MOLECULAR ASSESSMENT OF ANTIMALARIAL DRUGS RESISTANCE IN *PLASMODIUM FALCIPARUM* AT NUBA MOUNTAINS, SOUTHERN SUDAN.

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A thesis submitted to the Graduate School in partial fulfillment for the requirement of the Degree of Master of Science in Biochemistry of Egerton University

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DECLARATION AND RECOMMENDATION

DECLARATION

This thesis and the results presented in it are entirely my own work except where indicated, and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to my loving parents, Udu Mohdhar and Maryam Mohammed
and
my son Muhsin and daughter Maryam

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I am very thankful to my loving husband Rashid for his understanding, support and encouragement while I was away in college and in Glasgow during the course of this study. I would like to express my sincere gratitude to my parents for the sacrifice they made hence giving me a chance to attain some education, support and always in their prayers.

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ABSTRACT

One of the major obstacles to the treatment of malaria is the emergence and spread of parasite resistant to antimalarial drugs. Both northern and southern Sudan is deploying artemisinin-based combinations against uncomplicated *Plasmodium falciparum* malaria. In this study, the efficacy of artesunate (AS) in combination with sulfadoxine-pyrimethamine (SP) or amodiaquine (AQ) was evaluated. This study aimed at determining the prevalence of antimalarial drug resistance markers in *P. falciparum* isolates from Nuba Mountains, which involves the detection of mutations at the *Pfcrt* and *mdr 1- 86* that associates with chloroquine resistance and amodiaquine resistance, and *dhfr* mutations which are associated with SP resistance.

Samples in this study were obtained from Nubian region of Sudan. A total of 160 field isolates that were from patients of between 6 months and 10 years were recruited for the study and four positive controls were analyzed, 80 of the patients were of AS/AQ treatment arm and the other 80 were treated with AS/SP combination. The investigation involved collecting blood samples from malaria patients, extracting the DNA of *Plasmodium falciparum* from these blood samples, amplifying the extracted DNA using PCR method and finally analysing the amplified products using RFLP for *Pfcrt* and *dhfr* gene markers and radioisotopic method to determine the prevalence of *mdr1- 86*.

Detection for mutations at the *P. falciparum* chloroquine transporter (*Pfcrt*) gene (associated with CQ resistance) from pre-treatment blood samples was analysed to correlate the existing use and resistance to Chloroquine. The *Pfcrt* K76T mutation occurred in 90.0% of infections, suggesting CQ would work poorly in this region. Furthermore, among patients with failure endpoints, genotyping of merozoite surface proteins 1 and 2 (*msp-1* and *msp-2*) was employed to compare the pre- and post-treatment to establish whether failure was due to true recrudescence or a novel infection.

The prevalence of the *dhfr* point mutation among the field isolates of *P. falciparum* was established, and the result correlated between these mutations and resistance of

isolates to antimalarial. Overall, 82.5% carried mutations at *dhfr,* but triple mutants were rare (3.1%). The dot-blot/ probe hybridization, which is more sensitive and specific, was adopted to determine multi-drug resistance (*mdr1-86*). Baseline dot-blot hybridisation of the *mdr1* 86 locus shows 33 (89.19%) carried parasites harboring the *mdr1*-86 Tyr resistance allele, while 3 (8.11%) carried *mdr1*-86 Asn sensitivity allele and 1 (2.7%) were of mixed infection, having both resistance and wild type allele.

The research presented here shows that the number of mutations present in *dhfr* has a significant effect on the type of treatment following SP chemotherapy. SP resistance may propagate rapidly, and AS + AQ is likely to be a better long-term option, provided AQ use is limited to the combination. The significance of the study shows that indeed combination of drugs improves SP therapy at the study site.

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LIST OF ABBREVIATIONS

ACT	Artemisinin based combination therapy
ACR	Adequate clinical response
AdoMetDC	D- adenosylmethionine decarboxylase
AIDS	Acquired immune deficiency syndrome
ARDS	Acute respiratory distress syndrome
Arg	Arginine
Asn	Asparagine
ATP	Adenosine triphosphate
CBRD	Center for Biotechnology Research and Development
Ci	Curie
CS	Circumsporozoites
CSO	Circumsporozoite proteins
CT	Combination therapy
CQ	Chloroquine
dd H_2O	Double distilled water
Dhfr	Dihydrofolate reductase
Dhfr-TS	Dihydrofolate reductase- thymidylate synthase
Dhps	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
DNA-ME-TRAP	Deoxyribonucleic acid-malaria epitopes-thrombospondin related adhesion protein
EIR	Entomological inoculation rate
EPSPS	5- enolpyruvyl shikimate 3-phosphate synthase
RPGS	Roly-poly glutamate synthetase
GDP	Gross domestic product
G6PD	Glucose 6 phosphate dehydrogenase
GLURP	Glutamate rich protein

HBr	Hepatitis B virus
HRPII	Histidine rich protein
HIV	Human immuno deficiency virus
IC_{50}	50% Inhibitory concentration
KEMRI	Kenya Medical Research Institute
K_i	Inhibition constant
MLP	Monophosphoryl lipid
MoH	Ministry of Health
Msp1, 2,	Merozoite surface proteins, 1 or 2.
MVA	Modified vaccinia virus Ankara
ODC	Ornithine decarboxylase
ORF	Open reading frame
PAR	Population at risk
PABA	Para amino benzoic acid
Pfmdr1	*Plasmodium falciparum* multi drug resistance gene-1
Pfcrt	*Plasmodium falciparum* chloroquine resistance gene
PBS	Phosphate buffered saline
pM	Pico moles
PY	Pyrimethamine
RBC	Red blood cells
SK	Shikimate kinase
SP	Sulfadoxine-pyrimethamine
Tyr	Tyrosine
UTL	Useful therapeutic life
WHO	World Health Organization

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

The existence of malaria is dated as early as 1897 even before it was found that mosquito bites were the cause of malaria (Ross, 1897). Burton (1856) in his "First step in Africa had this to say: According to the Somali people, the mosquito bite brings deadly fever. The superstition probably arise from the fact that mosquito and fever become formidable at the same time"

One of the major contributors of the increase of disease burden is drug resistance, which has threatened the use of chemotherapy as a weapon against *falciparum* malaria. Several countries have already abandoned Chloroquine (CQ) in favor of Sulfadoxine-Pyrimethamine (SP) (Fansidar®). This is due to the emergence and worsening rise of CQ resistance (Sansoetti *et al*., 1985; Delfini, 1989; Rapouda *et al*, 1997; Shretta *et al*., 2000). However CQ is still being used in Sudan as second line treatment for malaria, and SP as first line drug for malaria treatment owing to the increasing number of CQ treatment failures. Despite countries changing treatment policies in favour of SP, development and rapid spread of SP resistant strains (White, 1998; Takechi *et al*., 2001) has threatened its use. This has necessitated that better treatment regimens be sought to replace SP. Combination therapy (CT) especially Artemisinin based combination therapy (ACT) seems to be the best option at the moment. This study evaluated the efficacy of Artesunate in combination with (SP) or Amodiaquine (AQ). Molecular techniques were employed to determine prevalence of CQ resistance, establish prevalence of the point mutations, and differentiate recrudescence and re-infection.

