point mutations with PCR, the mutant base is located at most, three bases from the 3' end of the primer. At the 3' end of the primer, annealing is more specific than the 5' end and also initiation of extension occurs at the 3' end. Therefore complementarity of bases is preserved with no altering of bases with the use of a polymerase with high proofreading ability.

2.8.3.1 Standard PCR amplification protocol

The standard PCR amplification protocol (Innis and Gelfand, 1986) will amplify most target DNA, however, optimal performance is sought by varying most parameters and conditions for each new application.

The standard PCR reaction mix comprises of the following:

- 1 x PCR reaction buffer
- 200µM each of dATP, dCTP, dGTP, dTTP (deoxyribonucleotides)
- 2.5units/ 100µl *Taq* DNA polymerase
- 0.5µM each of forward and reverse primers (synthetic oligonucleotides)
- 1ng- 1µl of template DNA

The standard PCR thermal cycling conditions as follows:

Denaturation	94^{O}C for 30 seconds
Primer annealing	(Tm - 5^{O}C) for 30 seconds
Primer extension	72^{O}C for 2 minutes

The reaction is usually concluded with a final extension step of 72^{O}C for 5 minutes to enable completion of all strands.

2.8.3.2 Nested PCR amplification protocol

This is a form of PCR amplification protocol that involves two rounds of amplification with the product or amplicon of the first amplification (described as 'outer PCR') used as template for the second amplification (described as 'inner PCR'). This nested approach has been shown to have an increased sensitivity of between 10-100 fold for detecting blood parasites (Snounou *et al.*, 1993). Several studies have demonstrated that this approach reveals genetic polymorphisms (Cortese and Plowe, 1999; Djimde *et al.*, 2001).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Area

The studies were carried out at Hohoe and Navrongo (Fig. 3.1). These were selected on the basis that they are located in two different ecological zones and also had the facilities to handle a minimum daily attendance of about 200 patients at the out patient department (OPD). The facilities were also to have reliable supply of water, electricity and adequate laboratory space for the study.

3.1.1 Hohoe District

The Hohoe district lies in the middle belt of the country with semi deciduous forest vegetation. The population of this district is estimated to be 110,000 (National Census, 1984). The district is divided into six sub-districts for health administration purposes. There is one government hospital in the district capital, one health centre, 17 health posts and a Roman Catholic Church clinic. Disease transmission is perennial with peaks occurring after the major rains in June to October. The major vector for disease transmission is *An. gambiae s. l.* but *An. funestus* predominates in the dry season. More than 95% of all infections are caused by *P. falciparum* whilst mixed infections with *P. malariae* are also seen (Afari *et al.*, 1992).

3.1.2. Navrongo District

The Kassena Nankana district lies in the northern part of the Guinea savannah belt and Navrongo is the district capital. The population is estimated to be about 140,000 living

in dispersed compounds (Binka *et al.*, 1994). The Navrongo War Memorial Hospital serves the whole district. Malaria transmission is intense and highly seasonal with *P. falciparum* being the predominant parasite. The main transmission vectors are *An. gambiae s.s.* and *An. funestus.*

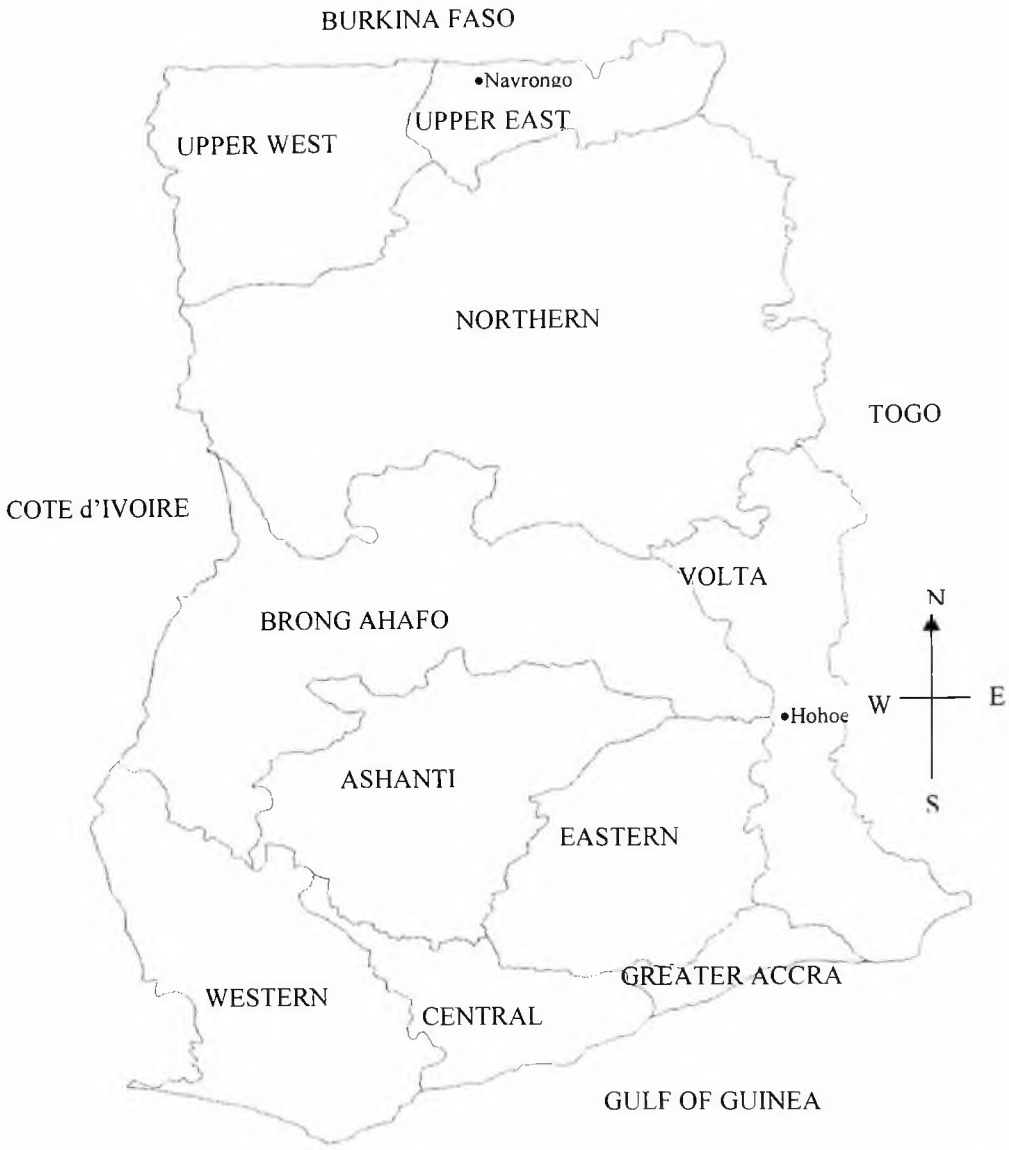

Figure 3.1 Map showing the location of the study sites Hohoe and Navrongo in Ghana.

3.2 Field Sample Collection

Children aged 5 years and below reporting with symptoms of malaria or history of fever (with temperatures of ≥37.5^{O}C in the past 48-72hours) and parasite count between 2000/μl and 100,000/μl were recruited for the study. The consents of parents or guardians of the children were sought before recruiting them into the study. Children who were unable to drink or breast-feed, vomiting intermittently, convulsing and unconscious were excluded from the study. The recruitment was carried out over a period of one year at both sites.

Blood films were prepared before treatment, stained with Giemsa and parasites counted. Venous blood was collected into heparinised tubes for *in-vitro* drug sensitivity testing. Blood blots were made on 110mm Whatman filter paper, dried and stored individually in plastic bags at room temperature for PCR analysis. The children were then treated with chloroquine at a standard dose of 25 mg/kg over 3 days (Appendix III). They were followed up daily to day 3 then on days 7 and 14. Blood film for parasite count and filter paper blood blot for PCR were collected on each follow-up day. Children with parasitaemia above 25% of pre-treatment level on day 3 were treated with Fansidar at 25mg/kg in one dose and were asked to report on day 7. Filter paper blood blots were kept at room temperature until ready for use.

Non-respondents were classified as RI, RII, RIII according to the classic parasitological definition by WHO (1996). According to the criteria, patients were classified as RI if there was initial clearance of parasites but parasitaemia recurred by day 14. Those who

had persistent parasitaemia with reduction to less than 25% of the initial level by day 3 were classified as RII whilst those who had persistent parasitaemia with no reduction in the level of parasitaemia or with a reduction to 25% or more of the initial level by day 3 were classified as RIII. A patient was however considered to be chloroquine sensitive if there was clearance of parasites initially and no parasitaemia was observed by day 14.

3.3 Chemicals and Reagents

The chemicals and reagents used for the present study are listed in Appendix I. The various buffers and solutions were prepared as described in Appendix II.

3.4 Laboratory Studies

3.4.1 Preparation and examination of blood slides

A trained technician did microscopic preparation and the examination of the blood slides using standard examination protocol.

3.4.2 *In vitro* susceptibility test of *P. falciparum* to Chloroquine

This test was performed following the modification done by Rieckmann (1978).

About 5ml of patient blood was collected into heparinised tubes and washed 4 times with neutral RPMI 1640 to remove WBCs leaving behind RBCs, some of which will be infected with the trophozoites stage of the parasite. Then 5µl of the washed parasitized RBC were dispensed into the wells of the microtitre plate containing 45µl of sterile parasite growth medium (Appendix II). The plate was tapped gently to ensure that the contents were mixed well. Then chloroquine at different concentrations of 1, 2, 4, 8, 16, 32 and 64 pmol were added. Some wells with no chloroquine served as controls. The microtitre plate was then covered with its lid and placed in a candle jar which has already been warmed in an oven. A candle was lit and placed in the jar with the exhaust cork opened. Thereafter, the jar was tightly closed when the candlelight was almost extinguished. The jar and contents were then placed in an incubator at 37.5^{O}C for 24 hours. After that, the jar was removed and opened in a sterile hood,the microtitre plate was removed and 10µl of radioactively labelled hypoxanthin (H^3) was added to each well and mixed gently. The plate was placed back in the candle jar and was put into the incubator for another 24 hours.

The cells were harvested after incubation using the Filtermate 196 harvester (Canbetta Comp). The levels of radioactivity were determined using a Matrix Direct Beta Counter 96 (Canbetta Comp).

The percentage inhibition of parasite growth was then calculated as follows:

$$\text{Inhibition} = \frac{CPM_O - CPM_C}{CPM_O} \times 100\%$$

where

CPM = counts per minute

CPM_O =CPM in wells without chloroquine

CPM_C = CPM in wells with concentration of chloroquine

The inhibition concentration (IC_{50}), which is the effective concentration at which 50% of the parasites are inhibited, was estimated from a plot of inhibition levels against chloroquine concentration.

3.4.3 The identification of *Plasmodium* species

3.4.3.1 Isolation of *Plasmodium* DNA from filter paper blood blot

Two methods for isolating of DNA from filter paper blood blots were used. The first which is the Chelex method (Wooden *et al.*, 1993) was used initially, but was later abandoned for the methanol fixation method (Cortese and Plowe, 1999) because the latter was relatively faster.

a) Chelex extraction method

About 3mm filter paper blood blots were individually cut out and transferred into a 1.5ml microfuge tube. Then 1ml of PBS (pH 7.4) and 50µl of 10% saponin were added, inverted three times and stored at $4^{O}C$ overnight. Each tube was centrifuged at 14,000rpm for 5secs and the reddish PBS/saponin supernatant discarded. Another 1ml of PBS was then added to the filter paper in the tube, inverted several times and incubated at $4^{O}C$ for 15mins after which the tube was centrifuged under the same conditions as above and the supernatant also discarded. Then 100µl of sterile water and 50µl of 20% Chelex were then added to the tube and vortexed. Parasite DNA was extracted by incubating the tube and its content at $95^{O}C$ for 10mins, vortexing at 2 minutes intervals. After incubation, the tube was centrifuged at 14,000rpm for 5mins and as much solution as possible was transferred into a 0.5ml tube, centrifuged again and the supernatant transferred into a fresh tube making sure the Chelex was not carried over. The content of the tube was stored at $-20^{O}C$ until ready for use.

b) Methanol fixation method

An approximately 3mm-square piece of blood-impregnated filter paper was cut out using a sterile razor (wiping off the razor with tissue paper soaked in 75% ethanol between cuttings). Then each piece of the filter paper blood blot was transferred into a 0.5ml PCR microfuge tube and 50µl of methanol was added making sure the paper was totally submerged. The tube was incubated for 15mins at room temp. After incubation, the methanol was pipetted out with care taken to retain the paper in the tube. The tube was left opened on the bench on its side to allow remaining methanol to evaporate. This usually took 15mins for the paper blot to be fully dried. 50µl of water was then added and the tube heated for 15mins at 95^{O}C with occasional vortexing at 2 minutes intervals during the incubation to improve DNA yield. It was then stored at –20^{O}C until ready for use.

3.4.3.2 Nested PCR method for *Plasmodium* species identification

The nested PCR method for the identification of human *Plasmodium* (Snounou *et al.*, 1993) was used. The initial amplification reaction involved the use of genus-specific oligonucleotide primer pair, rPLU5 and rPLU6 to amplify DNA targets from the four malaria species. The PCR product obtained was used as the template for the nested PCR using species-specific oligonucleotide primer pairs for *Plasmodium* species. The primer pairs were rFAL1 and rFAL2 for *P. falciparum*, rMAL1 and rMAL2 for *P. malariae*, rOVA1 and rOVA2 for *P. ovale* and rVIV1 and rVIV2 for *P. vivax* (Table 1).

All the reactions were carried out in a final volume of 20µl which contained 1xPCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 2mM $MgCl_2$, 200µM dNTP mix, 125nM of each primer, 0.5U *Taq* polymerase and 3µl of DNA template for primary amplification. This was thoroughly mixed and overlaid with 20µl of mineral oil to avoid evaporation and refluxing of the reaction mixture. For the nested reaction, the mix remained the same except that 2µl of the PCR product was used as DNA template.

For both the primary and secondary amplifications, PCR was carried out using a PTC-100 thermal cycler (MJ Research, USA) with cycling parameters of an initial melt at $94^{O}C$ for 2mins followed by 30cycles of $94^{O}C$ for 30secs (denaturation), $58^{O}C$ for 30secs (annealing), $72^{O}C$ for 2mins (extension) and a final cycle of $72^{O}C$ for 5mins.

3.4.3.3 Analysis of PCR products

Following the PCR, 10µl of the PCR products were electrophoresed on a 2% agarose gel stained with 5µg/ml ethidium bromide. The electrophoresis was run in 1x TAE buffer at 80 volts for 1 hour. The gel was visualised under a Dual Intensity UV transilluminator (UVP) after which a photograph of the gel was taken using a Polaroid camera with Polaroid type 667 films (Sigma). The sizes of the PCR products were estimated by comparison with the mobility of a standard of known band sizes. The diagnostic sizes expected of the PCR amplified fragments are 205bp for *P. falciparum*, 144bp for *P. malariae*, 800bp for *P. ovale* and 120bp for *P. vivax*.

Table 3.1

DNA sequences of the synthetic oligonucleotide primers for PCR identification of *Plasmodium* species

Name of Primer*	DNA Sequence (5' – 3')	Melting temp. ($Tm^{O}C$)
rPLU5 (f)	CCT GTT GTT GCC TTA AAC TTC	58
rPLU6 (r)	TTA AAA TTG TTG CAG TTA AAA CG	58
rFAL1 (f)	TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT	76
rFAL2 (r)	ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC	86
rMAL1 (f)	ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC	82
rMAL2 (r)	AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA	72
rOVA1 (f)	ATC TCT TTT GCT ATT TTT TAG TAT TGG AGA	76
rOVA2 (r)	GGA AAA GGA CAC ATT AAT TGT ATC CTA GTG	74
rVIV1 (f)	CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC	82
rVIV2 (r)	ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA	86

*Where f is forward and r is reverse

3.4.4 Detection of *Plasmodium falciparum* genetic mutations associated with antimalarial drugs resistance.

3.4.4.1 Chloroquine resistance associated genetic mutations

The same methods as described in 3.4.3.1.1 and 3.4.3.1.2 above were used to isolate *P. falciparum* DNA from filter paper blood blot.

a) *Pfcrt* 76

The approach used by Cortese and Plowe (1999) for amplification of the DNA sequences of interest was used. With this approach, an initial PCR to amplify the region with the mutation, then two more PCRs, one to detect either mutant or wildtype and the second for the restriction analysis with *ApoI* to confirm the presence or absence of mutation. For the details of primers sequences see Table 3.2.