1.1.1 The malaria problem

Until the late 1960's, malaria was not only found in tropical areas of Africa, Latin America and Asia but also in large parts of Europe, in parts of the USA and in Northern Australia. Malaria now exists in 100 countries but is mainly confined to poorer tropical areas of Africa, Asia and Latin America, with 2,400 million persons, about a third of the World's population is at risk. More than 90% of malaria cases and the great majority of

malaria deaths occur in tropical Africa mainly due to *P. falciparum* infections. Each year an estimated 300 to 660 million clinical cases of malaria occur worldwide and an estimate of between 1.5 and 2.7 million deaths are attributed to malaria, 75% of which are African children below the age of 5 years (Radloff, 1996; Nchinda, 1998; Snow *et al.*, 1999; Bremen, 2001; Snow *et al.*, 2005).

Malaria is not only a direct cause of death, but also contributes indirectly to death due to respiratory infections, diarrhoea and malnutrition by impairing immunity. Over 60 percent of the Sudan population constituting approximately 10 million Sudanese are at the risk of the disease, with approximately 8.2 million clinical cases and 34,000 deaths annually of children below age five (MSF, 2002; MOH Kenyan/Sudanese, 2004).

1.1.2 Malaria burden

Malaria has economic implications over and above the losses occasioned by the morbidity and mortality of the disease (AID, 1995). A comparison of income in malaria prone and non-prone countries indicates that average gross domestic product (GDP) in malaria countries in 1995 was US$ 1,526 compared with US$ 8,268 in countries without intensive malaria which is more than five fold difference (Gallup and Sachs, 2001). The annual economic burden of malaria in 1995 was estimated as US$8 billion, for Africa alone (Foster and Phillips, 1998). Households in Africa spend between US$2 and US$25 on malaria treatment, and between US$0.20 and US$15 on prevention, each month (WHO, 1999). This calls for an urgent need to control this debilitating infection. The emergence and rapid spread of resistant strains to some antimalarial drugs including chloroquine, a cheap and safe drug, has worsened the malaria burden. Resistance of *P. falciparum* to antifolate chemotherapy is a significant problem where combinations such as Sulfadoxine/Pyrimethamine are used in the treatment of chloroquine-resistant malaria. Antifolate resistance has been associated with variant sequences of dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*), the targets of pyrimethamine and sulfadoxine respectively, but these variations have not been fully studied in Southern Sudan. Resistance to chloroquine is estimated at 66-87% (Shretta *et al.*, 2000), while resistance to SP is over 40% (Omar *et al.*, 2001 a, b; MSF, 2002). However, this has not been established in all the regions in the country especially in the epidemic areas,

implying that successful chemotherapeutic intervention cannot be employed. Thus one of the major obstacles to the control of malaria is the emergence and spread of parasites resistant to anti-malarial drugs and lack of suitable epidemiological drug resistance monitoring techniques. When malaria infection is viewed as a risk factor, increasing parasite exposure has been shown to account for over 60% of the variation in all-cause mortality in young African children (Snow *et al.*, 2005).

A hidden burden is borne by patients who survive the severe consequences of infection and are left with debilitating sequel, such as plasticity or epilepsy (Mung'ala *et al.*, 2004), or subtler consequences including behavioral disturbances or cognitive impairment (Holding and Kitsao-Wekelo., 2004).

1.2 Statement of the problem

Malaria is a major public health problem placing almost half of the World population at risk, with estimated mortality of one million in Africa alone annually. Unless new strategies are deployed to combat malaria, the already enormous health and economic burden related to the disease in tropical countries is bound to worsen. The main obstacle to malaria control is the emergence of drug resistant strains of *Plasmodium falciparum*. The use of combinations of antimalarial drugs reduces the risk of selecting for resistant mutants of the plasmodial parasites. Massive economic assistance will be needed to detect and treat adequately the estimated 515 million cases of malaria per year, but without radical action there is no prospect of 'Rolling Back' malaria (Nosten and Brasseur, 2002; Snow *et al.*, 2005). The combined effects of climate change, population growth and urbanisation on the Population at risk (PAR), the number of people living in areas of climatic suitability for stable *P. falciparum* malaria transmission, of *falciparum* malaria in Africa has been stipulated. The results suggest that the PAR will change from approximately 0.63 billion in 2005, to 0.87 billion in 2015 and 1.15 billion in 2030 (Hay *et al.*, 2006; Snow *et al.*, 2006).

Every year in tropical countries, *P. falciparum* the most dangerous of the four species affecting man, causes almost 270 million deaths in Africa. In Sudan 100% of the population is at risk with 55% at endemic risk.

There is therefore need to asses the mutation profiles of the parasites resistant to Sulfadoxine/ Pyrimethamine (SDX/PRY) drugs from the endemic area to enhance the prediction of treatment outcomes in a given treatment including the use of Artemisinin based Combination Therapy (ACT).

1.3 Justification

The Nuba Mountains region of central Sudan has an estimated population of 1 million, and has been severely affected by warfare. Since the early 1990s, as a result of heavy fighting, its population has suffered severe nutritional and health crises. *Plasmodium falciparum* is the dominant species (99.0%). No data on molecular analysis are available for the Nuba Mountains.

Due to the magnitude of the disease, the dangers posed by the development of drug resistant strains and the promise of Artemisinin combination therapy, it is important to evaluate the efficacy of Artesunate plus SP and Artesunate and AQ, and determine which of this combination is a viable option for treating uncomplicated *falciparum* malaria at Nuba. The multiplicity of infection is one of the important factors associated with drug resistance.

1.4 Objectives

1.4.1 General objective

To assess the prevalence of antimalarial drug resistance markers in *P. falciparum* isolates from Nuba Mountains, Southern Sudan.

1.4.2 Specific objectives

- To determine the prevalence of *Pfcrt* and *mdr1*-86 mutations in *Plasmodium falciparum.*
- To determine the prevalence of *dhfr* mutations in *Plasmodium falciparum.*
- To differentiate recrudescence from new infection using MSP-I and MSP-II gene marker.

1.5 Null Hypothesis

The prevalence of point mutations in the *Pfcrt, mdr1 and dhfr* genes responsible for resistance to 4-aminoquinoline and antifolate drugs respectively, used in malaria treatment in Nuba Mountains is low.

CHAPTER TWO

2.0 LITERATURE REVIEW

In the present era, malaria is one of the major public health problems. The changing face of worldwide malaria distribution over a century is presented in the Figure 2.0 (Hay *et al.*, 2006). Approximately 40% of the world's populations, mostly those living in the world's poorest countries, are at risk of malaria. Currently malaria is found throughout the tropical and sub-tropical regions of the world and causes more than 300 million acute illnesses and at least one million deaths annually (Hay *et al.*, 2006).

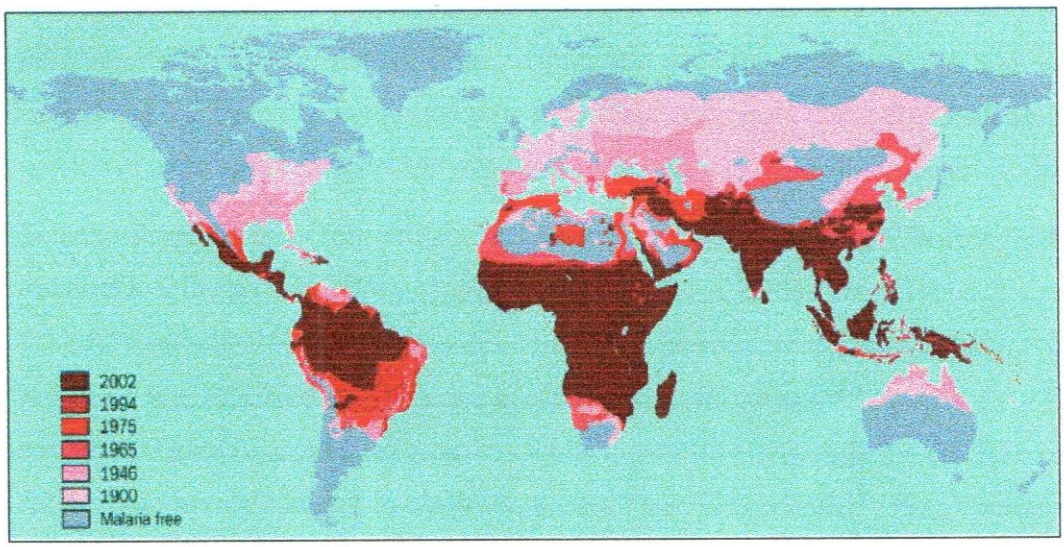


Figure 2.0 : World distribution of malaria over a century (Hay *et al.*, 2006)

2.1 Background to the disease

Malaria is an ancient disease known to mankind for thousands of years. Man and malaria seem to have evolved together and it was always part of the fluctuating fortunes of nations. The first confirmed human case of malaria dates from 450 AD where Polymerase Chain Reaction (PCR) was used to identify *Plasmodium* DNA in skeletal remains (Whitfield, 2002). Epidemiological studies have shown that some red blood cells (RBC) abnormalities confer natural resistance against malaria. The beneficial effect of the genetic disease in people who inherit the genetic factor from only one parent (heterozygous) is protective against malaria whereas the harmful effects of the disease are confined to the much smaller number of people who inherit the genetic factor from both

parents (homozygous). For example β-thalassemia, sickle cell anemia, other hemoglobinopathies like hemoglobin C (HbC) and E (HbE) and RBC enzyme deficiencies like glucose-6-phosphate dehydrogenase deficiency (Ruwende *et al.*, 1995) have been linked to varying degree of protection against severe malaria.

2.2 Current available treatment and prevention against malaria.

2.2.1 Drugs

Anti-infective drugs interfere with aspects of parasite metabolism that differ significantly from the human host (Clough and Wilson, 2001). Eukaryotic protozoal endoparasites like *Plasmodium* species impose a pharmacological challenge in this respect. The deadliest apicomplexan parasite, therefore, has always been studied extensively for finding newer drug targets. Being a disease of the developing and underdeveloped nations, malaria has never been a lucrative target for the pharmaceutical agencies. Of the 1393 new drugs registered between 1975 and 1999 only four were anti-malarials (Trouiller *et al.*, 2002).

To complicate the issue further there is the problem of drug resistance. Resistance to chloroquine, the principal drug of choice against malaria, started since 1960s. It unveiled the ease with which resistance may develop to anti-malarial agents. Chloroquine resistance was followed by resistance to antifolate combinations. At present, the exceptional pace of antifolate resistance limits the useful therapeutic life (UTL) of a sulfadoxine-pyrimethamine combination to about five years (White, 1992; Wernsdorfer, 1994). In order to avoid rapidly growing resistance to the available handful of anti-malarial drugs, multi-drug combination therapy is preferred to monotherapy. The existing drugs belong to four families of compounds namely quinolines, antifolate, artemisinin and miscellaneous (Butler, 2002). The chemical nature of currently available drugs and their utility in current malaria treatment are discussed below.

i): Quinolines: Quinine and synthetic quinine analogues belong to this class. These drugs accumulate in the acid food vacuoles (Krogstad and Schlesinger, 1987) where proteolysis of the globin moiety of ingested red cell haemoglobin provides the growing parasite with essential amino acids. The haem is detoxified by the formation of haemozoin which is a polymer of haem units linked between the central ferric ion of one haem and a carboxylate side-group oxygen of another (Slater *et al.*, 1991). The haem polymerization

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Quinine is a natural white crystalline alkaloid extracted from the bark of the South American cinchona tree. It acts mainly on trophozoite blood stage and also kills gametocytes of *P. vivax*, *P. ovale* and *P. malariae* (Panisko and Keystone, 1990). It was the first drug used in malaria and later superseded by chloroquine. However with the emergence of chloroquine resistance, quinine is now the drug of choice for severe chloroquine-resistant malaria caused by *Plasmodium falciparum*.

Chloroquine (CQ) is N'-(7-chloroquinolin-4-yl)-N, N-diethyl-pentane-1, 4-diamine, a synthetic quinine analogue, and has faster onset of action compared to quinine. Chloroquine is still the drug of choice for uncomplicated malaria. The resistance to chloroquine is associated with point mutations in *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) gene which increases chloroquine efflux from the food vacuole and renders the drug ineffective (Fidock *et al.,* 2000). Resistant parasites also often possess mutated ATP-binding cassette transporter genes reffered to as *Plasmodium falciparum* multi-drug resistance 1 *(Pfmdr1*). All the other drugs are considered for cases of chloroquine-resistant malaria depending on the sensitivity of the parasite species and the severity of the disease.

Mefloquine, 2, 8-bis (trifluoromethyl) quinolin-4-yl]-(2-piperidyl) methanol, is another synthetic quinine analogue and can be used in case of chloroquine resistant malaria. It is the drug of choice for chloroquine- resistant *P. vivax* malaria.

Primaquine is N-(6-methoxyquinolin-8-yl) pentane-1, 4-diamine, another synthetic quinine analogue which is active against exo-erythrocytic stages of *P. vivax* and *P. ovale*

and thus is the drug of choice for malaria recrudescence caused by these species. It can also effectively kill gametocytes of *P. falciparum*.

There are other newer generations of synthetic quinine derivatives like amodiaquine, a 4-aminoquinoline, piperaquine, a bisquinoline, among others. These are mainly used for combination therapies of chloroquine resistant malaria (Greenwood *et al.*, 2005).

ii) Antifolate combinations: These drug combinations act by inhibiting the dihydropteroate synthase (DHPS) domain of the bifunctional 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPK-DHPS) enzyme and the dihydrofolate reductase (DHFR) domain of the bifunctional DHFR-thymidylate synthase (TS) enzyme. These four enzyme domains catalyze four steps of the *de novo* folic acid biosynthesis pathway in *Plasmodium falciparum* (Hyde, 2005). The tetra-hydrofolate produced at the end of these reactions is an essential co-factor for thymidylate synthesis in dividing parasites, necessary for DNA replication. Antifolate combinations exert their therapeutic effect by interrupting the folate synthesis pathway in parasites.

The sulphadoxine-pyrimethamine combination was the most effective and cheap therapy after the emergence of chloroquine resistance. The molecular target for pyrimethamine is the dihydrofolate reductase domain of DHFR-TS enzyme and the target for sulfadoxine is the DHPS domain of the PPK-DHPS enzyme. Emergence of resistance against this combination has now rendered it ineffective to use on its own. Resistance is linked to three or four point mutations in the *dhfr-ts* gene and one or two point mutations in the *ppk-dhps* genes (Hastings, 2004). The combination is, however, used in conjunction with other available drugs.