The initial amplification reaction involved the use of the oligonucleotide primer pair, CRTP1 and CRTP2 to amplify a 537bp region around the mutation K76T. The reaction volume of 25µl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 2.5mM $MgCl_2$, 200µM of dNTP mix, 0.1µM of each primer, 0.625U of *Taq* polymerase and 5µl of DNA template. It was overlaid with 20µl of mineral oil. PCR assay were carried out using a Hybaid thermal cycler (Hybaid, UK) with cycling parameters of an initial melt at $94^{O}C$ for 3mins followed by 30 cycles of $94^{O}C$ for 30secs (denaturation), $56^{O}C$ for 30secs (annealing), $72^{O}C$ for 1min (extension) followed by a final extension of $72^{O}C$ for 3mins.

The oligonucleotide primers pairs CRTP3 and CRTP4m were used to amplify mutant alleles and CRTP3 and CRTP4w for wildtype alleles, both with an expected size of 366bp. The reaction volume of 25µl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 1.5mM $MgCl_2$, 200µM of dNTP mix, 0.1µM of each primer, 0.625U of *Taq* polymerase and 0.5µl of amplicon DNA. It was overlaid with 20µl of mineral oil. The cycling conditions were an initial melt at 94^{O}C for 3mins followed by 25 cycles of 94^{O}C for 30secs (denaturation), 50^{O}C for 30secs (annealing), 64^{O}C for 1min (extension) and a final extension of 64 ^{O}C for 3mins.

The confirmatory PCR used primers CRTD1 and CRTD2 to obtain a 134bp fragment for restriction digestion analysis. The reaction volume of 25µl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 2.5mM $MgCl_2$, 200µM of dNTP mix, 0.1µM of each primer, 0.625U of *Taq* polymerase and 0.5µl of amplicon DNA. The cycling conditions were an initial melt at 95^{O}C for 5 mins followed by 25 cycles of 92^{O}C for 30secs, 48^{O}C for 30secs, 65^{O}C for 30secs and a final extension of 65 ^{O}C for 3mins.

The restriction enzyme *ApoI* was used and the digestion was performed in a 20µl volume, which contained 5µl of the PCR product, 2.5µl of 4U/µl enzyme (0.5U) and 2.5µl of buffer. The mix was incubated at 50^{O}C for 6 hours. Then the restriction products were run on 2% ethidium bromide agarose gel. The restriction site and the expected product sizes after digestion are shown in Table 3.3.

b) *Pfmdr1* 86

The primary amplification reaction involved the use of oligonucleotide primer pair, MDR-1 and MDR-2 to amplify a 603bp fragment which had the mutation at codon 86. The reaction volume of 25µl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 2.5mM $MgCl_2$, 200µM of dNTP mix, 1.0µM of each primer, 0.625U of *Taq* polymerase and 5µl of DNA template. The cycling parameters were an initial melt at 95^{O}C for 3mins followed by 30cycles of 92^{O}C for 30secs (denaturation), 48^{O}C for 45secs (annealing), 65^{O}C for 1min (extension) and a final extension of 65^{O}C for 5mins.

The secondary amplification reaction involved primer pair MDR-3 and MDR-4, which amplified a 521bp, fragment if the mutation was present. A reaction volume of 25µl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 2.5mM $MgCl_2$, 200µM of dNTP mix, 1.0µM of each primer, 0.625U of *Taq* polymerase and 0.5µl of amplicon DNA. The cycling parameters were an initial melt at 95^{O}C for 3mins followed by 15 cycles of 92^{O}C for 30secs (denaturation), 48^{O}C for 30secs (annealing), 65^{O}C for 45secs (extension) and a final extension of 65^{O}C for 5mins.

Table 3.2

DNA sequences of synthetic oligonucleotide primers for the detection of mutations associated with chloroquine resistance.

Gene loci	Primer	DNA Sequence (5' – 3')	Melting temp. ($Tm^{o}C$)
pfcrt 76	CRTP1 (f)	CCG TTA ATA ATA AAT ACA CGC AG	62
	CRTP2 (r)	CGG ATG TTA CAA AAC TAT AGT TAC C	68
	CRTP3 (f)	TGA CGA GCG TTA TAG AG	50
	CRTP4m (r)	GTT CTT TTA GCA AAA ATT G	48
	CRTP4w (r)	GTT CTT TTA GCA AAA ATC T	48
	CRTD1 (f)	TGT GCT CAT GTG TTT AAA CTT	56
	CRTD2 (r)	CAA AAC TAT AGT TAC CAA TTT TG	58
*pfmdr*1 86	MDR-1 (f)	ATG GGG TAA AGA GCA GAA AGA	60
	MDR-2 (r)	AAC GCA AGT AAT ACA TAA AGT CA	60
	MDR-3 (f)	TGG TAA CCT CAG TAT CAA AGA A	60
	MDR-4 (r)	ATA AAC CTA AAA AGG AAC TGG	56

Where f is forward and r is reverse

Table 3.3

Recognition sites of restriction enzyme and product sizes for *pfcrt* 76

Restriction enzyme	Recognition site	Product sizes (bp)		Allele cut
		Before digestion	After digestion	
ApoI	5' Pu↓AATT Py	134	100 + 34	wildtype
	3' Py TTAA↑Pu			

3.4.4.2 Fansidar resistance associated genetic mutations

a) *Dhfr* 51, 59 and 108

Primary amplification reaction involved the use of oligonucleotide primer pair, AMP1 and AMP2 to amplify a 720bp of the *dhfr* coding region. The reaction volume of 25µl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 3.5mM $MgCl_2$, 200µM of dNTP mix, 1.0µM of each primer, 0.625U of *Taq* polymerase and 5µl of DNA template. The cycling parameters were an initial melt at 95^{O}C for 3mins followed by 30cycles of 92^{O}C for 30secs, 45^{O}C for 45secs, 72^{O}C for 45secs and a final extension of 72^{O}C for 3mins.

The PCR product obtained was used as the DNA template in two separate reactions using primer pair MUM-D and FR-51MB1 for mutant allele and MUM-D and FR-51WB1 for wildtype allele. Both have an expected size of 238bp. Each reaction volume of 25µl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 2.5mM $MgCl_2$, 200µM of dNTP mix, 0.5µM of each primer, 0.625U of *Taq* polymerase and 0.5µl of amplicon DNA. The cycling parameters were an initial melt at 95^{O}C for 3mins followed by 15 cycles of 92^{O}C for 30secs, 54^{O}C for 30secs, 72^{O}C for 1min and a final extension of 72^{O}C for 3min.

The second nested PCR involved using primer pairs SP1 and FR59m for the codon 59 mutant allele and SP1 and FR59w for wildtype allele in two separate reactions. Both have an expected size of 190bp. The reaction volume of 25µl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 1.5mM $MgCl_2$, 200µM of

dNTP mix, 0.5μM of each primer, 0.625U of *Taq* polymerase and 0.5μl of amplicon DNA. The cycling parameters were an initial melt at 95^{O}C for 3mins followed by 15 cycles of 92^{O}C for 30secs, 54^{O}C for 30secs, 72^{O}C for 30secs and a final extension of 72^{O}C for 3min.

The third secondary amplification reaction involved primer pairs SPI and DIA-12 and SPI and DIA-9 for the two codon 108 mutant alleles and SPI and DIA-3 for wildtype allele in three separate reactions. All three have an expected size of 337bp. Each reaction volume of 25μl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 1.5mM $MgCl_2$, 200μM of dNTP mix, 0.5μM of each primer, 0.625U of *Taq* polymerase and 0.5μl of amplicon DNA. The cycling parameters were an initial melt at 95^{O}C for 3mins followed by 15 cycles of 92^{O}C for 30secs, 55^{O}C for 45secs, 72^{O}C for 45secs and a final extension of 72^{O}C for 3min.

b) *Dhps* 437 and 540

The primary amplification reaction involved the use of oligonucleotide primer pair, DHPS-1 and DHPS-2 to amplify a 1328bp of the *dhps* coding region. The reaction volume of 25μl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 3.5mM $MgCl_2$, 200μM of dNTP mix, 1.0μM of each primer, 0.625U of *Taq* polymerase and 5μl of DNA template. The cycling parameters were an initial melt at 95^{O}C for 3mins followed by 30cycles of 92^{O}C for 30secs, 45^{O}C for 45secs, 65^{O}C for 1min and a final extension of 65^{O}C for 3mins.

The first nested PCR involved primer pairs 185S and 437M-A for the codon 437 mutant allele and 185S and 437W-2C for wildtype in two separate reactions. Both have an expected size of 333bp. Each reaction volume of 25µl contained 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 2.5mM $MgCl_2$, 200µM of dNTP mix, 0.5µM of each primer, 0.625U of *Taq* polymerase and 0.5µl of amplicon DNA. The cycling parameters were an initial melt at 95^{O}C for 3mins followed by 30 cycles of 92^{O}C for 30secs, 48^{O}C for 45secs, 65^{O}C for 1min and a final extension of 65^{O}C for 3min.

The second nested PCR involved primer pairs 185S and 540M for the codon 540 mutant allele and 540W and 218A for the wildtype in two separate reactions. Expected sizes for the wildtype and the mutant are 636bp and 561bp respectively. Each reaction volume of 25µl contains 1x PCR buffer (50mM KCl, 20mM Tris-HCl, pH 8.3, 0.1mg/ml of gelatin), 2.5mM $MgCl_2$, 200µM of dNTP mix, 0.5µM of each primer, 0.625U of *Taq* polymerase and 0.5µl of amplicon DNA. The cycling parameters were an initial melt at 95^{O}C for 3mins followed by 30 cycles of 92^{O}C for 30secs, 52^{O}C for 45secs, 72^{O}C for 1min and a final extension of 72^{O}C for 3min.

Table 3.4

DNA sequences of the synthetic oligonucleotide primers for the detection of mutations associated with Fansidar resistance.

Gene locus	Primer	DNA Sequence (5' – 3')	Melting temp. (Tm °C)
dhfr	AMP1 (f)	TTT ATA TTT TCT CCT TTT TA	40
	AMP2 (r)	CAT TTT ATT ATT CGT TTT CT	48
51	MUM-D (f)	TTT ATC CTA TTG CTT AAA GGT TTA	60
	FR-51MB1 (r)	GGA GTA TTA CCA TGG AAA TGT CT	64
	FR-51WB1 (r)	GGA GTA TTA CCA TGG AAA TGT CA	64
59	SPI (f)	ATG ATG GAA CAA GTC TGC GAC	62
	FR59M (r)	ATG TTG TAA CTG CAC G	46
	FR59W (r)	ATG TTG TAA CTG CAC A	44
108	SPI (f)	ATG ATG GAA CAA GTC TGC GAC	62
	DIA-12 (r)	GAA TGC TTT CCC AGT	44
	DIA-9 (r)	GAA TGC TTT CCC AGG	46
	DIA-3 (r)	GAA TGC TTT CCC AGC	46
dhps	DHPS-1 (f)	TTT TAG AGA TCC ACA AGA	48
	DHPS-2 (r)	TTA AAA CAT CCA AAA CC	44
437	185S (f)	TGA TAC CCG AAT ATA AGC ATA ATG	64
	437M-A (r)	TTT GGA TTA TGG TAT AAC AAA AGT CC	68
	437W-2C (r)	TTT GGA TTA TGG TAT AAC AAA AGT CG	68
540	185S (f)	TGA TAC CCG AAT ATA AGC ATA ATG	64
	540M (r)	CTA GAT TAT CAT AAT TTG TTA GTA C	62
	540W (f)	GGA AAT CCA CAT ACA ATG GAA A	60
	218A (r)	ATA ATA GCT GTA GGA AGC AAT TG	62

CHAPTER 4

RESULTS

4.1 Study Population

A total of 199 patients comprising 103 from Hohoe and 96 from Navrongo were selected for this study. The details of the demographic information, parasitaemia levels during pre and post treatment, and PCR data for each patient are given in Appendix V.

The 103 patients studied at Hohoe comprised of 48 (46.6%) males and 55 (53.3%) females. The mean age of the study population was 27.5 months with a standard deviation of 14.8 (range 6-59 months) and figure 4.1 shows the age distribution. Thirty-nine patients (37.8%) responded adequately to treatment with chloroquine whilst the non-respondents were 64 (62.1%). On the basis of parasitologic clearance, out of the 64 non-respondents 35 (55%) were class I (RI) resistant, 21 (33%) were RII and the rest 8 (13%) were RIII (Table 4.1).

Of the 96 patients studied at Navrongo, 52 (54%) were males and 44 (46%) were females. The mean age of the study population was 28.7 months with a standard deviation of 15.0 (range: 6-59 months) and figure 4.2 shows the age distribution. Sixty-nine (69%) patients responded adequately to chloroquine whilst treatment failed in 30 (31%) of them. Of these 30 non-respondents, 13 (43%) were RI, 10 (33%) were RII and 7 (23%) were RIII (Table 4.1).

4.2 Parasitaemic Profile of the Study Population

The pre-treatment parasitaemic profile of respondents and non-respondents at the two sites are shown in Table 4.2. The geometric mean parasitaemia of the Hohoe patients was 15,348parasites/μl of blood. The majority (56%) of the patients with adequate treatment response had pre-treatment parasitaemia below 10,000 parasites/μl whilst majority (64%) of the non-respondents had levels higher than 10,000 parasites/μl. There was no significant difference in the mean parasitaemia in the respondents and non-respondents groups ($p = 0.44$).

For Navrongo, the geometric mean parasitaemia was 22,089 parasites/μl with the majority (70%) of respondents having levels above 10,000 parasites/μl. Eighty percent of the non-respondents had pre-treatment parasitaemia above 10,000 parasites/μl. There was no significant difference between the mean parasitaemia in the respondents and non-respondents groups ($p = 0.52$).

Table 4.1

Distribution of sexes and treatment outcome of the study sites

	Hohoe	Navrongo
No. of males	48	52
No. of females	55	44
No. of patients sensitive to chloroquine	39	66
No. of patients with the classes of resistance		
RI	35	13
RII	21	10
RIII	8	7

Table 4.2

Parasitaemic profiles of patients before treatment with chloroquine

Parasitaemia (parasite/µl)	Number of patients			
	Hohoe (n = 103)		Navrongo (n = 96)	
	Respondents	Non-respondents	Respondents	Non-respondents
<2000	0 (0)	1 (0.97)	2 (2.1)	0 (0)
2001-5000	16 (15.5)	17 (16.5)	10 (10.4)	3 (3.1)
5001-10,000	6 (5.8)	6 (5.8)	8 (8.3)	3 (3.1)
10,001-20,000	7 (6.7)	7 (6.7)	10 (10.4)	7 (7.3)
20,001-50,000	4 (3.8)	11 (10.4)	19 (20.0)	7 (7.3)
50,001-100,000	4 (3.8)	11 (10.4)	13 13.5)	6 (6.3)
≥100,001	2 (1.9)	12 (11.6)	4 (4.2)	4 (4.2)

Percentages are in brackets.

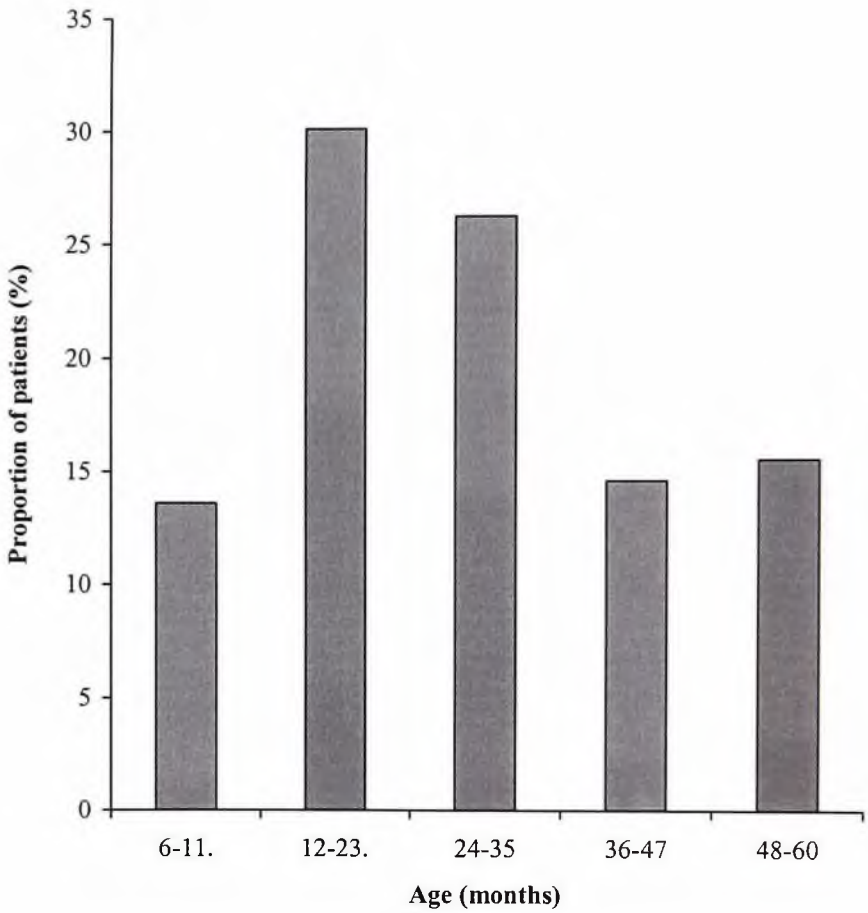

Figure 4.1 Age distribution of patients recruited for the study at Hohoe

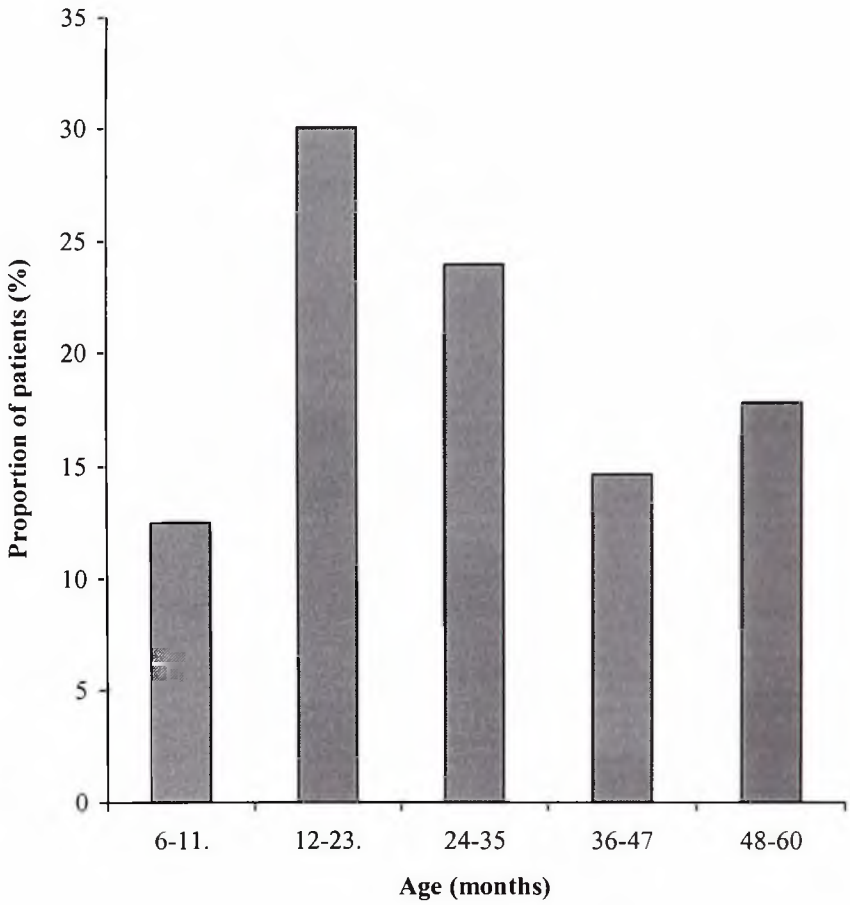

Figure 4.2 Age distribution of patients recruited for the study at Navrongo

4.3 *In vitro* Chloroquine Sensitivity Studies

A total of 26 isolates were successfully tested at the two sites (Appendix V). Eight of these were from Hohoe and 18 from Navrongo. Mean percentage inhibition of growth of *P. falciparum* isolates at chloroquine concentrations of 1, 2, 4, 8, 16, 32 and 64 pmol respectively for the two sites are shown in Table 4.3. The IC_{50} value determined was $1.5x10^{-6}$ mol/litre for the 26 isolates.

Out of the 8 isolates from Hohoe, 7 (86%) were sensitive to chloroquine and one showed resistance even at concentrations above 8.0 pmol. Overall, the mean percentage inhibition at 8pmol was 89.1%, which indicate a high level of sensitivity of *P. falciparum* to chloroquine in the area.

For Navrongo, 12 (67%) of the 18 isolates tested were sensitive to chloroquine whilst 6 (33%) were observed to be resistant. The mean inhibition at 8pmol was 67.8%.

Table 4.3.

Chloroquine inhibition levels of Plasmodium falciparum isolates from Hohoe and Navrongo.

Site	No. of patients	Mean percentage inhibition at concentrations of chloroquine (pmol)						
		1	2	4	8	16	32	64
Hohoe	8	15	29.6	36.9	89.1	100	100	100
Navrongo	18	7.6	12.6	24.6	67.8	72.9	87.4	100

4.4 PCR Detection of *Plasmodium* Species

The results obtained demonstrated that PCR quality grade DNA was obtained using the two rapid DNA extraction methods. In all, parasite DNA was extracted from 385 filter paper blood blot samples. The nested PCR amplifications for detection and identification were all successful. The diagnostic sizes of the PCR products were 205bp for *P. falciparum* and 144bp for *P. malariae*.

At Hohoe, *P. falciparum* was identified as single infections in 98 (95.2%) of the patients and as mixed infection with *P. malariae* in 5 (4.8%). Neither of the other two human parasites, *P. ovale* and *P. vivax* was found.

At Navrongo, *P. falciparum* was identified as single infections in 86 (89.6%) of the patients and as mixed infection with *P. malariae* in 10 (10.4%). No *P. ovale* and *P. vivax* were found.

4.5 Distribution of *P. falciparum* Genotypes Associated with Chloroquine Resistance

The PCR based assay of *P. falciparum* genotypes, *pfcrt* and *pfmdr1* were all successful. A total of 2865 individual PCR assays were performed.

4.5.1 *Pfcrt* 76

At Hohoe, the baseline prevalence (Day 0) of the *pfcrt* T76 within the patients was 82.5% (85/103). The details of how the mutation was distributed within respondents and non-respondents are shown in Table 4.4. Of the 39 respondents, 5 had the mutant allele only, 12 had the wildtype only and 22 had both alleles, whilst for the 64 non-respondents, 31 had the mutant only, 6 had the wildtype only and 27 had both alleles. The distribution of the mutation in the Day 0 specimens of the different non-respondent categories RI, RII and RIII were 86% (30/35), 95% (20/21) and 100% (8/8) respectively. The prevalence of the mutation in the 46 non-respondents whose parasitaemia had not cleared from Day 7 post-treatment was 100% (46/46); 43 had the mutant only and 3 had mixed alleles. The allelic frequencies of *pfcrt* 76 at Day 0, 7 and 14 samples of the non-respondents are shown in Figure 4.3. The sensitivity and specificity of the detection of resistance were 71% and 84% respectively. The presence of *pfcrt* T76 was found to be strongly associated with the development of chloroquine resistance (odds ratio = 12.40, $p = 0.0001$).

At Navrongo, the baseline prevalence of the *pfcrt* T76 mutation within the patients was 43.8% (46/96). The distribution of the mutation within respondents and non-respondents are shown in Table 4.4. Of the 66 respondents, 24 had the mutant only, 37 had the

wildtype only and 4 had both alleles, whilst for the 30 non-respondents 12 had the mutant only, 16 had the wildtype only and 2 had both alleles. The distribution of the mutation among the different categories of the non-respondents RI, RII and RIII at Day 0 were 69% (9/13), 33% (3/10) and 29% (2/7) respectively. The prevalence of the mutation in the 28 non-respondents whose parasitaemia had not cleared from Day 7 post-treatment was 75% (21/28); 20 had the mutant only and one had mixed alleles. None of the alleles was detected in the remaining 7 (i.e. PCR negative). The allelic frequencies of *pfcrt* 76 in Day 0, 7 and 14 specimens of the non-respondents are shown in Figure 4.4. The sensitivity and specificity of detection of resistance was 61% and 57% respectively. There was no association between the presence of the mutation and treatment outcome (odds ratio = 1.16, p = 0.75).

4.5.2 *Pfmdr*1 86

At Hohoe, the baseline prevalence of the *pfmdr1* Y86 among patients was 82.5% (84/103). Details on the distribution of the mutation within respondents and non-respondents are shown in Table 4.5. Of the 39 respondents, 30 had the mutant and 9 had the wildtype, whilst for the 64 non-respondents, 57 had the mutant allele and 7 had the wildtype allele. The distribution of the mutation among the different categories of the non-respondents RI, RII and RIII at Day 0 were 83% (29/35), 95% (20/21) and 100 (8/8) respectively. The prevalence of the mutation in the 46 non-respondents whose parasitaemia had not cleared from Day 7 post-treatment was 100% (46/46). The allelic frequencies of *pfmdr*1 86 in Days 0, 7 and 14 samples of the non-respondents are shown in Figure 4.5. The sensitivity and specificity of detection of resistance was 89.1% and

33.0% respectively. The association between the mutation and treatment outcome was significant (odds ratio = 3.62, p = 0.01).

At Navrongo, the baseline prevalence of *pfmdr1* Y86 was 61.5% (59/96). Of the 66 respondents, 38 had the mutant only and 28 had the wildtype allele whilst for the 30 non-respondents, 21 had the mutant and 9 had the wildtype. The distribution of the mutation among the three non-respondent categories, RI, RII and RIII at Day 0 were 69% (9/13), 60% (6/10) and 86% (6/7) respectively. The prevalence of the mutation in the 28 non-respondents whose parasitaemia had not cleared from Day 7 post-treatment was 68% (19/28). None of the alleles was detected in the remaining 9 (i.e. PCR negative). The allelic frequencies of *pfmdr1* 86 in Days 0, 7 and 14 samples of the non-respondents are shown in Figure 4.6. The sensitivity and specificity of detection of the mutation were 70.0% and 42.4% respectively. The association between the presence of mutation and treatment outcome was not significant (odds ratio = 1.72, p = 0.25).

4.5.3 Double mutations (both *pfcrt* T76 and *pfmdr1* Y86)

At Hohoe, the baseline prevalence of the double mutations within the patients was 73.8% (76/103). The details of the distribution of the mutation are shown in Table 4.6. Of the 39 respondents, 56% (22/39) had both mutations whilst for the 64 non-respondents 84% (54/64) had both mutations. The distribution of both mutations in the Day 0 specimen of the different non-respondent categories RI, RII and RIII were 83% (29/35), 95% (20/21) and 100% (8/8) respectively. The prevalence of both mutations in the 46 non-respondents whose parasitaemia had not cleared from Day 7 post-treatment was 61% (28/46). The remaining 18 had either *pfcrt* T76 or *pfmdr*1 Y86 but not both.

There was a strong association between treatment outcome and the presence of both mutations (odds ratio = 4.17, p = 0.002).

At Navrongo, the baseline prevalence of the double mutations within the patients was 36.5% (35/96). Of the 66 respondents, 36% (24/66) had both mutations whilst for the 30 non-respondents, 37% (11/30) had both mutations. The distribution of both mutations in the Day 0 specimen among the different non-respondent categories RI, RII and RIII were 69% (9/13), 33% (3/10) and 29% (2/7) respectively The prevalence of both mutations in the 28 non-respondents whose parasitaemia had not cleared from Day 7 post-treatment was 39.3% (11/28). Of the remaining 17, 10 had either *pfcrt* T76 or *pfmdr*1 Y86 and none of the mutations was detected in 7 samples (i.e. PCR negative). There was no association between clinical outcome and the presence of both mutations (odds ratio = 1.01, p = 0.98).

Table 4.4

Prevalence of *pfcrt* codon 76 alleles in patients at pre-treatment from Hohoe and Navrongo

Alleles	Prevalence of *pfcrt* 76 alleles (%)			
	Hohoe		Navrongo	
	Respondents (n = 39)	Non-respondents (n = 64)	Respondents (n = 66)	Non-respondents (n = 30)
Mutant only	12.8	48.4	36.4	40.0
Wildtype only	30.8	9.4	56.1	53.3
Mixed	56.4	42.2	6.1	6.7

Table 4.5

Prevalence of *pfmdr1* codon 86 alleles in patients at pre-treatment from Hohoe and Navrongo

Alleles	Prevalence of *pfmdr*1 86 alleles (%)			
	Hohoe		Navrongo	
	Respondents (n = 39)	Non-respondents (n = 64)	Respondents (n = 66)	Non-respondents (30)
Mutant	69.2	89.1	57.6	70.0
Wildtype	30.8	10.9	42.4	30.0

Table 4.6

Prevalence of double mutations, *pfcrt*T76 and *pfmdr*1Y86, at pre and post-treatment in patients from Hohoe and Navrongo.

Site	Prevalence of double mutations (*pfcrt* T76 + *pfmdr1* Y86)/%			
	Respondents	Non-respondents		
	Day 0	Day 0	Day 7	Day 14
Hohoe	54.0	84.0	9.0	61.0
Navrongo	36.0	37.0	39.3	32.0

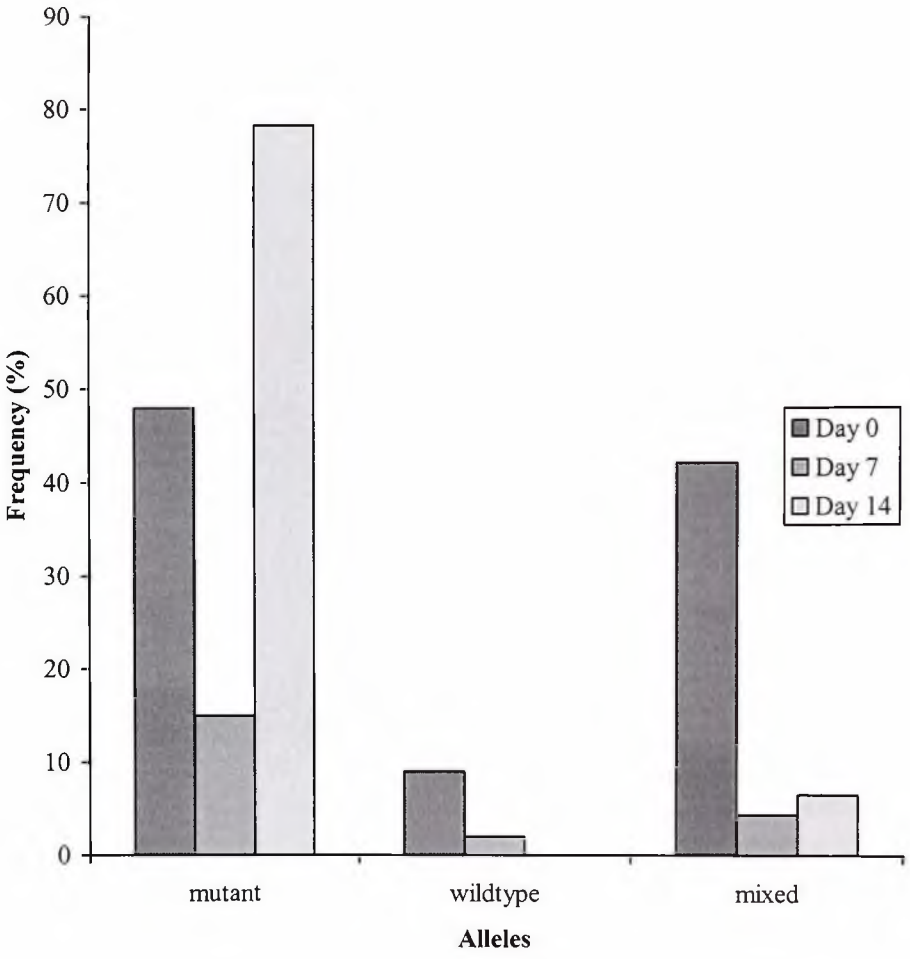

Figure 4.3 Allelic frequencies of *pfcrt* 76 of non-respondents from Hohoe at pre and post-treatment