Proguanil is a pro-drug and most of its activity is exerted through its active metabolite cycloguanil which is also an inhibitor of the dihydrofolate reductase domain of DHFR-TS enzyme. Studies have indicated that proguanil may also possess intrinsic anti-parasitic activity (Fidock and Wellems, 1997). It is mainly used for malaria prophylaxis.

iii) Artemisinins: Artemisinin was isolated from the Chinese shrub *Artemisia annua*.

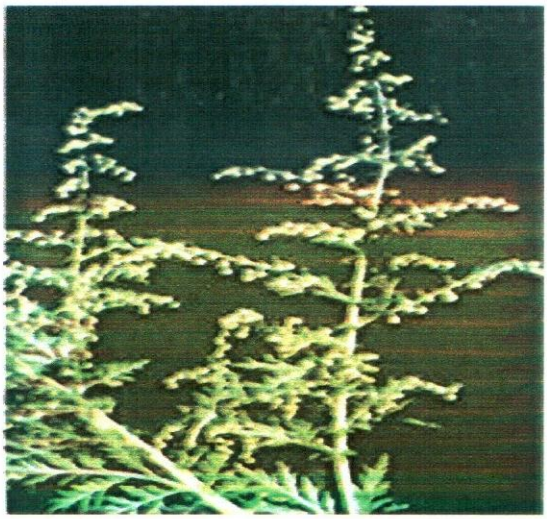


Figure 2.2.1 Artemisia *annua* herb

Its therapeutic effect has been proposed to occur through the generation of reactive oxygen species detrimental to the parasites (Krungkrai and Yuthavong, 1987). Artemisinin and its synthetic analogues are possibly activated by intraparasitic heme or iron to release toxic free radicals (Meshnick *et al.,* 1991) which function as alkylating agents, damaging heme and parasite proteins. However, a sarcoplasmic and endoplasmic reticulum (ER) Ca^{2+} ATPase (SERCA)-type protein encoded by a gene denoted *pfatp6* has also been suggested as a major chemotherapeutic target for this class of drugs (Eckstein-Ludwig *et al.,* 2003). Thus it remains to be definitely shown how these drugs work. Dihydroartemisinin, the active metabolite of artemisinin and synthetic derivatives like artemether, arteether and artesunate are used for the artemisinin based combination therapy (ACT) with other class of drugs including, antifolates, and amino alcohol derivatives, among others. Though highly effective, ACT use is ten times more expensive and therefore their use is limited (Greenwood *et al.,* 2005). ACT is presently the treatment of choice for multi-drug resistant malaria. Though sporadic clinical artemisinin resistance has been reported it has not been established by molecular biological studies (Noedl, 2005).

iv) Miscellaneous: Atovaquone, 2-[*trans*-4-(4'-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone, and a ubiquinone analogue, is an inhibitor of mitochondrial electron transport chain (Fry and Pudney, 1992), probably by inhibiting cytochrome bc_1 complex (Vaidya *et al.*, 1993).

Resistance is mediated by single point mutations in the cytochrome-b gene (Schwobel *et al.*, 2003). It is useful as a combination with other drugs for therapeutic or prophylactic purposes.

Amino alcohols: Lumefantrine, a fluorine derivative of aminoalcohol, Halofantrine, a 9-phenanthrenemethanol are used alongside artemisinin derivatives in ACT (Basco *et al.*, 1998). Pyronaridine is an acridine derivative and blood schizonticide; however its clinical use is limited.

2.3 *Plasmodium falciparum*

Plasmodium falciparum has a synchronized life cycle in the human body which is the only intermediate host for the species. Apart from anemia which is common in any form of malaria, the cytoadherence of parasitised RBCs to visceral capillary endothelium of lungs, kidney and brain is a typical feature of *Plasmodium falciparum* malaria and can be fatal. Clogging of cerebral blood vessels leads to the deadly cerebral malaria which can leave permanent neurological sequel in case of recovery.

Apart from its medical importance there is an established *in vitro* culture system (Trager and Jensen, 1976) for *Plasmodium falciparum*. Moreover, the nearly completed genome sequence of the parasite along with the facilities of genetic manipulation for this human malaria species made it the species of choice for this study.

2.4 The life cycle

The malaria parasite lifecycle comprise of two phase, namely, the asexual and sexual phase. The former occurs in humans and the later in mosquito therefore, there is an alteration of generation of lifecycle of the malaria parasite.

In the asexual phase, the parasite multiplies by division or splitting, a process reffered to as schizogony. Schizogony occurs in two locations, namely, in the RBC (erythrocyte schizogony) and in the liver cells (exoerythrocytic schizogony). The products of schizogony, whether erythrocytic or exoerythrocytic are called merozoites.
The asexual phase take place in the female *Anopheles* mosquito even though the sexual forms of the parasite (gametocytes) originate in the human RBCs. Maturation and fertilization takes place in the mosquito giving rise to a large number of sporozoites hence the phase of the sexual cycle is called sporogony (WHO, 1987; Paniker, 1989).

2.4.1 The human phase

The human is infected through a mosquito bite. The sporozoites present in the salivary gland of the mosquito are transferred into the blood capillaries. These then pass into the bloodstream reaching the liver and thus entering the hepatocytes.

2.4.1.1 The exoerythrocytic (tissue) stage

In the hepatocytes, the sporozoites start the pre-erythrocytic schizogony where they undergo repeated nuclear division and each daughter nucleus is surrounded by a cytoplasm. Within 6-16 days, the schizonts mature and burst releasing thousands of merozoites (WHO 1987; Paniker, 1989).

2.4.1.2 Erythrocytic stage

The merozoites released by the pre-erythrocytic schizonts invade the RBCs, they develop into ring forms. The ring forms develop and enlarge in size becoming irregular in shape while showing amoeboid motility. These are called amoeboid forms, which then forms trophozoites on development. The trophozoites eventually segments to form schizonts. Mature schizonts rapture open, releasing merozoites and these repeat the cycle of RBCs infection. This process of erythrocytic schizogony is repeated sequentially leading to progressive increase in intensity of parasitemia.

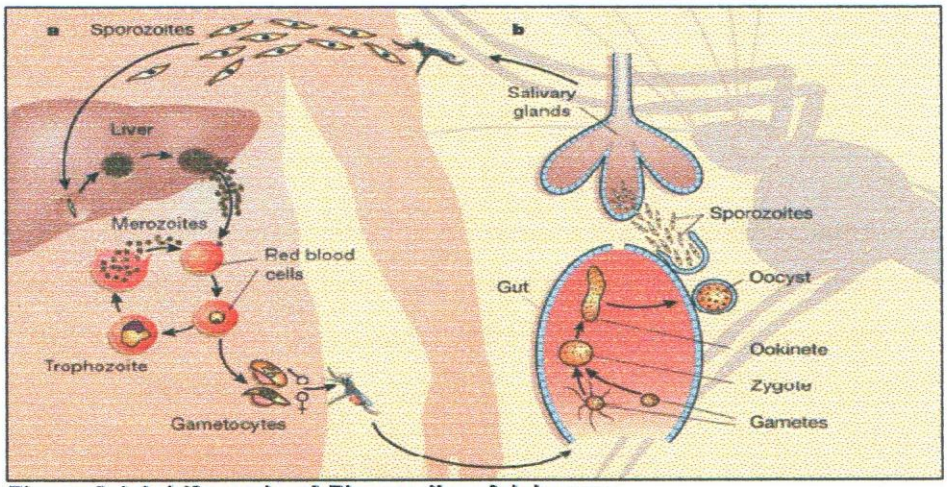


Figure 2.4.1: Life cycle of *Plasmodium falciparum*.

a) The asexual schizogony in the human host, b) sexual development in the mosquito vector (reproduced from http://www.sanger.ac.uk/PostGenomics/plasmodium/presentations/plasmodium_lifecycle.shtml).