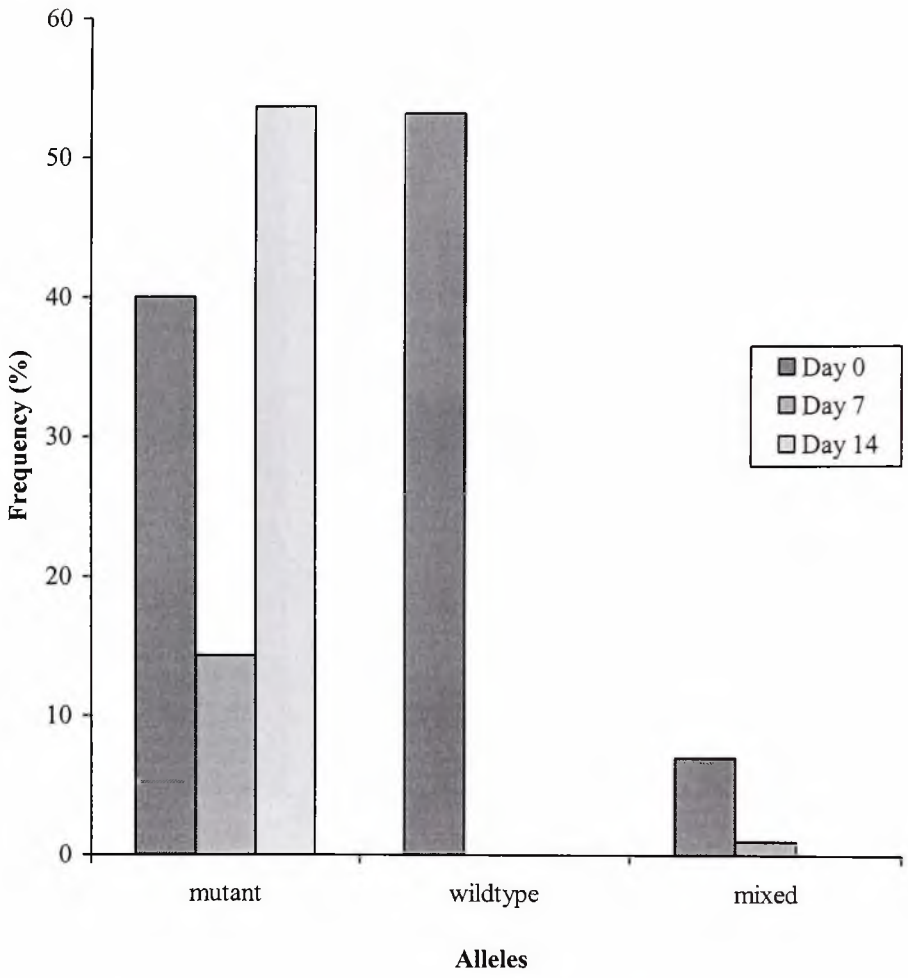

Figure 4.4 Allelic frequencies of *pfcrt* 76 of non-respondents from Navrongo at pre and post-treatment

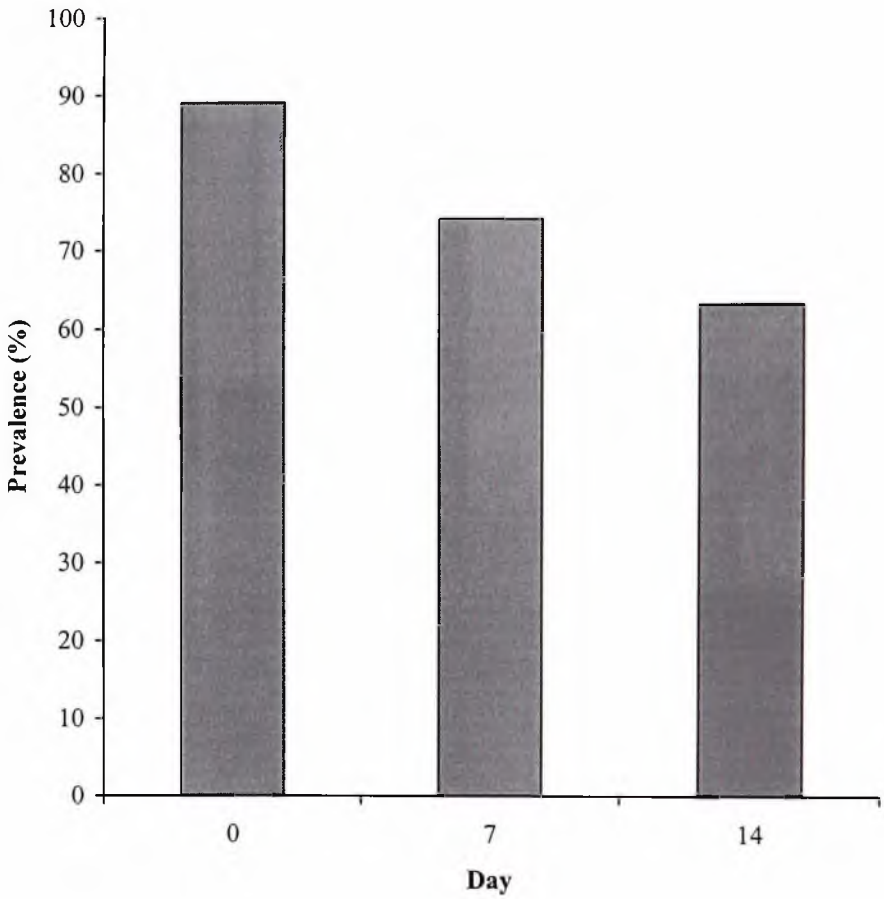

Figure 4.5 Prevalence of *pfmdr1* 86 mutation of non-respondents from Hohoe at pre and post-treatment

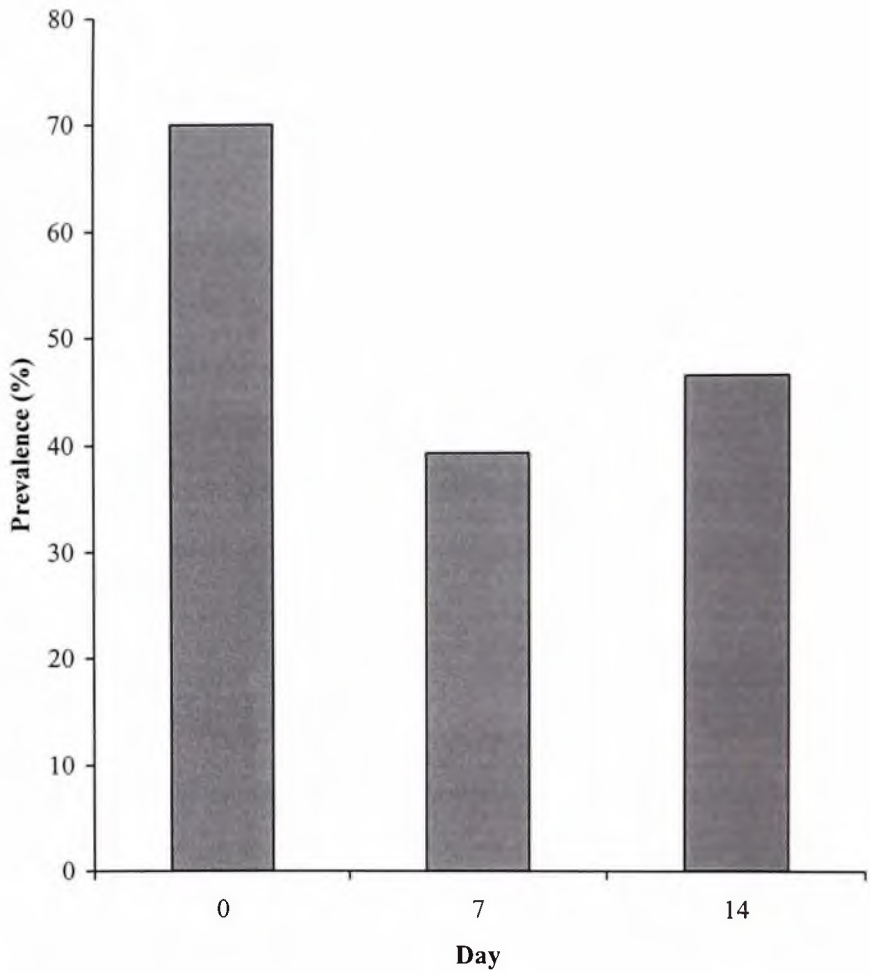

Figure 4.6 Prevalence of *pfmdr1* 86 mutation of non-respondents from Navrongo at pre and post-treatment

4.6 Distribution of *P. falciparum* Genotypes Associated with Fansidar Resistance

The PCR based assay of *P. falciparum* genotypes, *dhfr* and *dhps* were all successful. In all, 4450 individual PCR assays were performed. It is worth noting that all chloroquine non-respondents who were given Fansidar showed adequate treatment response.

4.6.1 *Dhfr* 51

At Hohoe, the baseline prevalence of the mutations at codon 51 within the patients was 44.6% (46/103). The details of the distribution of alleles of this codon within the malaria patients are shown in Table 4.7. In all, 40% (41/103) of the samples analysed were PCR negative (no allele detected by PCR). Of the 14 patients given Fansidar, 8 had mixed alleles, 1 had the mutant allele and 5 were PCR negative at Day 0. At post-treatment, 2 of them had mixed alleles, 4 had the mutant only and 2 had the wildtype only.

At Navrongo, the baseline prevalence of the mutation within the patients was 6.3% (6/96). The details of the distribution of the alleles of codon 51 are shown in Table 4.7. Sixty-five percent (62/96) of the samples analysed were PCR negative. Three patients were given Fansidar and at pre-treatment, 1 had the wildtype only and the 2 were PCR negative. At post-treatment, it was as above.

4.6.2 *Dhfr* 59

The baseline prevalence of the mutation at codon 59 within the patients at Hohoe was 57.3% (59/103). The details of the distribution of the alleles of this codon are shown in Table 4.8. In all 35% (36/103) were PCR negative. Of the 14 patients given Fansidar, at pre-treatment, 8 had mixed alleles, 4 had the mutant allele and 2 were PCR negative. At

post-treatment, 1 had mixed alleles, 2 had the mutant only and 2 had the wildtype.

At Navrongo, the baseline prevalence of the mutation at codon 59 was 28% (27/96). The details of the distribution of the alleles of this codon are shown in Table 4.8. Sixty-nine percent (66/96) of the samples were PCR negative. Of the 3 patients given Fansidar, at pre-treatment, 1 had the mutant only and the 2 were PCR negative. At post-treatment, it was the same.

4.6.3 *Dhfr* 108

At Hohoe, the baseline prevalence of the mutation at codon 108 within the patients was 66% (68/103). The details of the distribution of the alleles of the codon 108 are shown in Table 4.9. Of the 103 samples analysed, 28.2% (29/103) were PCR negative. Of the 14 patients given Fansidar, at pre-treatment, 9 had mixed alleles, 2 had the mutant allele and 3 were PCR negative. At post-treatment, 1 had mixed alleles and 4 had the mutant only and 2 had the wildtype only.

At Navrongo, the baseline prevalence of the mutation was 31.3% (30/96). Details of the distribution of alleles are shown in Table 4.9. Of the 96 samples analysed, 59.4% (57/96) were PCR negative. Of the 3 patients given Fansidar, at pre-treatment, 1 had the mutant only and the 2 were PCR negative. At post-treatment, 1 had the wildtype and 1 had the mutant only.

4.6.4 Triple mutation (*dhfr* 51, 59 and 108)

At Hohoe, the baseline prevalence of the presence of all three mutations in the *dhfr* region within the patients was 33% (34/103) [Table 4.10]. Of the 14 patients given Fansidar, at pre-treatment, 12 had the triple mutations. At post-treatment, 3 had the triple mutations.

At Navrongo, the baseline prevalence was 5.2% (5/96) within the patients. Of the 3 patients given Fansidar, at pre-treatment, none had the triple mutations.

4.6.5 *Dhps* 437

The baseline prevalence of the mutation at codon 437 within the patients at Hohoe was 76% (78/103). The details of the distribution of the alleles are shown in Table 4.11. Of the 103 samples analysed 18.5% (19/103) were PCR negative. Of the 14 patients given Fansidar, at pre-treatment, 4 had mixed alleles, 6 had the mutant allele, 1 had the wildtype and 3 were PCR negative. At post-treatment, 1 had mixed alleles, 2 had the mutant only, 7 had the wildtype only.

For Navrongo, the baseline prevalence of the mutation was 9.4% (9/96). Details of the distribution of alleles are shown I Table 4.11. About 72% of the samples were PCR negative. Of the 3 patients given Fansidar, at pre-treatment, 1 had the wildtype only and the 2 were PCR negative. At post-treatment, none of them had any of the alleles.

4.6.6 *Dhps* 540

At Hohoe, the baseline prevalence of the mutation at codon 540 within the patients was 53.4% (55/103). The details of the distribution of the alleles are shown in Table 4.12. Twenty-two percent (23/103) were PCR negative. Of the 14 patients given Fansidar, at pre-treatment, 9 had mixed alleles, 1 had the wildtype allele and 4 were PCR negative. At post-treatment, 5 had mixed alleles, 3 had the mutant only, 2 had the wildtype only.

At Navrongo, the baseline prevalence of the mutation within the patients was 6.3%(6/96). Details of the distribution of alleles are shown in Table 4.12. Seventy-two percent (69/96) were PCR negative. Of the 3 patients who were given Fansidar, none of them had any of the codon 540 alleles at both pre and post-treatment.

4.6.7 Double mutations (*dhps* 437 and 540)

At Hohoe, the baseline prevalence of patients with both codons 437 and 540 mutations was 51.5% (53/103) [Table 4.13]. Of the 14 patients given Fansidar, at pre-treatment, 8 had the double mutations. At post-treatment, 3 of them had the double mutations.

For Navrongo the baseline prevalence was 3.1% (3/96). All the 3 patients who were given Fansidar did not have both mutations at both pre and post-treatment.

4.6.8 Quintuple mutations (*dhfr* 51, 59, 108, *dhps* 437 and 540)

At Hohoe, the baseline prevalence of patients with all five mutations associated with Fansidar resistance was 31.1% (32/103)[Table 4.14]. Of the 14 patients given Fansidar, at pre-treatment, 8 had the quintuple mutations. At post-treatment, 1 had the quintuple

mutations

For Navrongo, the baseline prevalence was 1.04% (1/96) [Table 4.14]. None of the 3 patients given Fansidar had the quintuple mutations either at pre or post-treatment.

Table 4.7

Baseline prevalence of alleles of the *dhfr* codon 51 in patients at Hohoe and Navrongo

Alleles	Prevalence of alleles of *dhfr* 51/%	
	Hohoe (103)	Navrongo (96)
Mutant only	12.0	2.0
Wildtype only	15.5	29.0
Mixed alleles	33.0	4.0

Table 4.8

Baseline prevalence of alleles of the *dhfr* codon 59 in patients at Hohoe and Navrongo

Alleles	Prevalence of alleles of *dhfr* 59/%	
	Hohoe	Navrongo
Mutant only	24.3	19.8
Wildtype only	7.7	3.0
Mixed alleles	33.0	8.3

Table 4.9

Baseline prevalence of alleles of the *dhfr* codon 108 in patients at Hohoe and Navrongo

Alleles	Prevalence of alleles of *dhfr* 108/%	
	Hohoe	Navrongo
Mutant only	18.4	7.3
Wildtype only	5.8	9.4
Mixed alleles	47.6	24.0

Table 4.10

Baseline prevalence of triple mutations of *dhfr* (51 + 59 + 108) in patients at Hohoe and Navrongo.