2.4.1.3 Gametogony

After many cycles of erythrocytic schizogony, some merozoites that infect RBC do not proceed to become schizonts but instead into sexually differentiated forms, the gametes. Gametocyte development generally takes place within the internal organs, such as spleen and bone marrow and only mature forms appear in peripheral circulation.

The mature gametocytes are round in shape, except in *P. falciparum*, where they are crescent shaped. The female macrogamates and male microgametes do not cause any clinical illness in the host but are essential for the transmission of the infection. They do not develop further in the vertebrate host and unless taken up by the vector mosquito, they die in a few days (WHO, 1987; Paniker, 1989).

2.4.2 The Mosquito phase

The gametocytes are transformed into micro and macrogametes. The gametes fertilize to form a zygote which then develops into an ookinete. The ookinete penetrate the mosquito gut wall and form the oocyst, which rest on the gut wall. The oocyst undergoes sporogony to form sporozoites and these find their way to mosquito salivary gland to be released during the mosquito blood meal. It is during this sexual cycle that genetic recombination occur which can possibly bring about mutations arising at different loci in

different parasite clones being combined in one parasite clone. This mutation could be studied and used as a possible marker in predicting drug resistance.

2.5 Malaria control

Eradication of malaria is desirable but not achievable. Malaria control is thus the option available. Malaria control simply implies reducing its health impact as much as possible through employment of resources that are available. Control of malaria is based on the following interventions:

1. Case management which involves diagnosis and treatment.
2. Vector control involving destruction of larval breeding sites, insecticide spraying inside houses, use of insecticide treated bed nets.
3. Prophylaxes which detect, contain and prevent epidemics at an early stage.
4. To strengthen local capacities in basic and applied research to permit and promote the regular assessment of countries malaria situation, in particular the ecological, social and economic determinants of the disease (WHO, 1993).

2.6 Diagnosis of malaria

2.6.1 Microscopic diagnosis

Direct microscopic examination of stained blood films is the current standard for definitive diagnosis in nearly all settings. Simple light microscopic examinations of Giemsa stained blood films is the most widely practiced and useful method of definitive diagnosis. While availability of microscopic diagnosis has been shown to reduce drug use in some trial setting (Jonkman *et al.*, 1995) in practice, results are often disregarded by clinicians (Barat *et al.*, 1999).

2.6.2 Clinical diagnosis

In highly endemic areas particularly in Africa, clinical or presumptive diagnosis is common. Diagnosis in this case is based on signs and symptoms. Lack of enough resources and trained health personnel, necessitates the employment of presumptive diagnosis as the only realistic option. This type of diagnosis therefore can identify most

patients who truly need antimalarials, but it is also likely to misclassify many who do not need it (Oliver *et al.*, 1991).

2.6.3 Antigen detection tests

These are rapid or 'dipsticks' tests. In this technique, parasite antigens are detected through rapid immuno-chromatographic techniques. Multiple experimental tests have been developed targeting a variety of parasite antigens (Mackey *et al.*, 1982; Fortier *et al.*, 1987; Khusmith *et al.*, 1987). Various kits are available which include ParaSight(R) Beckton-Dickinson, Malaquick(R) ICT to perform rapid diagnostic tests. These are based on the detection of histidine rich protein 2 (HRP II) of *P. falciparum*. These tests yield rapid and highly sensitive results of *P. falciparum* infection as compared with light microscopy (WHO, 1996; Craig and Sharp, 1997). A test based on detection of a specific parasite enzyme including lactate dehydrogenase, (pLDH) has been developed such as optimal(R) Flow Inco. Portland, OR, USA and reportedly only detects viable parasites, which if true eliminates prolonged periods of false positivity post treatment (Beck, 1999; Palmer *et al.*, 1999; Piper *et al.*, 1999).

2.6.4 Molecular diagnosis

Parasite nucleic acids are detected using polymerase chain reaction (PCR). This technique is more accurate than microscopy. However, it is expensive, and requires a specialised laboratory even though technical advances will likely result in field-operated PCR machines. Species-specific primers have been developed for each of the four species causing human malaria. PCR techniques have profound use in the detection of mixed infection or differentiating between infecting species when microscopic examination is inconclusive (Beck, 1999). PCR also permits genetic analysis of strains (Brown *et al.*, 1992). Improved PCR techniques could prove useful for conducting molecular epidemiological investigations of malaria clusters or epidemics (Freeman *et al.*, 1999).

2.6.5 Serology

Specific serological markers have been identified for each of the four species of human malaria. A positive test generally indicates past infection. Serology is not useful for diagnosing acute infections because detectable levels of antimalarial antibodies do not appear until weeks into infection and persist long after parasitemia has resolved. Moreover, the test is relatively expensive and not widely available.

2.7 Definition of Antimalarial drug resistance

Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and / or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject"
This definition was later changed in reference to the drug in question by indicating that the drug must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bruce-Chwatt, 1986). This was interpreted by a majority of other researchers to refer only to the persistence of parasite after antimalarial treatment other than prophylaxis failure. The continued presence of parasitaemia after antimalarial treatment is an indicator for early warning of the occurrence of drug resistance (Lobel *et al.*, 1986)

2.8 Malaria treatment failure

There are several factors that are able to contribute to malaria treatment failure. These include the following: incorrect dosing, no-compliance with duration of dosing regimen, poor drug quality, drug interactions, poor drug metabolism and misdiagnosis. These factors at the same time may contribute to the development and strength of true drug resistance by elevating the rate at which the parasites are exposed to sub-optimal drug levels. Therefore, drug resistance can cause treatment failure but not at all treatment failure is a result of drug resistance (Bloland, 2001).
Combination therapy has been suggested to confront *P. falciparum* resistant malaria, but the suitable combination therapy for sub Saharan Africa is not yet clear. Since drug treatment is the only efficacious control strategy used in Sudan, monitoring of levels of resistance is important.

2.9 Mechanism of antimalarial drug resistance

The way in which parasites can acquire resistance depends on the mechanism by which the drug kills them. This is an important factor in determining how fast parasite resistance can occur.

2.9.1 Chloroquine resistance

The mechanism of chloroquine resistance is not yet clear but the formation of a complex with haem is thought to be important (Mungithin *et al.*, 1998). The molecular basis of drug resistance to CQ is now partially understood, although there is still some debate regarding the contribution of mutations in different genes to resistance. Initial reports associated resistance with mutations in the gene *P. falciparum* multi drug resistance 1(*pfmdr1)* (Foote *et al.*, 1989) association between *pfmdr1* point mutations and *in vivo* or *in vitro* CQ resistance were inconsistent. An early study suggested that it was possible to predict whether an isolate was resistant or sensitive to CQ by the presence of defined mutations in *pfmdr1* (Foote *et al.*, 1990). Studies have established an association between the presence of a mutation at codon-86 causing an amino acid substitution from aspharagine in the wild type to tyrosine in the mutant form and *in vivo* CQ resistance, with sub-Saharan isolates (Basco *et al.*, 1995a; Adagu *et al.*, 1996). A similar association was not observed with culture-adapted Sudanese (Awadh El Kariem *et al.*, 1992) and Thai isolates (Wilson *et al.*, 1993). Other studies using fresh clinical isolates showed that the mutant's alleles of *pfmdr1* encoding tyr-86 could be used to predict *in vivo* drug resistance to CQ (Duraisingh *et al.*, 1997). However, extensive field studies (Wellems *et al.*, 1990; Adagu *et al.*, 1999; Bhattacharya *et al.*, 1999; McCutcheon *et al.*, 1999; Price *et al.*, 1999; Duraisingh *et al.*, 2000; Pillai *et al.*, 2001) produce conflicting results, casting doubts on a direct causal association between polymorphism in *pfmdr1* and CQ resistance. Analysis of a genetic cross between CQ-sensitive and CQ-resistant parasites clones demonstrated that *pfmdr1* mutations were not linked to the resistance phenotype (Wellems *et al.*, 1990). Transfections studies that followed showed that substitution of the mutant *pfmdr1* with the wild type sequence in resistant parasites decreases chloroquine resistance from high to moderate levels (Reed *et al.*, 2000). Therefore it

appears that, although mutation in *pfmdr1* may not be absolutely required for chloroquine resistance, polymorphisms in this gene may play a role in modulating the resistance phenotype (Wellem *et al.*, 1991; Su *et al.*, 1997; Reed *et al.*, 2000; Dorsey *et al.*, 2001). Linkage analyses of a genetic cross identified two loci, known as candidate gene 2 (*cg2)* and *P. falciparum* chloroquine resistance transporter (*Pfcrt)* implicated in chloroquine resistance (Su *et al.*, 1997; Fiddock *et al.*, 2000b; Djimde *et al.*, 2001). Inheritance of a 36 kb segment of chromosome 7 from CQ resistant parents in the cross conferred resistance in the progeny clones. DNA sequencing and searches for open reading frame (ORF) encoding more than 100 amino acids within this segment identified nine ORFs. Of these, one ORF, denoted cg2, contains multiple and complex polymorphisms associated with CQ resistance both in the progeny clones of the genetic cross, and also in a group of isolates from Asia and Africa (Su *et al.*, 1997). However larger scales field studies failed to demonstrate a clear association between the complex polymorphism within cg2 and CQ resistance (Adagu *et al.*, 1999; Basco *et al.*, 1999; Durand *et al.*, 1999; McCutcheon *et al.*, 1999; Price *et al.*, 1999; McCutcheon *et al.*, 2000; Pillai *et al.*, 2001). Transfection-based studies confirmed that the *cg2* gene was not the gene responsible for the CQ resistance (Fidock *et al.*, 2000a). Extra analysis of the initial 36 kb locus comprising the cg2 gene led to the discovery of a highly interrupted gene, denoted *Pfcrt*. Polymorphism in this gene were absolutely associated with CQ resistance *in vitro* in forty known parasite lines (Fidock *et al.*, 2000b). A mutation in codon 76, causing an amino acid change from lysine (K) to threonine (T) was identified as the key mutation necessary for resistance. Transformation of a CQ-sensitive *P. falciparum* clone using plasmid constructs that causes expression of a *Pfcrt* sequence with the K76T codon change produced a highly CQ resistant line. However, the resistance parasites ended up losing the transfection DNA and instead acquired a novel mutation encoding K76I in the chromosomal *Pfcrt* gene. The change in the sequence directly contributed to the change in CQ phenotype (Fidock *et al.*, 2000b). There is therefore strong evidence that an amino acid change at position 76 in *Pfcrt* is associated with CQ resistance in Africa and Asian *P. falciparum* isolates. This is supported by field studies showing a strong link between the presence of *Pfcrt* 76 mutations and clinical CQ resistance (Djimde *et al.*, 2001). Further *in vivo* studies on field isolates have shown that the *Pfcrt* 76 mutation is often

also present in CQ-sensitive isolates (Dorsey *et al.*, 2001; Mayor *et al.*, 2001; Pillai *et al.*, 2001; Chen *et al.*, 2002; Kyosiimire *et al.*, 2002). The *Pfcrt* mutant allele is always present in CQ treatment failure samples. This therefore suggests that the *Pfcrt* 76 mutation may be necessary but is not always sufficient for CQ resistance *in vivo*.
It has been suggested that CQ resistance is a multigenic phenomenon, involving changes to *Pfcrt* and other genes such as *Pfmdr1* (Reed *et al.*, 2000). Support for this hypothesis is provided by field studies in which clinical treatment failure with CQ is significantly associated with parasites with the mutated forms of both *Pfcrt* 76 and *Pfmdr1* 86 (Babiker *et al.*, 2001; Dorsey *et al.*, 2001; Mockenhaupt *et al.*, 2001). In addition, the relatively long time taken for CQ resistance to arise and spread could indicate that multiple mutations are required.

2.9.2 Pyrimethamine resistance

Sulfadoxine-pyrimethamine (SP)-(Fansidar®) is a synergistic combination of drugs that interact with two enzymes of the folate biosynthetic pathway. Dihydrofolate reductase (DHFR) is the biochemical target for pyrimethamine, and also of cycloguanil, the biological active metabolite for proguanil. In *P. falciparum*, two genes involved in the pyrimidine biosynthesis pathway, DHFR and thymidylate synthase (TS) are contiguous and are expressed as bifunctional enzymes DHFR-TS (Bzik *et al.*, 1987). Pyrimethamine binds to the parasite DHFR with a high affinity, inhibiting enzyme activity, resulting in the depletion of dTMP and the disruption of DNA synthesis (Hyde, 1990). The selective activity of pyrimethamine had usually been attributed to higher affinity of the drug for *Plasmodium* DHFR-TS than for human DHFR (Hitchings, 1960; Foote *et al.*, 1990; Peterson *et al.*, 1990,). Recent work has shade light on the differential specificity of the drug for parasite and human DHFR. The DHFR-TS of both species binds its cognate messenger RNA (mRNA) and inhibits its own translation. Pyrimethamine treatment removes translational repression of the human enzyme, and more DHFR is produced to partially overcome the drug barrier. However, in *Plasmodium* DHFR-TS, mRNA binding is not coupled to enzyme active sites. Therefore antifolate treatment does not relieve translational inhibition and the parasites do not have the ability to make fresh enzyme (Zhang and Rathod, 2001).