Site	Prevalence of triple mutations (*dhfr* 59, 59 and 108)/%
Hohoe	33.0
Navrongo	5.2

Table 4.11

Baseline prevalence of alleles of the *dhps* codon 437 in patients at Hohoe and Navrongo

Alleles	Prevalence of alleles of *dhps* 437	
	Hohoe	Navrongo
Mutant only	38.8	0
Wildtype only	5.8	18.8
Mixed alleles	36.9	9.4

Table 4.12

Baseline prevalence of alleles of the *dhps* codon 540 in patients at Hohoe and Navrongo

Alleles	Prevalence of alleles of *dhps* 540	
	Hohoe	Navrongo
Mutant only	1.0	0
Wildtype only	24.3	21.0
Mixed alleles	52.4	6.3

Table 4.13

Baseline prevalence of double mutations of *dhps* (437 + 540) in patients at Hohoe and Navrongo

Site	Prevalence of double mutations (*dhps* 437 and 540)
Hohoe	51.5
Navrongo	3.1

Table 4.14

Baseline prevalence of quintuple mutations (*dhfr* 51 + 59 + 108 + *dhps* 437 + 540) in patients at Hohoe and Navrongo

Site	Prevalence of quintuple mutations (*dhfr* 59, 59, 108, *dhps* 437 and 540)
Hohoe	31.1
Navrongo	1.04

4.7 Comparison between Molecular Analysis, *In vivo* and *In vitro* Outcomes

Generally, the results of the *in-vivo* studies correlated with that of the *in vitro* at Hohoe (Table 4.15). One of the isolates, which was sensitive *in-vivo* showed resistance in the *in vitro* testing. The presence of *pfcrt* T76 and *pfmdr1* Y86 were observed in pre-treatment samples of both *in vivo* sensitive and resistant infections and also in the post-treatment samples of the resistant infections.

For Navrongo, the outcomes of both *in-vivo* and *in vitro* analysis were generally similar (Table 4.15). However, all RII infections were sensitive *in-vitro* and 3 of the sensitive infections *in vivo* were resistant *in vitro*. For all RIII infections, there was growth of the parasite *in vitro* even at concentrations of 8-32pmol but were totally inhibited at 64pmol. *Pfcrt* T76 was not detected in any of the pre-treatment samples analysed but was detected in post-treatment samples of the *in-vivo* chloroquine resistant infections. For *pfmdr*1 Y86, there was detection in 15 of the 18 samples and in all resistant infections.

Table 4.15

Outcomes of *in-vivo* and *in-vitro* analysis for the selected samples from Hohoe and Navrongo

***In vivo* outcome**	***In-vitro* outcome**			
	Hohoe		**Navrongo**	
	S	**R**	**S**	**R**
Sensitive	6	1	9	3
Resistant	1	0	3	3

Where S and R are sensitive and resistant respectively

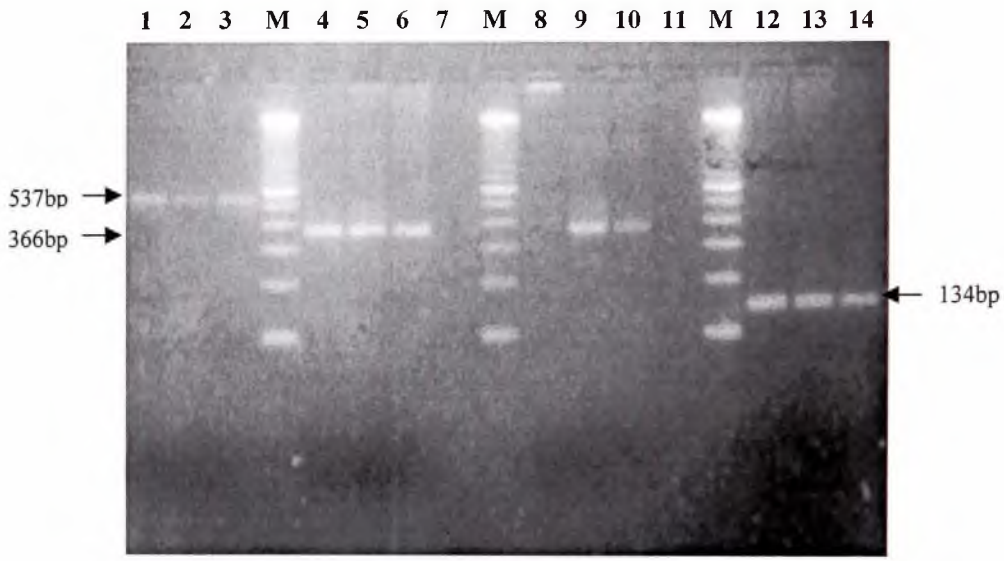

Fig 4.7 Agarose gel electrophoresis of PCR products obtained from the nested amplification of alleles of *pfcrt* codon 76. Lanes 1-3 = primary amplification using primers CRTP1 and CRTP2, Lanes M = 100bp molecular weight marker, Lanes 4-6 = secondary amplification using primers CRTP3 and CRTP4m, Lanes 8-10 = secondary amplification using CRTP3 and CRTP4w, Lanes 12-14 = secondary amplification with primers CRTD1 and CRTD2, Lanes 7 and 11 are negative controls.

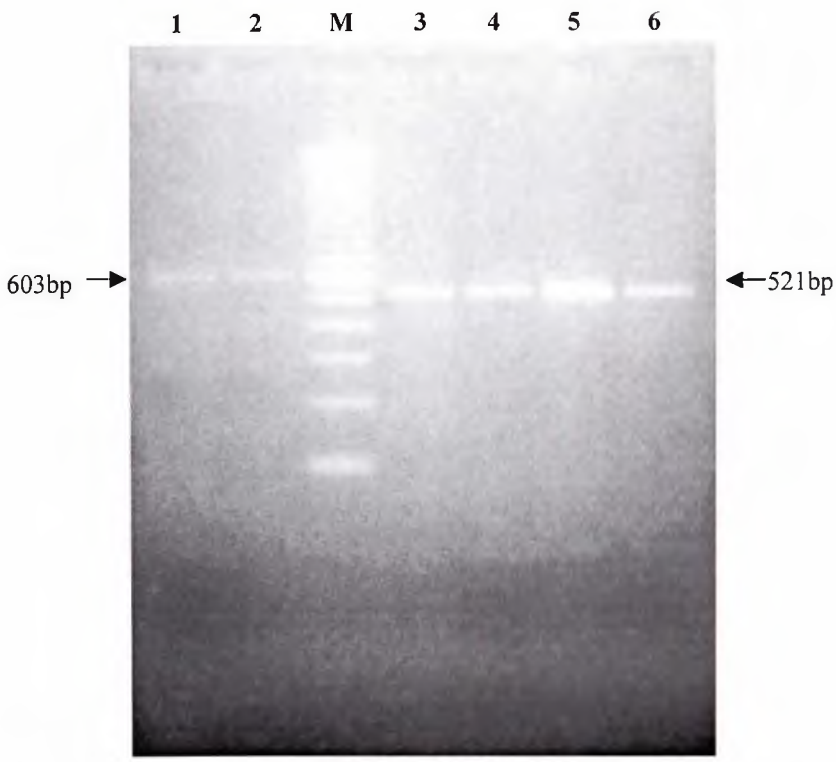

Fig 4.8 Agarose gel electrophoresis of PCR products obtained from the nested amplification of alleles of *pfmdr1* codon 86. Lanes 1-2 = primary amplification using primers MDR-1 and MDR-2, Lane M = 100bp molecular weight marker, Lanes 3-6 = secondary amplification with primers MDR-3 and MDR-4.

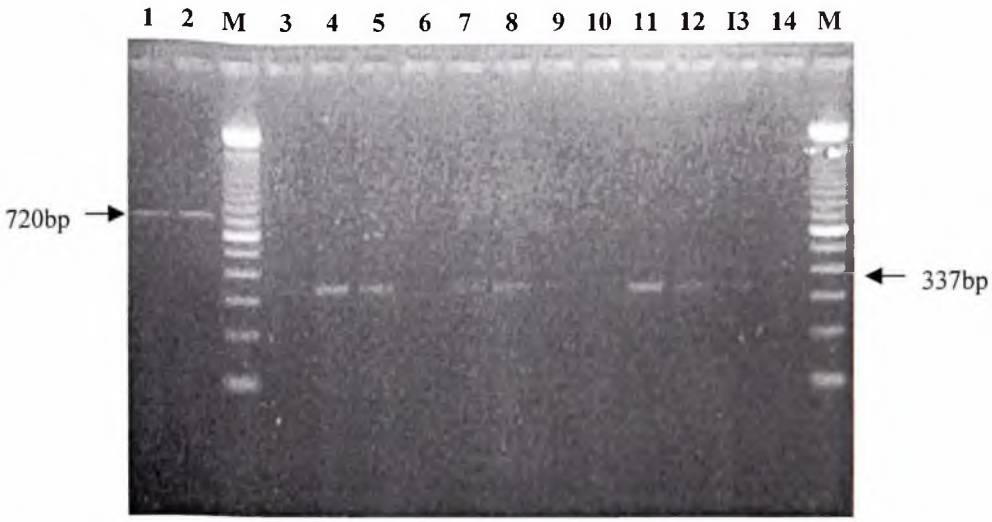

Fig 4.9 Agarose gel electrophoresis of PCR products obtained from the nested amplification of alleles of *dhfr* codon 108. Lanes 1-2 = primary amplification using primers AMP1 and AMP2, Lanes M = 100bp molecular weight marker, Lanes 3-5 = secondary amplification with primers SP1 and DIA-12, Lanes 7-9 = secondary amplification with primers SP1 and DIA-9, Lanes 11-13 = secondary amplification with primers SP1 and DIA-3, Lanes 6,10 and 14 are negative controls.

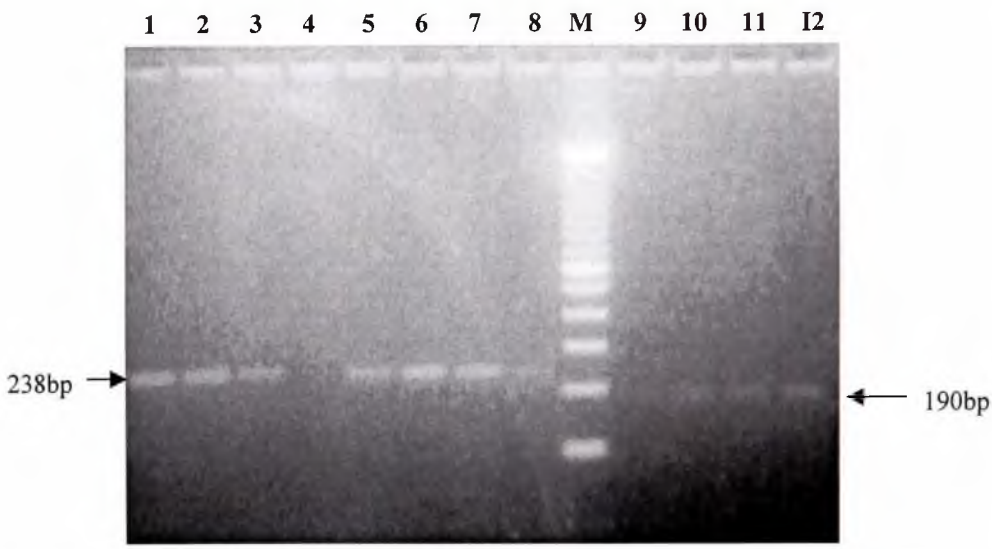

Fig 4.10 Agarose gel electrophoresis of PCR products obtained from the nested amplification of alleles of *dhfr* codons 51 and 59. Lanes 1-3 = secondary amplification for codon 51 using primers MUM-D and FR-51MB1, Lanes 5-8 = secondary amplification for codon 51 using primers MUM-D and FR-51WB1, Lane M = 100bp molecular weight marker, Lanes 9-10 = secondary amplification for codon 59 with primers SP1 and FR59m, Lanes 11-12 = secondary amplification for codon 59 with primers SP1 and FR59w, Lane 4 is a negative control.

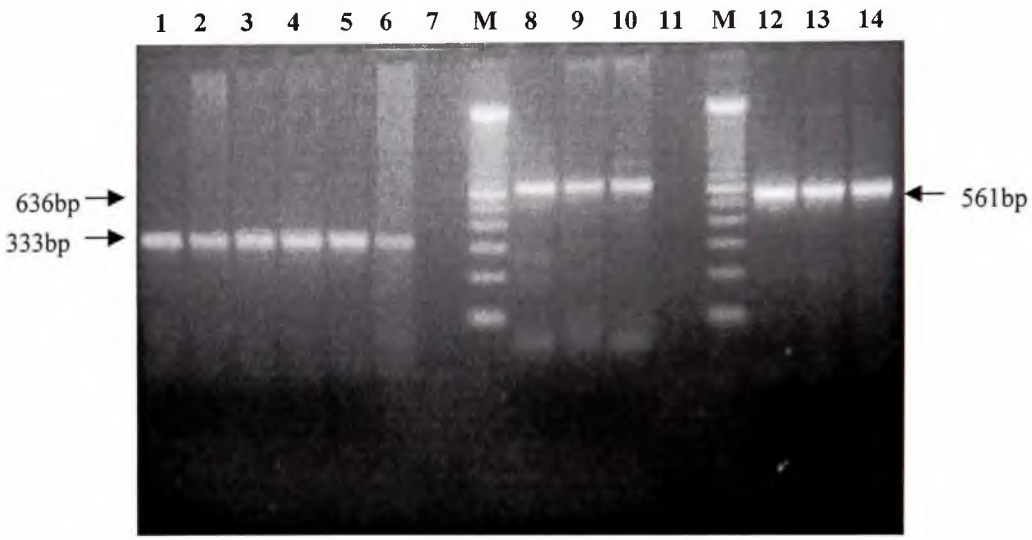

Fig 4.11 Agarose gel electrophoresis of PCR products from the nested amplification of alleles of dhps codons 437 and 540. Lanes 1-3 = secondary amplification for codon 437 using primers 185S and 437M-A, Lanes 4-6 secondary amplification for codon 437 using primers 185S and 437W-2C, Lanes M = 100bp molecular weight marker, Lanes 8-10 = secondary amplification for codon 540 using primers 218A and 540W-S, Lanes 12-14 = secondary amplification for codon 540 with primers 185S and 540M-A, Lanes 7 and 11 are negative controls.

CHAPTER 5

DISCUSSION AND CONCLUSION

This study investigated the species of *Plasmodium* responsible for malaria in patients and compared the *in-vivo* and *in-vitro* classification of resistance in 26 isolates at two selected sentinel sites in Ghana. Also, the relationship between responses to the antimalarial drugs and the presence of mutations at several gene loci, *pfcrt* and *pfmdr1* for chloroquine as well as *pfdhfr* and *pfdhps* for Fansidar in *P. falciparum* were investigated.

The results obtained revealed that the majority of the malaria cases (94.2% and 89.6% for Hohoe and Navrongo respectively) were caused by *P. falciparum*. Mixed infections of *P. falciparum* and *P. malariae* were also recorded, occurring in 4.8% and 10.4% at Hohoe and Navrongo respectively. The findings are similar to those of previous studies in Ghana which found that about 95% of all infections in the country is caused by *P. falciparum* and the rest by *P. malariae* and *P. ovale* in that order of importance (Ahmed, 1989; Binka *et al.*, 1994). Surprisingly, no significant differences were found between the parasitaemia of respondents and non-respondents at the study sites ($p = 0.44$ and 0.51 for Hohoe and Navrongo respectively).

Results from this study suggest that the presence of *P. falciparum pfcrt* 76 genotypes can be used to predict chloroquine resistance levels at some (Hohoe) but not all sites (Navrongo) in the country. A high frequency of the *pfcrt* mutant gene (82%) was observed in Hohoe, which was reflected in a high rate (62.1%) of chloroquine treatment

failure. The relatively higher frequency of the mutation as compared to that observed in the *in vivo* outcome of treatment supports the findings of Djimde *et al.* (2001) that clinical failure to chloroquine occurs at rates lower than the frequency of *pfcrt* T76 allele.

A significant association was found between treatment outcome and the presence of *pfcrt* T76 (odds ratio 12.40, p-value 0.0001) in Hohoe and moreover, all the non-respondents were found to be infected with *pfcrt* T76 parasites at post-treatment. The observations made in Hohoe are consistent with findings in Mali, Mauritania, Sudan and Cameroon, where the high prevalence of the resistant genotypes was associated with treatment failures (Djimde *et al.*, 2001; Jelinek *et al.*, 2001; Babiker *et al.*, 2001; Basco and Ringwald, 2001), which lends support to the belief that T76 plays an essential role in chloroquine resistance.