Resistance to pyrimethamine is linked to a point mutation in DHFR leading to an amino acid substitution at codon 108 from serine to aspharagine (Peterson *et al.*, 1988, Peterson *et al.*, 1990). Resistance to cycloguanil has been linked to paired mutations in codon 16 (alanine to valine) and 108 (serine to threonine) (Foote *et al.*, 1990; Peterson *et al.*, 1990). Other mutations at position 51 (asparagine to isoleucine), 59 (cysteine to argenine) and 164 (isoleucine to leucine) are associated with higher levels of pyrimethamine and cycloguanil resistance (Foote *et al.*, 1990, Peterson *et al.*, 1990). Following the introduction of pyrimethamine monotherapy, resistance was reported within a year (Maberti, 1960), most likely as the result of the simple resistance mechanism. Pyrimethamine is now only used in synergistic combinations with sulfadoxine or other antifolate (Chulay *et al.*, 1984), so it is not possible to correlate *in vivo* pyrimethamine treatment failure with DHFR genotype. However parasites taken from infected individuals can be tested for their pyrimethamine susceptibility *in vitro*. In these tests, resistance is denoted by a 50% inhibitory concentration (IC_{50}) value of > 100nM (de Picoulas *at al.*, 1996). Such studies reveal a very strong link between mutations in DHFR and pyrimethamine and cycloguanil resistance. For pyrimethamine, DHFR Asn108 is almost completely associated with resistance to pyrimethamine, and the presence of parasites encoding DHFR Ser108 alone is a good indicator of sensitivity *in vitro* to pyrimethamine (Foote *et al.*, 1990; Hyde *et al.*, 1990; Peterson *et al.*, 1990; Basco *et al.*, 1995b; Basco *et al.*, 1996; de Picoulas *et al.*, 1996; Reeder *et al.*, 1996; Zindrou *et al.*, 1996 and Khan *et al.*, 1997). Geometric means IC_{50} were over 1000-fold higher for parasites with DHFR Asn108 from African patients compared to parasites with DHFR Ser108 (IC_{50} of 4050 nM for Asn 108 and 12.5 nM for Ser 108). The central mutation implicated in pyrimethamine resistance is at position 108 (Cowman *et al.*, 1988; Peterson *et al.*, 1988; Zolg *et al.*, 1990), a finding that was confirmed by transfection studies (Wu *et al.*, 1996). It is believed that additional mutations in DHFR in codon 51 and 59, combined with the Asn 108 change, confer higher levels of resistance. Kinetics analysis of the *P. falciparum* DHFR-TS enzyme after expression in *Escherichia coli* confirmed the role of these mutations in different levels of resistance. The inhibition constant (K_i) values for the double mutants Asn108/Arg59 and Asn108/Ile51 are 72 nM and 37 nM respectively, compared to 13 nM for the single mutated allele encoding Asn108

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(Sirawaraporn *et al.*, 1997). The inhibition constant for parasites carrying the triple mutant Asn108/Ile51/Arg59 was 120 nM (Sirawaraporn *et al.*, 1997) indicating a much higher level of resistance as compared to the single and double mutated alleles. Transfection experiments have also been performed using DHFR as a selectable marker that confers resistance to pyrimethamine (Wu *et al.*, 1996). Pyrimethamine-sensitive parasites transformed with the DHFR allele from parasite Dd2, the triple mutant form of DHFR Asn108/Ile51/Arg59, yielded drug-resistant parasites with an IC_{50} of 16 nM. Transformation with the DHFR gene from parasite HB3 a single mutant from Asn108 yielded parasites with an IC_{50} value of 1.0 nM. Investigating the effect of an additional mutation in DHFR at codon 51 was also done by trasfection of *P. falciparum* DHFR alleles into yeast (Wooden *et al.*, 1997). This suggests that the double mutant from DHFR Asn108/Ile51 (*pf-dhfr*-Mikenga, resistant construct) causes a further ten-fold increase in IC_{50} compared to the single mutant form DHFR Asn108 (pf-dhfr-Honduras, a resistance construct), which itself produces a ten-fold increase in IC_{50} compared to the wild type form DHFR Ser108 (pf-dhfr-D6, a sensitive construct).

2.9.3 Sulfadoxine resistance

Sulfadoxine, the component of the combination drug SP, is also never used as monotherapy for malaria. The information on the resistance mechanism to sulfadoxine alone has never been obtained from *in vitro* work. The molecular target of sulfadoxine is the enzyme DHPS, a part of bifunctional protein with hydroxymethylpterin pyrophosphate. Sulfadoxine is a structural homologue of 4-amino benzoic acid (PABA) the natural substrate of DHPS (Brooks *et al.*, 1994; Triglia and Cowman, 1994) and is a competitive inhibitor of the enzyme. Mutations in the DHPS gene are found in natural populations, and have been correlated with resistance to SP *in vivo*, for example, mutations leading to amino acid changes at positions 437 (alanine to glycine), 436 (serine to phenylalanine), 613 (alanine to serine), and 581 (alanine to glycine) (Plowe et al., 1997; Triglia *et al.*, 1997; Wang *et al.*, 1997a).

Some studies have attempted to correlate *in vitro* sulfadoxine resistance and DHPS mutations with isolates taken from the field and with laboratory lines, but these have been

inconclusive (Table 2.9.4(a): a, b, d and f). *In vitro* assay for sulfadoxine have been done under varying folate conditions (Wang *et al.*, 1997b), and some parasites isolates are known to use host folate to antagonise sulfa drugs irrespective of their DHPS genotype (Millious *et al.*, 1985; Watkins *et al.*, 1985). Sulfadoxine *in vitro*-drug testing is technically difficult but can be done using culture medium depleted of folate and PABA that antagonise sulfadoxine (Zindrou, 1985; Chulay *et al.*, 1996; Millious *et al.*, 1996; Watkins *et al.*, 1996).

2.9.4 SP resistance

The association of mutation in both DHFR and DHPS with resistance to SP in malaria parasites has been investigated (Hyde *et al.*, 1990; Brooks *et al.*, 1994). Previous studies have analysed the correlation between *in vitro* sulfadoxine resistance and DHFS genotype separate from *in vitro* pyrimethamine resistance and DHFR genotype. Generally, laboratory adapted strains of *P. falciparum* have been used, although field isolates have occasionally also been studied. It is not clear what role mutation in the DHFR and DHPS plays in *in vivo* pyrimethamine and sulfadoxine resistance, as these drugs are never used alone (Hyde *et al.*, 1990; Wang *et al.*, 1997a). Although an *in vitro* SP susceptibility test has been described, the 'folate effect' described above can complicate their interpretation, and most studies have used samples from clinical SP treatment failure. The molecular criteria for *in vitro* antifolate resistance have not been confirmed or correlated with *in vivo* response of patients treated with SP (Rallon *et al.*, 1999). Furthermore, the DHFR and DHPS allelic combination that may be associated with SP therapeutic failure are not yet clearly known.

Controversies have arises between different studies showing evidence for and against mutations in DHFR and DHPS being implicated in SP resistance of *P. falciparum* strains. Studies dealing with the determination of genetic changes as the basis of clinical resistance have been carried out in West Africa (Plowe *et al.*, 1995; Plowe *et al.*, 1996). These studies have confirmed that the molecular diagnostic techniques used are appropriate for epidemiological studies of resistance where it is not possible using standard *in vitro* and *in vivo* methods for detecting resistance. They also show that surveillance studies carried out at intervals to determine resistance levels in different

regions using these molecular assays may assist health officials in changing antimalarial drug treatments policies accordingly. Arguments have been made for limiting the distribution and the use of specific antimalarial drugs, but evidence that such an approach can slow down the spread of resistance has been lacking (Price and Nosten, 2001).

Several studies have been carried out to investigate the role of mutations in DHFR and DHPS in clinical SP resistance. Field tests carried out in Kenya both *in vivo* and *in vitro* have suggested that pyrimethamine resistance alone associated with mutation in DHFR is sufficient to cause SP failure (Nguyen-Dinh *et al.*, 1982). This was supported by a study done in Tanzania, which showed evidence in *in vivo* selection under SP drug pressure of parasites with alleles encoding the DHFR Asparagine 108 mutant (Curtis *et al.*, 1996). An *in vivo* study carried out in Mali showed that SP selected for DHFR mutants rapidly, with Arginine 59 (55.0%) and Asparagine 108 (55.0%) being more common in post-treatment isolates, supporting their role in SP failure (Diourte *et al.* , 1999). However, because there was a low level of *in vivo* SP resistance in the study area, it was not possible to identify any group of DHFR or DHPS mutations that were predictive of clinical SP failure. A study carried out in Cameroon showed that the triple DHFR mutant encoding Asn 108/Arg 59/Ile 51 was strongly associated with *in vivo* resistance to SP (Basco *et al.*, 2000).