However, 27 patients carrying mutant parasites responded satisfactorily to chloroquine. Of these, 5 patients had the mutant only and 22 had mixed alleles of *pfcrt* 76, which is almost 69% (27/39) of the total number of respondents. A similar observation was also made in the Mali study where Djimde *et al.* (2001) found 37% (40/107) of the respondents having the mutant allele. The possible explanations for this observation could be that, mutant parasites as compared to the wildtype parasites were few in numbers in these patients and therefore were taken care of by the host's enhanced immunity. However, it is difficult to explain away the 5 cases which had only the mutant parasites at pre-treatment, though it is likely that other factors including low parasitaemia and host immunity played a role in their elimination. Another interesting

observation was that *pfcrt* T76 was not detected in 5 (4.8%) cases at pre-treatment at Hohoe but was found in post-treatment samples. Here also, three possible explanations could have accounted for this. Firstly, the resistant parasites could have been in the liver or sequestrated in deep tissues during pre-treatment and have then emerged into the blood stream after treatment. Secondly, it could have been due to reinfection. However, reinfection is unlikely by day 14 because the incubation period of the parasite and the time taken to appear in the blood after an infective bite is averagely about 12 days (White, 1996). Thirdly, it could have been due to mixed infections consisting mainly of sensitive parasites with a very small population of resistant parasites, which were not detected by PCR since this method is known to be poor at detecting minority alleles (at <0.1%) [Ranford Cartwright, 2001]. With the administration of chloroquine, the sensitive parasites were cleared whilst the resistant parasite population persisted resulting in treatment failure and subsequent detection of the mutant parasites in the post-treatment samples. The detection of low levels mutant parasites can only be achieved by employing quantitative PCR, which is very expensive to undertake and therefore was not done.

With regards to the other chloroquine resistant gene *pfmdr*1, the prevalence of the mutation among the Hohoe patients was 82% and a positive association was found between the *pfmdr*1 Y86 and chloroquine treatment outcome (odds ratio 3.62; p-value 0.01). Moreover, the mutant parasites were persistent in all the non-respondents at post-treatment. Similar findings have been made by researchers in Mauritania, Sudan and Kenya (Jelinek *et al.*, 2001; IAEA, 2001).

The presence of both mutant forms of the *pfcrt* 76 and *pfmdr*1 86 were observed in 74% of the patients. Both mutations were found to be strongly associated with treatment failure in Hohoe (odds ratio 4.17, p-value 0.001) and similar findings have been reported by Jelinek *et al.* (2001) in Mauritania. Since *pfmdr1* and *pfcrt* are on different chromosomes, their selection cannot be attributed to physical linkage. Rather it could be that *pfmdr1* confers some advantage to the parasite in the presence of chloroquine by augmenting the level of resistance due to *pfcrt* mutation (additive effect).

At Navrongo, a different scenario was observed. The prevalence of *pfcrt* T76 in the patients was low (44%) and no association was found between the mutation and clinical outcome (odds ratio 1.16, p-value 0.75). Also, *pfcrt* T76 was not detected in 10 (10.4%) non-respondents at pre-treatment but was however detected in them at post-treatment. Although there was no association between the *pfcrt* mutation and treatment outcome, the mutant alleles were found after treatment in all the non-respondents. Djimde *et al.* (2001) had suggested that other mutations at different codons of the *pfcrt* gene such as I74, E75, S220 and T356 may be important for chloroquine resistance therefore it is likely that these or other mutations may be responsible for resistance in the parasites at Navrongo. Further studies are required to investigate the presence of these other mutations and to determine their association to chloroquine resistance in Navrongo.

The prevalence of the *pfmdr*1 mutation was 62% and similarly no association was found between *pfmdr*1 mutation and clinical response in Navrongo (odds ratio 1.72, p-value 0.24). This finding has also been reported in Mali and Cameroon (Djimde *et al*, 2001; Basco and Ringwald, 1998). Moreover, no association was found between the presence

of both mutations, *pfcrt T76* and *pfmdr1* Y86 with clinical response (odds ratio 1.01, p-value 0.97).

There were marked differences in treatment responses at the study sites with significantly more non-respondents in Hohoe (62%) than at Navrongo (31%). The differences in the observed resistance between the urban (Hohoe) and the rural community (Navrongo) could be attributed to self-medication due to easy accessibility to chloroquine in the former. Most often patients resort to buying from chemical sellers to avoid spending too much time and money at health centres and hospitals. Failure to adhere to prescription for treatment (non-compliance) by patients could also be largely responsible for the occurrence of drug resistance. The consequences of inappropriate use of the drug lead to an increase in drug pressure, resulting in the selection for resistant strains of the parasites. Hohoe is urban in its settings and access to drugs is easier. This is most likely the reason for the selection of mutant *pfcrt* T76 parasites and therefore an association between this genotype and clinical outcome but not at Navrongo, which is relatively rural in comparison.

Parasite antimalarial drug resistance is known to occur naturally. Genetically diverse wildtype populations of *P. falciparum* have heterogenous sensitivity to antimalarial drugs (Thaithong, 1983). Additionally, resistance may arise from spontaneous chromosomal point mutations, which are independent of drug pressure. Once formed, these more resistant mutants have a survival advantage in the presence of antimalarial drugs (Curtis and Otoo, 1986; Cross and Singer, 1991). Resistant parasites may also be selected when parasites are exposed to subtherapeutic drug concentrations which are

below the concentrations that kill the parasites. This occurs if there is widespread use of the drug at inadequate doses. Resistance is also very likely if the drug is eliminated slowly from the body because new parasites introduced to low drug concentrations are not eliminated (White, 1992). There is an assertion that high transmission rates favour the spread of drug resistance when combined with heavy drug pressure which is itself the most important factor in the survival of drug resistant mutants (Mackinnon, 1997). This was observed at the two study sites. Hohoe is characterised by perennial malaria transmission and this could be the reason for the spread of resistant parasites but not at Navrongo with an intense but seasonal transmission.

The alleles of *dhfr* and *dhps* associated with response to pyrimethamine and sulphadoxine were also characterised in the present study.. The presence of mutations in codons 51, 59 and 108 of the *dhfr* gene was detected in pre-treatment *P. falciparum* isolates as well as in some post-treatment samples of non-respondents to chloroquine. Out of the total number of isolates analysed in both sites (103 from Hohoe and 96 from Navrongo), about 40% were PCR negative for *dhfr* codons 51, 59 and 108 at both sites. Similarly, about 30% were PCR negative in the case of *dhps* codons 437 and 540. The reason may be that the regions amplified in this study, which has been found to be associated with Fansidar sensitivity in other countries like Mali, Tanzania, Cambodia, Vietnam and others (Plowe *et al.*, 1999) may differ in sequence from what is present at the two sites, hence the primers used may be inappropriate for the detection.

The results from this study showed that the presence of *dhfr* and *dhps* mutations does not accurately predict Fansidar sensitivity or resistance *in vivo* in Hohoe and Navrongo. It is

possible that these mutations may become more useful in the future with increasing treatment failure. However, it is worth noting that all chloroquine treatment failures responded adequately to Fansidar treatment. This gives credence to the usefulness of Fansidar as the second line antimalarial drug in Ghana The observed prevalence of the mutations in these settings, in the face of very low Fansidar resistance, suggests that *in-vivo* Fansidar resistance may evolve more quickly than predicted should the drug become the first line antimalarial to replace of chloroquine in the country.

This study has demonstrated that the method of sequential processing of whole blood spots and performance of PCR is valuable in detecting mutations associated with drug resistance but very expensive. Samples collected from patients using minimally invasive technique (blood spots) can therefore be used for extensive hospital or field surveys of the relationship between mutations in *P. falciparum* and antimalarial drug resistance. Although, the molecular technique described is relatively easy and rapid to perform, the relevance of genetic mutation as reliable genetic markers of *in vivo* antimalarial drugs resistance in Ghana needs further detailed investigation.

The results of the *in-vitro* chloroquine sensitivity test correlated very well with that of the *in-vivo* observation thus, supporting the idea that *in-vitro* sensitivity is a useful epidemiological tool. However, the *in-vitro* drug sensitivity testing does not predict clinical response to treatment since it does not take into account individual differences in antimalarial pharmacokinetics, immunity or stage of disease.

The estimated IC_{50} for chloroquine was $1.5x10^{-6}$M indicating that isolates encountered in this study were fairly sensitive to the drug. All resistant cases at RIII level *in-vivo* showed isolate *in-vitro* inhibition at concentrations higher than the cut off 8pmol concentration indicative of chloroquine resistance.

To summarise, the present study has shown that the *pfcrt* and *pfmdr1* genotyping could be useful in predicting the level of chloroquine resistance at Hohoe but not at Navrongo. The likely reason being that the level of drug pressure is higher at urbanised Hohoe than in the relatively rural Navrongo, which was reflected in the observed low prevalences of both the *pfcrt* and *pfmdr1* mutations at the latter study site. Other *pfcrt* mutations in the *P. falciparum* genome also need to be investigated in isolates at Navrongo to find out if they could predict the level of chloroquine resistance. However at both sites, all the non-respondents harboured *pfcrt* T76 parasites, providing evidence and thus confirming the results of other studies that this mutation is associated with chloroquine resistance. For Fansidar, the low prevalences of *dhfr* and *dhps* mutations observed revealed that there is low-level use of the drug especially in Navrongo whilst for Hohoe with a relatively high prevalence of the quintuple mutations is indicative of a rapid emergence and spread of Fansidar resistance in the event of drug policy change.

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APPENDICES

Appendix I

PREPARATION OF STANDARD SOLUTIONS

The following standard solutions were prepared using sterilised double distilled water. Where appropriate, the solutions were autoclaved at 121lb/sq for 15minutes in an Eyela Autoclave (Rikikkaki, Tokyo).

DNA Extraction

10% saponin

10g of saponin was dissolved in 100ml of sterilised doubled distilled water

1x PBS (Phosphate buffered saline)

One tablet dissolved in 200ml of water to obtain 0.01M phosphate buffer, 0.0027M potassium chloride, pH 7.4 at 25^{O}C.

20% Chelex

20g of Chelex-100 was dissolved in 100ml of double distilled water and autoclaved.

Gel Electrophoresis (agarose)

Ethidium bromide (10mg/ml)

1g of ethidium bromide was completely dissolved in 100ml of sterilised double distilled water and stored in the dark at 4^{O}C.

50x TAE buffer

242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA, pH adjusted to 7.7 and the volume made to 100ml with doubled distilled water.

0.5M EDTA (pH 8.0)

186g of EDTA, dissolved in 800ml double distilled water, pH adjusted with NaOH pellets and stored at room temperature.

Gel Loading Buffers

6x Bromophenol blue

0.25% bromophenol blue, 40% sucrose in water and stored at $4^{O}C$.

5x Orange G

20% (w/v) Ficoll, 25mM EDTA, 2.5% (w/v) Orange G stored at room temperature.

DNA Molecular Weight Marker

100bp ladder

The first band is 100bp, the subsequent bands measure 200, 300, 400, …, 1000bp.

Appendix II

CHEMICALS AND REAGENTS

DNA Extraction and PCR

10x PCR buffer (Sigma)

Magnesium chloride (Sigma)

Taq DNA polymerase (Sigma)

Deoxyribonucleotide triphosphates, dATP, dCTP, dGTP and dTTP (Amersham Pharmacia Biotech).

Oligonucleotide primers (Oswel)

Saponin (Sigma)

Silica gel (Sigma)

Absolute ethanol (Sigma)

Mineral oil

Chelex-100 (Sigma)

Bromophenol blue (Sigma)

Agarose (molecular biology grade, Sigma)

Methanol (Sigma)

Tris(hydroxymethyl)aminomethane (Trizma base, Sigma)

Ethylene diamine tetra acetate disodium salt (EDTA, Sigma)

Ethidium bromide (Sigma)

100bp molecular weight markers (Gibco-BRL)

Ficoll (Sigma)

***In vitro* Drug Sensitivity Test**

Neutral RPMI 1640 (without glutamine but with bicarbonates, Sigma)

Glutamine

Glucose

HEPES

AB sera

Gentamycine

Chloroquine phosphate powder

Radiolabelled Hypoxanthin mono-hydrochloride

COMPOSITION OF RPMI 1640

From Gibco catalogue

Component	Amount (mg/l)
Inorganic salts	
$Ca(NO_3)_2.4H_2O$	100.0
KCl	400.0
Mg_2SO_4	48.84
NaCl	6000.0
$NaHCO_3$	2000.0 (added before use)
Na_2HPO_4	800.0
Amino acids	
L-Arginine (free base)	200.0
L-Asparagine	50.0
L-Aspartic acid	20.0
L-Cysteine	65.0 (2HCl)
L-Glutamic acid	20.0
L-Glutamine Glycine	300.0
L-Histidine (free base)	10.0
L-Hydroxyproline	15.0
L-Isoleucine (allo-free)(	20.0
L-Leucine (methionine free)	50.0
L-Lycine-HCl	40.0
L-Methionine	15.0
L-Phenylalanine	15.0
L-Proline (hydroxy-proline free)	20.0
L-Serne	30.0
L-Threonine (allo-free)	20.0
L-Tryptophan	5.0
L-Tyrosine	28.94 (sodium salt)
L-Valine	20.0
Vitamins	
Calcium pantothenate	0.20
Choline chloride	3.00
Folic acid	1.0
Isoinositol	35.0
Nicotinamide	1.0
P-Aminobenzoic	1.0
Pyridoxine-HCl	1.0
Riboflavin	0.20
Thiamine-HCl	1.0
Vitamin B12	0.005
Others	
Glucose	2000.0
Glutathione	1.0
Phenol red	5.0

Appendix III

RECRUITMENT AND CASE RECORD FORM

DISTRICT:.....DATE:.....
NAME:.....STUDY NO.:.....
AGE (MTHS):.....SEX:.....WEIGHT(KG):.....
HOME ADDRESS:.....LANDMARK:.....
GUARDIAN'S NAME:.....OCCUPATION:.....
FAMILY HEAD:.....
ANTI-MALARIAL INTAKE DURING THE PAST WEEK:.....

	DAY 0	DAY 1	DAY 2	DAY 3	DAY 7	DAY 14	+DAY 21
DATE							
FEVER (Yes/No)							
VOMITING (Yes/No)							
DIARRHOEA (Yes/No)							
CONVULSION (Yes/No)							
*UNABLE TO STAND/SIT UP. (Yes/No)							
*UNABLE TO DRINK/ BREASTFEED/EAT (Yes/No)							
*LETHARGIC/UNCONSCIOUS (Yes/No)							
AXILLARY TEMP. (^{O}C)							
RESPIRATORY RATE							
PARASIT COUNT/WBC							
DRUG							
DOSE							
HB. (g/dl)							

* Danger signs-: Do not recruit if they are present on Day 0, and remove from study and refer if present after Day 0.

+ For those given alternative drug on Day 14

Comments:.....

.....

CONSENT FORM: MAPPING THE RESPONSE OF *PLASMODIUM FALCIPARUM* TO CHLOROQUINE AND OTHER ANTIMALARIAL DRUGS IN GHANA.

I_____ being the mother/father/legal guardian of _____, am being asked to allow _____ to participate in a medical study called "Mapping the Response of *Plasmodium falciparum* to Chloroquine and other anti-malarial drugs in Ghana".

It has been explained to me that the study is being conducted in 6 districts across Ghana and that it is a collaborative study between Ministry of Health, Accra, the Noguchi Memorial Institute for Medical Research (NMIMR), Legon, the University of Ghana Medical School (UGMS), Korle-Bu and the Institute de Medicine Tropicale du Service de Sante des Armees (IMTSSA), Marseille, France. The information gathered would be used to determine how useful chloroquine is for treatment of malaria in Ghana.

It has been explained to me that my child/ward will be one of several children in the country (about 300 in this district) being asked to volunteer for the study. If I agree for my child/ward to join the study s/he will be asked to give a small blood sample (less than 1 ml) before treatment is given for malaria. The treatment given will be chloroquine and I should not give any other medication to my child/ward without making the investigators aware.

I understand that I will bring my child/ward to the hospital for review by the investigators two days, a week and two weeks from today. At each of these visits, my child/ward will be examined and a small volume of blood taken from my child/ward by finger prick to determine whether treatment given him or her is effective for treating the malaria infection. Finger pricks may cause mild pain during the short time it takes to insert the lancet and withdraw the blood. There is a very small risk of infection with finger pricks but the investigators have informed me that this is very unlikely to occur. The study investigators will take all available precautions such as using sterile equipment only once, and alcohol swabs to protect my child/ward. If I choose not to let my child/ward participate in the study s/he will be treated in the usual way and the decision not to let my child/ward participate will in no way affect the care that s/he will be given in this hospital. If my child/ward is less than 6 months and more than 5 years or very ill or anaemic, s/he will not be allowed to participate in the study. These restrictions are necessary for the safety of the children.

During the study if my child/ward is found not be responding to the chloroquine treatment, other drugs will be given that will cure the child of the infection. These will include but not limited to Fansidar and Quinine. In the event that my child/ward is given another drug besides chloroquine s/he will be required to come to the clinic to be examined to ensure that the infection is completely cured.

I have been informed that the risks of participating in the study are those of finger pricks as mentioned before. The benefits of participating in the study include monitoring of the child/ward to ensure that the malaria infection is completely cured and the treatment of

any other illness while the child/ward is under supervision. If I have any question or concerns about the study I can contact any of the following persons,

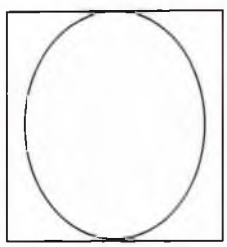
1 The Medical Officer in charge at the district hospital
2 The Regional Director of Health Services
3 Dr. Kwadwo A. Koram at the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon (tel no. 021 500374) or in person at the district hospital
4 Dr. Richard Y. Osei, Malaria Control Programme, Ministry of Health, Accra or in person at the district hospital.
5 Prof. David Ofori-Adjei, University of Ghana Medical School, Korle-Bu, Accra

I understand that joining the study is voluntary (I do not have to let my child/ward join). If I allow my child/ward to join, I will be expected to bring the child for all the follow up visits but I may withdraw my child/ward's participation at any point without any penalty or loss of benefits to which I'm otherwise entitled. The doctors may also withdraw my child/ward without my consent if they fel this is necessary for the child's safety. The information in my child/ward's records is confidential, but may be examined by representatives of the Ghanaian Ministry of Health, the NMIMR, UGMS or by doctors providing care for the child.

The study described above has been explained to me and I voluntarily agree to let my child/ward participate in the study. I have had the opportunity to ask questions and understand that future questions I have will be answered by one of the study investigators.

NAME: _____ DATE: _____
(MOTHER/FATHER/GUARDIAN)

SIGNATURE/RT. THUMPRINT _____



WITNESS NAME: _____ DATE: _____

WITNESS SIGNATURE: _____

INVESTIGATOR'S NAME: _____ DATE: _____

INVESTIGATOR'S SIGNATURE: _____

Guidelines for the oral administration of malaria drugs

Chloroquine doses in mg/kg body weight using 500mg salt tablets

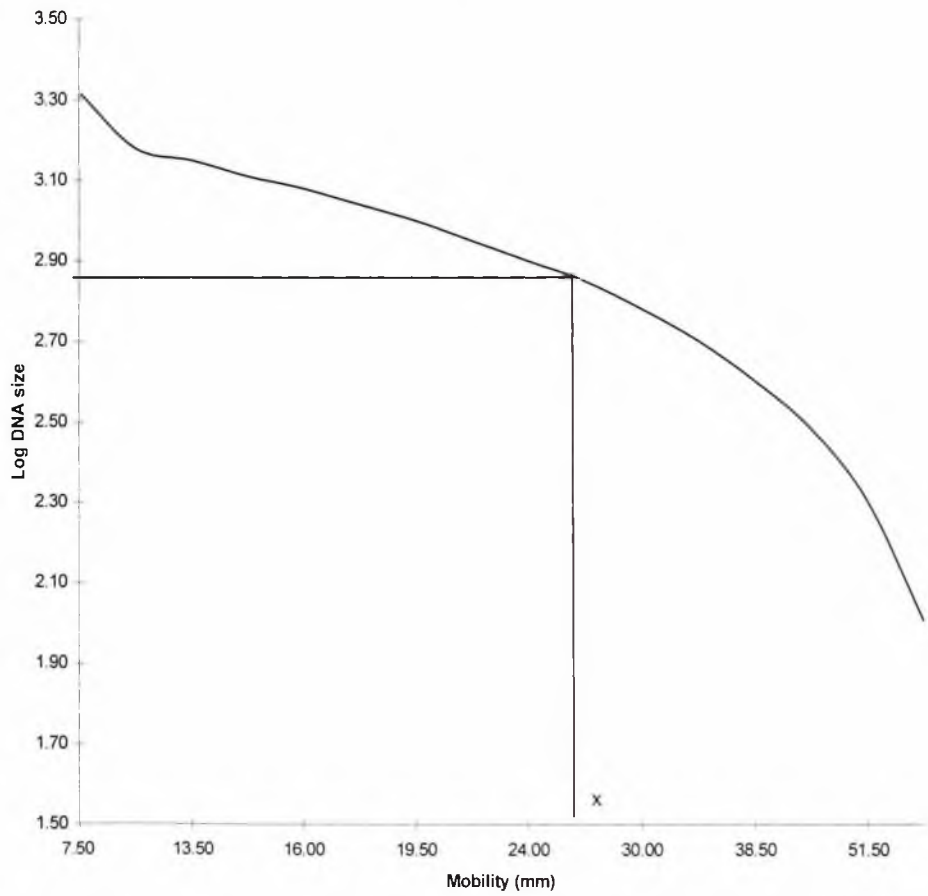
WEIGHT RANGE/kg	DAY 1	DAY 2	DAY 3
3.4 – 7.4	½	½	¼
7.5 – 9.9	¾	¾	½
10.0 – 14.4	1	1	½
14.5 – 18.4	1½	1½	¾
18.5 – 34.9	2½	2½	1

Sulphadoxine/Pyrimethamine (Fansidar) doses in mg/kg body weight using 500mg S + 25mg P tablets.

WEIGHT RANGE/kg	FANSIDAR
5.0 – 6.0	¼
7.0 – 11.0	½
12.0 – 17.0	¾
18.0 – 22.0	1
23.0 – 25.0	1 ¼

Appendix IV

AN EXAMPLE PLOT OF LOG MOLECULAR WEIGHT AGAINST MOBILITY TO DETERMINE SIZES OF PCR PRODUCTS



The size of a DNA fragment that has a mobility x is estimated by extrapolating on to the Y-axis. The anti-log of the figure where it transects the Y-axis is the molecular weight of the fragment

Appendix V

DATA FROM FIELD AND LABORATORY WORK

I) Demographic data and parasitaemia for Hohoe and Navrongo

Definition of codes and numbers in the data

Site code:

1 – Hohoe

3 – Navrongo

Age in months

Sex:

1 – Male

2 – Female

Trt resp. – Treatment response

1 – Sensitive

2 – Resistant

Class – Classification of resistance

0 – Sensitive

1 – RI

2 - RII

3 - RIII

d-0 – Day 0

d-3 – Day 3

d-7 – Day 7

d-14 – Day 14

d-21 – Day 21

Site code	idno.	age	sex	trtresp	class	Parasite count/µl d-0	d-3	d-7	d-14	d-21
1	3	21	1	2	1	3480	40	0	1400	0
1	4	9	2	2	3	2680	1360	0	10680	2840
1	10	14	2	2	3	2000	9960	0	0	0
1	12	11	2	2	1	12680	120	40	20160	0
1	23	36	2	2	3	19520	2200	7440	13000	1680
1	33	58	2	2	1	96800	0	0	2560	0
1	59	14	1	2	1	32000	0	280	920	0
1	60	8	2	2	1	14400	40	800	40800	0
1	130	28	2	2	3	44800	15200	0	0	0
1	132	48	1	2	1	56800	0	0	9880	0
1	141	20	1	2	2	25600	5600	0	0	0
1	149	48	1	2	1	4800	0	0	2320	0
1	153	18	2	2	1	79200	280	0	1160	0
1	257	28	2	2	1	24080	0	0	3560	120
1	290	16	1	2	1	3400	0	0	120	46720
1	303	7	1	2	2	50400	160	0	40600	0
1	305	13	2	2	2	29600	320	1400	3440	160
1	307	23	1	2	1	46800	0	0	0	2680
1	313	56	2	2	2	122240	440	160	1200	0
1	318	48	2	2	1	138960	0	40	9920	0
1	324	17	1	2	2	72080	520	640	4880	0
1	329	36	2	2	2	116080	18600	440	4960	0
1	353	30	2	2	2	151200	1480	0	2000	0
1	357	59	1	2	1	127720	200	0	9800	0
1	362	30	2	2	2	3920	360	600	0	40
1	364	15	1	2	3	125520	52000	0	0	61200
1	371	23	2	2	2	2280	360	1040	560	0
1	374	28	2	2	1	22720	0	0	160	0
1	376	20	2	2	1	5920	0	4400	0	0
1	379	9	2	2	1	120000	0	0	240	1880
1	383	54	2	2	3	32840	8760	48800	0	0
1	397	13	1	2	2	272000	160	0	360	0
1	455	8	2	2	2	92600	3480	0	1000	400
1	468	20	2	2	1	34160	0	0	0	5200
1	479	26	1	2	1	56800	0	0	10640	0
1	482	42	1	2	1	72000	0	0	0	69240
1	552	24	2	2	2	124600	4080	0	91200	0
1	558	12	1	2	1	576040	0	0	5360	0
1	584	52	2	2	1	8160	0	0	0	130000
1	585	25	2	2	1	25120	0	480	320	0
1	586	28	2	2	1	93040	0	0	800	26200
1	610	8	2	2	3	9680	15400	0	1120	8440
1	620	36	2	2	1	15880	0	0	0	560
1	684	18	2	2	2	4040	120	0	200	40

Site code	idno.	age	sex	trtresp	class	Parasite count/µl d-0	d-3	d-7	d-14	d-21
1	685	31	1	2	1	120000	0	0	17280	0
1	690	34	2	2	1	3440	0	0	1400	0
1	700	9	1	2	1	6960	0	0	0	440
1	703	20	1	2	1	3360	0	0	0	1160
1	707	14	1	2	2	2440	120	240	120	0
1	709	6	2	2	2	2480	0	0	40	120
1	710	9	2	2	2	2480	80	40	600	0
1	713	13	2	2	2	22000	840	720	0	40
1	730	33	1	2	2	3400	1080	120	41600	9600
1	731	28	2	2	1	4880	0	0	0	19840
1	733	17	1	2	1	15360	0	0	0	160
1	735	15	2	2	3	14000	1920	2320	0	6200
1	741	16	1	2	1	6320	40	0	0	1520
1	742	29	2	2	1	8960	0	0	200	1160
1	744	27	2	2	2	2360	200	0	0	4800
1	745	24	1	2	2	3720	240	0	0	24200
1	747	12	2	2	1	80960	0	40	80	0
1	945	12	1	2	1	128000	26520	0	0	0
1	949	21	2	2	1	4400	3040	0	0	0
1	956	59	2	2	2	10600	0	0	1280	0
3	14	26	2	2	1	6480	0	0	7480	0
3	36	19	2	2	2	87200	240	0	5520	0
3	66	25	2	2	1	12080	0	0	360	0
3	154	9	1	2	1	8840	0	0	14880	0
3	155	24	1	2	1	2560	0	0	13920	0
3	157	19	2	2	2	17120	160	0	21040	0
3	162	11	2	2	3	72800	16280	0	14440	0
3	170	45	1	2	2	12720	120	0	2720	0
3	182	27	1	2	1	13320	0	0	0	720
3	237	37	2	2	1	16520	0	0	5120	0
3	240	30	2	2	1	28920	0	3360	1120	0
3	248	9	1	2	2	20840	840	0	2240	0
3	255	21	1	2	1	55280	0	0	2520	0
3	258	22	1	2	2	75360	8240	0	1920	0
3	263	40	1	2	1	20240	0	0	7320	0
3	269	24	2	2	1	35360	0	0	1040	0
3	279	33	2	2	1	39440	0	8240	0	0
3	313	19	1	2	1	2080	0	0	8600	0
3	315	34	1	2	2	380800	4800	0	2320	0
3	319	.	2	2	1	3440	0	0	9280	0
3	337	25	1	2	2	108800	28320	0	0	0
3	341	15	2	2	3	34640	5080	8240	8360	0
3	343	16	1	2	3	174400	1520	0	39440	0
3	403	14	2	2	3	8240	640	0	16800	0

Site code	idno.	age	sex	trtresp	class	Parasite count/µl d-0	d-3	d-7	d-14	d-21
3	411	23	2	2	3	127680	0	80240	159680	0
3	412	23	2	2	3	87320	0	11280	12960	0
3	420	15	1	2	2	17280	0	22400	0	0
3	445	33	2	2	2	16960	4800	5840	0	0
3	447	49	1	2	2	62800	120	12640	0	0
3	450	43	2	2	3	28400	0	0	3280	0
1	13	36	1	1	0	28000	0	0	0	0
1	14	24	1	1	0	4760	0	0	240	0
1	16	59	1	1	0	3240	0	0	0	0
1	46	30	1	1	0	5000	0	0	0	0
1	47	22	2	1	0	16800	0	0	0	0
1	54	42	1	1	0	34000	0	0	0	0
1	550	16	1	1	0	96800	0	0	0	0
1	551	19	1	1	0	4080	0	0	0	0
1	567	56	2	1	0	95200	800	2680	0	0
1	572	48	2	1	0	15200	80	0	0	0
1	599	24	2	1	0	2560	0	0	0	0
1	605	36	1	1	0	130000	4880	0	0	0
1	609	6	2	1	0	33440	0	0	0	0
1	663	25	2	1	0	2760	0	0	0	0
1	695	20	2	1	0	4400	0	0	40	0
1	697	29	2	1	0	8800	0	0	0	0
1	698	38	1	1	0	16600	0	120	0	0
1	699	27	1	1	0	2120	0	0	0	0
1	708	36	1	1	0	10360	160	0	0	0
1	712	8	2	1	0	22520	80	0	0	0
1	714	40	2	1	0	3760	0	0	0	0
1	729	56	1	1	0	2040	360	40	0	0
1	732	43	2	1	0	2240	0	0	0	0
1	738	38	1	1	0	5960	0	0	0	0
1	739	51	1	1	0	9680	0	0	40	0
1	740	13	2	1	0	7520	0	0	0	0
1	743	30	2	1	0	11800	80	0	0	0
1	944	25	1	1	0	3600	0	0	0	0
1	946	46	1	1	0	91400	0	0	0	0
1	947	35	1	1	0	10400	600	0	0	0
1	948	15	2	1	0	2320	680	0	0	0
1	950	49	1	1	0	5040	0	0	0	0
1	951	49	2	1	0	5040	0	0	0	0
1	952	25	1	1	0	3320	0	0	0	0
1	954	10	1	1	0	3000	0	0	0	0
1	955	10	1	1	0	17600	0	0	0	0
1	957	42	1	1	0	51120	0	0	0	0
1	958	37	1	1	0	2440	0	0	0	0

Site code	idno.	age	sex	trtresp	class	Parasite count/µl d-0	d-3	d-7	d-14	d-21
1	959	35	1	1	0	140000	0	0	0	0
3	3	51	1	1	0	27320	120	0	0	0
3	5	58	1	1	0	19560	0	0	0	0
3	6	33	2	1	0	23880	0	0	0	0
3	17	36	1	1	0	2480	0	0	0	0
3	45	24	2	1	0	83560	80	0	0	0
3	61	51	1	1	0	86720	0	0	0	0
3	67	6	1	1	0	3320	0	0	0	0
3	77	32	1	1	0	37120	280	0	0	0
3	83	17	2	1	0	23560	0	0	0	0
3	145	59	2	1	0	30720	0	0	0	0
3	159	13	1	1	0	8240	0	0	0	0
3	164	58	2	1	0	9160	0	0	0	0
3	166	14	2	1	0	73040	1120	0	0	0
3	171	20	1	1	0	3320	0	0	0	0
3	173	27	1	1	0	1600	0	0	0	0
3	176	48	2	1	0	35240	0	0	0	0
3	177	12	1	1	0	24680	0	0	0	0
3	183	21	2	1	0	16040	0	0	0	0
3	185	51	1	1	0	8120	0	0	0	0
3	188	32	1	1	0	74720	0	0	0	0
3	189	49	2	1	0	3080	19200	0	0	0
3	197	14	2	1	0	76920	6520	0	0	0
3	198	16	2	1	0	24080	0	0	0	0
3	202	10	1	1	0	13080	0	0	0	0
3	205	21	2	1	0	4920	0	720	0	0
3	207	9	1	1	0	73040	720	0	0	0
3	234	42	1	1	0	8240	0	0	0	0
3	238	59	1	1	0	24960	0	0	0	0
3	243	39	2	1	0	12760	0	0	0	0
3	247	50	2	1	0	25120	0	0	0	0
3	251	52	1	1	0	16600	0	0	0	0
3	254	52	2	1	0	32080	0	0	0	0
3	256	32	1	1	0	12360	0	0	0	0
3	260	50	2	1	0	2840	0	0	0	0
3	264	40	1	1	0	18720	0	0	0	0
3	268	27	1	1	0	37040	1120	0	0	0
3	273	10	1	1	0	690440	8640	0	0	0
3	276	7	2	1	0	4960	0	0	0	0
3	304	15	2	1	0	95200	0	0	0	0
3	323	35	2	1	0	42880	0	0	0	0
3	329	12	2	1	0	2560	0	0	0	0
3	332	36	2	1	0	113600	0	0	0	0
3	339	14	1	1	0	78720	0	0	0	0

Site code	idno.	age	sex	trtresp	class	Parasite count/µl d-0	d-3	d-7	d-14	d-21
3	342	56	1	1	0	29040	0	0	0	0
3	404	6	2	1	0	6160	0	0	0	0
3	405	17	2	1	0	20240	0	0	0	0
3	406	25	1	1	0	16080	1120	0	0	0
3	407	24	1	1	0	7480	0	0	0	0
3	409	31	1	1	0	80240	0	0	0	0
3	413	19	1	1	0	3520	0	0	0	0
3	414	50	2	1	0	49120	0	0	0	0
3	416	42	1	1	0	31840	0	0	0	0
3	423	37	2	1	0	9280	0	440	0	0
3	424	10	1	1	0	32800	0	0	0	0
3	426	41	1	1	0	2480	0	0	0	0
3	428	8	1	1	0	1560	0	0	0	0
3	431	24	2	1	0	71200	0	0	0	0
3	436	51	1	1	0	72800	0	0	0	0
3	437	20	1	1	0	110720	1520	0	0	0
3	438	46	1	1	0	76480	0	0	0	0
3	441	38	1	1	0	8400	0	0	0	0
3	442	34	2	1	0	71600	0	0	0	0
3	443	15	1	1	0	18800	160	0	0	0
3	444	11	2	1	0	10200	80	0	0	0
3	448	14	1	1	0	32320	0	0	0	0
3	451	20	1	1	0	242400	0	0	0	0

II) Data from PCR assays at pre-treatment for Hohoe and Navrongo

Definitions of codes and numbers in the data.

Site code

1 – Hohoe

3 – Navrongo

Id no. – Identification number

Trt. Resp. – Treatment response

1 – Sensitive

2 – Resistant

pfcrt – pfcrt 76

pfmdr1 – pfmdr1 86

dr-51 – dhfr 51

dr-59 – dhfr 59

dr-108 – dhfr 108

ds-437 – dhps 437

ds-540 – dhps 540

For all the gene codons

0 – no alleles

1 – mutant allele only

2 – wildtype allele only

3 – mixed alleles

Site				Mutation specific PCR for the four gene loci at day 0					
code	id no.	trt resp.	pfcrt	pfmdr1	dr-51	dr-59	dr-108	ds-437	ds-540
1	3	2	1	1	0	0	0	0	0
1	4	2	1	1	0	0	0	0	0
1	10	2	3	1	0	0	0	0	0
1	12	2	1	1	3	3	3	3	2
1	23	2	1	1	3	3	3	1	3
1	33	2	3	1	3	3	3	3	3
1	59	2	3	1	2	1	3	1	3
1	60	2	1	1	2	3	3	1	3
1	130	2	1	1	0	1	3	1	3
1	132	2	1	1	1	3	3	1	3
1	141	2	3	1	3	3	3	3	3
1	149	2	3	1	1	3	3	1	3
1	153	2	1	1	3	3	3	1	3
1	257	2	1	1	3	3	1	1	3
1	290	2	1	0	3	2	3	1	3
1	303	2	1	1	3	3	3	3	3
1	305	2	1	1	3	3	3	1	3
1	307	2	1	1	3	2	3	1	3
1	313	2	1	1	3	3	3	1	3
1	318	2	1	1	3	3	3	3	3
1	324	2	1	1	3	3	3	1	3
1	329	2	3	1	2	3	3	1	2
1	353	2	3	1	3	3	3	1	3
1	357	2	3	1	2	3	3	3	3
1	362	2	1	1	3	3	3	3	3
1	364	2	3	1	3	3	3	1	3
1	371	2	1	1	3	1	3	1	2
1	374	2	1	1	1	1	1	1	3
1	376	2	1	1	3	1	3	1	3
1	379	2	1	1	3	3	3	1	3
1	383	2	1	1	1	1	1	1	3
1	397	2	2	1	3	1	3	1	3
1	455	2	1	1	0	0	0	0	0
1	468	2	1	1	1	1	2	1	3
1	479	2	2	0	1	0	0	0	2
1	482	2	2	0	2	0	2	1	2
1	552	2	3	1	1	1	3	1	3
1	558	2	3	1	3	0	3	1	3
1	584	2	3	1	3	3	3	1	3
1	585	2	1	1	3	3	3	3	3
1	586	2	3	1	3	3	3	1	3
1	610	2	1	1	0	0	0	0	0

Site code	id no.	trt resp.	pfcrt	pfmdr1	Mutation specific PCR for the four gene loci at day 0 dr-51	dr-59	dr-108	ds-437	ds-540
1	620	2	3	0	3	0	0	0	0
1	684	2	1	1	3	1	1	3	2
1	685	2	2	1	0	0	0	0	2
1	690	2	2	1	0	0	0	3	2
1	700	2	3	1	0	0	0	0	0
1	703	2	3	1	1	3	1	1	3
1	707	2	1	1	2	1	1	1	1
1	709	2	3	1	0	0	0	0	0
1	710	2	3	1	3	1	1	1	3
1	713	2	1	1	3	3	3	2	3
1	730	2	1	1	0	2	0	3	2
1	731	2	3	1	2	2	3	0	2
1	733	2	3	1	1	3	3	3	3
1	735	2	3	1	3	3	3	3	3
1	741	2	3	1	2	0	3	3	3
1	742	2	3	0	0	0	0	3	2
1	744	2	3	1	2	3	2	3	2
1	745	2	3	1	1	2	1	1	3
1	747	2	3	1	1	3	3	2	2
1	945	2	2	0	0	1	2	3	0
1	949	2	1	1	0	3	1	0	0
1	956	2	3	0	2	1	3	1	0
3	14	2	1	1	2	1	3	0	0
3	36	2	3	0	2	3	3	0	2
3	66	2	1	1	2	3	2	0	0
3	154	2	1	0	0	0	0	0	0
3	155	2	2	1	0	0	0	0	0
3	157	2	2	0	0	0	0	0	0
3	162	2	1	1	0	0	0	0	0
3	170	2	2	0	0	0	0	0	0
3	182	2	2	0	0	0	0	0	0
3	237	2	1	1	0	1	3	0	0
3	240	2	1	1	0	1	1	0	0
3	248	2	1	1	1	1	1	0	0
3	255	2	1	1	0	1	0	0	2
3	258	2	3	1	2	3	3	0	0
3	263	2	1	1	0	0	0	0	0
3	269	2	1	0	0	0	0	0	0
3	279	2	1	1	0	0	0	0	0
3	313	2	2	1	0	0	0	0	0
3	315	2	2	1	0	0	0	0	0
3	319	2	2	0	0	0	0	0	0

Site code	id no.	trt resp.	pfcrt	Mutation specific PCR for the four gene loci at day 0 pfmdr1	dr-51	dr-59	dr-108	ds-437	ds-540
3	337	2	2	0	0	0	0	0	0
3	341	2	1	1	2	1	3	0	2
3	343	2	2	0	2	3	2	0	0
3	403	2	2	1	2	0	2	0	2
3	411	2	2	1	3	1	1	2	2
3	412	2	2	1	3	0	3	2	3
3	420	2	2	1	2	1	1	2	0
3	445	2	2	1	2	0	2	3	2
3	447	2	2	1	2	1	3	3	2
3	450	2	2	1	0	0	0	2	0
1	13	1	1	0	0	0	0	3	3
1	14	1	2	1	0	1	3	1	3
1	16	1	2	0	0	0	0	3	0
1	46	1	3	1	2	2	1	1	3
1	47	1	3	1	3	2	3	3	3
1	54	1	0	0	0	0	0	0	0
1	550	1	3	1	2	3	1	3	3
1	551	1	3	1	2	3	3	3	3
1	567	1	3	1	3	0	1	3	2
1	572	1	3	1	1	1	3	1	3
1	599	1	2	0	0	2	1	3	0
1	605	1	2	0	0	1	0	3	2
1	609	1	1	0	0	0	0	3	0
1	663	1	2	0	0	0	0	3	3
1	695	1	1	1	3	1	1	3	3
1	697	1	1	1	0	1	0	3	0
1	698	1	3	1	0	0	0	3	2
1	699	1	3	1	0	0	0	3	2
1	708	1	3	0	0	0	0	3	0
1	712	1	3	1	0	0	0	3	3
1	714	1	3	1	0	0	0	3	2
1	729	1	3	1	3	0	1	1	3
1	732	1	2	0	0	0	0	0	0
1	738	1	1	0	0	0	2	0	2
1	739	1	2	1	0	0	0	1	2
1	740	1	3	1	3	3	3	3	3
1	743	1	3	1	0	0	0	3	3
1	944	1	2	0	0	1	3	2	0
1	946	1	3	1	2	0	3	3	3
1	947	1	2	1	0	1	3	0	3
1	948	1	2	1	0	3	1	0	2
1	950	1	2	0	2	0	0	0	0

Site				Mutation specific PCR for the four gene loci at day 0					
code	id no.	trt	resp.pfcrt	pfmdr1	dr-51	dr-59	dr-108	ds-437	ds-540
1	951	1	3	1	2	0	2	0	0
1	952	1	3	1	0	0	3	2	0
1	954	1	3	1	0	1	1	2	2
1	955	1	2	1	0	0	1	1	2
1	957	1	3	1	0	1	3	1	2
1	958	1	3	1	0	1	1	2	0
1	959	1	3	1	0	1	3	3	2
3	3	1	1	1	0	0	0	0	0
3	5	1	2	0	0	0	0	0	0
3	6	1	2	0	0	0	0	0	0
3	17	1	1	1	0	0	0	0	0
3	45	1	1	1	0	0	0	0	0
3	61	1	2	0	0	0	0	0	0
3	67	1	1	0	0	0	0	0	0
3	77	1	2	0	0	0	0	0	0
3	83	1	1	1	0	0	0	0	0
3	145	1	2	0	2	0	2	0	0
3	159	1	1	1	0	0	0	0	0
3	164	1	2	0	0	0	0	0	0
3	166	1	1	1	0	0	0	0	0
3	171	1	1	1	0	0	0	0	0
3	173	1	2	0	0	0	0	0	0
3	176	1	2	0	0	0	0	0	0
3	177	1	2	1	0	0	0	0	0
3	183	1	2	0	0	0	0	0	0
3	185	1	2	0	0	0	0	0	0
3	188	1	2	0	0	0	0	0	0
3	189	1	2	0	0	0	0	0	0
3	197	1	2	0	0	0	0	0	0
3	198	1	2	0	0	0	0	0	0
3	202	1	2	0	0	0	0	0	0
3	205	1	1	1	0	0	0	0	0
3	207	1	0	0	0	0	0	0	0
3	234	1	3	1	0	0	0	0	0
3	238	1	3	1	0	0	0	0	0
3	243	1	1	1	0	0	0	0	0
3	247	1	1	1	0	0	0	0	0
3	251	1	3	1	0	0	0	0	0
3	254	1	2	0	0	0	0	0	0
3	256	1	2	0	0	0	0	0	0
3	260	1	1	0	0	0	0	0	0
3	264	1	1	1	0	0	0	0	0

Site code	id no.	trt resp.	pfcrt	pfmdr1	Mutation specific PCR for the four gene loci at day 0 dr-51	dr-59	dr-108	ds-437	ds-540
3	268	1	1	1	0	0	0	0	0
3	273	1	1	1	0	0	0	0	0
3	276	1	1	1	0	0	0	0	0
3	304	1	3	1	2	2	2	0	2
3	323	1	2	0	2	3	3	0	2
3	329	1	1	0	0	3	3	0	2
3	332	1	1	1	2	2	2	0	0
3	339	1	1	0	2	1	0	0	0
3	342	1	1	1	0	0	0	0	0
3	404	1	2	1	2	1	3	2	2
3	405	1	2	1	2	1	3	2	2
3	406	1	2	1	3	1	3	3	3
3	407	1	2	1	0	0	1	2	0
3	409	1	2	1	2	1	1	2	2
3	413	1	1	1	0	0	1	2	3
3	414	1	2	0	0	0	0	2	0
3	416	1	2	0	2	0	2	2	0
3	423	1	1	1	3	1	3	2	2
3	424	1	2	1	2	1	0	3	3
3	426	1	2	1	2	0	3	0	0
3	428	1	2	1	0	3	3	2	3
3	431	1	2	0	2	3	3	2	2
3	436	1	2	1	2	0	3	3	3
3	437	1	2	1	2	1	3	3	2
3	438	1	2	0	0	0	2	2	0
3	441	1	1	1	2	0	3	2	2
3	442	1	1	1	1	1	3	2	2
3	443	1	2	1	2	0	3	2	2
3	444	1	2	0	0	2	0	3	0
3	448	1	2	1	2	0	3	3	2
3	451	1	2	1	2	1	3	3	2

III) Data from *in-vitro* chloroquine sensitivity test on *P. falciparum* isolates from Hohoe and Navrongo

Definitions of codes and numbers in the data

Site code

1 – Hohoe

3 – Navrongo

Id no. – Identification number

Trt. Resp. – *In-vivo* treatment response

1 – Sensitive

2 – Resistant

Concentration of chloroquine pmol

1 pmol

2 pmol

4 pmol

8 pmol

16 pmol

32 pmol

64 pmol

In-vitro response

S – Sensitive

R - Resistant

Site Code	id no.	trt resp	Percentage inhibition at concentrations of chloroquine pmol 1	2	4	8	16	32	64	In-vitro resp
1	946	1	32	37.6	46	100	100	100	100	S
1	947	1	18	12.5	16.7	100	100	100	100	S
1	949	2	11	9.1	14.4	100	100	100	100	S
1	951	1	25.2	22.7	18	100	100	100	100	S
1	952	1	0	0	0	100	100	100	100	S
1	954	1	2.9		0	12.5	100	100	100	R
1	958	1	3.8	25.3	100	100	100	100	100	S
1	959	1	27.2	100	100	100	100	100	100	S
3	403	2	0	0	0	0	0	0	100	R
3	404	1	11	7	0	100	100	100	100	S
3	405	1	0	1	7	100	100	100	100	S
3	406	1	0	8	1	2	4	21	100	R
3	407	1	41	43	100	100	100	100	100	S
3	409	1	3	2	18	100	100	100	100	S
3	411	2	0	11	26	9	100	100	100	R
3	412	2	0	0	0	0	0	100	100	R
3	414	1	0	0	100	100	100	100	100	S
3	418		0	0	0	0	0	53		R
3	420	2	36	31	64	100	100	100	100	S
3	424	1	0	0	0	3	9	100	100	R
3	437	1	0	0	4	100	100	100	100	S
3	440		0	7	14	100	100	100	100	S
3	443	1	0	3	0	100	100	100	100	S
3	445	2	9	7	7	100	100	100	100	S
3	447	2	32	100	100	100	100	100	100	S
3	448	1	5	8	2	100	100	100	100	S