There is also evidence that mutations in both DHFR and DHPS are necessary for *in vivo* SP resistance. Studies carried out in West and East Africa, the Middle East and Vietnam (Schapira *et al.*, 1986; Wang *et al.*, 1997a) suggest that DHFR 108 and DHPS 437 changes are necessary for SP treatment failure. The study by Schapira *et al.*, (1986) also performed *in vitro* sulfadoxine tests on parasites from SP-resistant cases and found that the *in vivo* results reflected the *in vivo* treatment failure. A study carried out in Peru (Kublin *et al.*, 1998) showed that a collection of mutation in DHFR (Asn 108/Ile 51/Leu 164) and DHPS (Gly 437/Glu 540/Gly 581) was strongly associated with *in vivo* SP resistance. In Vietnam, a combination of multiple DHFR antifolate resistant mutations coupled with the 437 Glycine mutation in DHPS correlated best (72%) with *in vivo* resistance to SP (Masimirembwe *et al.*, 1999). In Kenya, a combination of the triple

DHFR mutant (Asn 108/Ile 51/ Arg 59) couple with the DHPS double mutant (Gly 437/Glu 540) was associated (55.2%) with *in vivo* resistance to SP (Nzila *et al.*, 2000). In other studies, however, mutations in DHFR and DHPS were not informative for predicting SP resistance. A study done in Colombia showed that DHFR alleles encoding Asn 108 were detected in 75% of clinical samples with adequate clinical response (ACR) and in all treatment failure samples, rendering it inadequate for predicting SP treatment failure (Rallon *et al.* , 1999). Studies carried out in Tanzania found no association between the presence of mutations at different loci of the DHFR gene before treatment and subsequent clinical SP resistance (Jelinek *et al.*, 1997).

It has also however been argued that DHPS mutations do not play a role in SP failure (Watkins *et al.*, 1997). A study carried out in Mali found no significant selection by SP for DHPS mutations, including no significant selection against the DHPS allele encoding Ala-436 (Diourte *et al.*, 1999). A study carried out in Cameroon to assess SP efficacy showed there was no effect on clinical outcome by the DHPS genotype (Basco *et al.*, 2000). From these studies and those listed in Table 1 and 2, it is difficult to judge whether molecular changes in the DHFR and / or DHPS correlate with *in vivo* treatment failure with SP. Table 2.9.4(a) and 2.9.4(b) summarize some of the studies performed to investigate the association between DHFR/DHPS mutations and SP resistance.

2.10 Drug combinations and resistance

In response to prevailing CQ resistance, Malawi, Kenya, Botswana and South Africa changed the first-line drug from CQ to SP and other countries have followed. However, the useful therapeutic life (UTL) predicted for SP has been very short probably because of its prolonged half-life, causing a higher probability of selecting resistant strains and consequent rapid development of resistance (Nzila *et al.*, 2000). This is why new alternative drugs or new approaches, such as drug combinations, are urgently needed. Drug pressure has been identified as one of the key factors in the emergence of resistance to antimalarial drugs. Selection of resistant strains can occur when a drug is misused (Wernsdorfer, 1991; Wernsdorfer, 1994) or used alone extensively. Readily absorbed drugs with a long half-life, such as mefloquine and sulphadoxine- pyrimethamine (SP), can permit effective single-dose treatment of malaria and the following

chemoprophylactic period prevents infection for several weeks. However, these drugs are likely to exert undesirable drug pressure for a long time once their concentrations drop below the critical threshold and may select resistant parasites. The contribution of monotherapy and misuse of antimalarial drugs to the emergence of drug resistance became particularly evident during the Global malaria eradication campaign launched in 1955 by WHO (Bruce-Chwatt 1986). This has been shown in Kenya where potent selective pressure for resistance operates even under conditions of supervised drug administration and optimal dosage (Watkins *et al.*, 1997). This is why drug combinations have recently been proposed to delay the emergence and spread of drug resistance (White and Olliaro 1996; White 1998; White 1999), an approach already used for highly drug-resistant infectious diseases such as tuberculosis, AIDS and leprosy.

The first consequence of using drugs in combination is that the starting frequencies of malaria parasites resistant to all the components are much reduced, such that the evolution of resistance is delayed compared with when its components are used alone. The second consequence is that the resistant combinations will be broken down much more frequently during recombination in meiosis. The greater the number of genes required to encode resistance, the greater the rate of loss (Hastings and D'Alessandro, 2001). In practice, the emergence and spread of resistant strains should be delayed and the useful therapeutic life of the combination should be much longer than its single components.

Combination therapy (CT) is defined as "the simultaneous use of two or more antimalarial drugs with different biochemical targets in the parasite or in the tissue hosting the parasite, which are synergetic, additive, or complimentary in their effect." CT can be either fixed combination where all components are co-formulated in a single tablet/capsule or free combination where the components are separate tablets/capsules but are co-administered.

Non-artemisinin based CT available in the market include, Chloroquine plus SP, Amodiaquine plus SP; Atovaquine plus Proguanil; Mefloquine plus SP and Chlorproguanil (Lapudrine) plus Dapsone. Combination therapies involving an Artemisinin derivative which have been recommended include, Mefloquine, SP and Amodiaquine with a derivative of Artemisinin (White *et al.*, 1999). Lumefantrine in

combination with Artemether (Coartem®) is another of such combination (Olliaro and Trigg, 1995; Van Vogt *et al.*, 1999). Others are on the pipeline include LapDap with Artesunate, Metakelfin with Artesunate (Cor arinate®) and Artesunate with Amodiaquine (Amornate®).

Table 2.9.4(a)

DHFR/ DHPS mutations and SP resistance *in vitro*

The listed references provide evidence of a correlation between mutations in DHFR and DHPS and resistance to pyrimethamine and sulfadoxine respectively

Evidence supporting the role of Asn-108 and other *DHFR* mutant codons in pyrimethamine *in-vitro* resistance	**Evidence supporting the role of Gly-437 and other *DHPS* mutant codons in sulfadoxine *in-vitro* resistance**
1. Basco *et al.*, 1996 Exp. Parasitol. 82: 97- 103	a. Basco *et al.*, 1998[a] Am. J. Trop. Med. Hyg. 58: 374-378
2. Basco *et al.*, 1995 Mol. Biochem. Parasitol. 69: 135-138	b. Brookes *et al.*, 1994 Eur. J. Biochem. 2: 397-405
3. Foote *et al.*, 1990 Pro. Natl. Acad. Sci. USA 87:3014-3017	c. Reeder *et al.*, 1996 Am. J. Trop. Med. Hyg. 55: 209-213
4. Hyde *et al.*, 1990 Pharmacol. Ther. 48: 45-49	d. Triglia *et al.*, 1994 Proc. Natl. Acad. Sci. USA. 91: 7149-7153
5. Khan *et al.*, 1998 Trans. R. Soc. Trop. Hyg. 91: 456-460	e. Triglia *et al.*, 1997 Proc. Natl. Acad. Sci. USA. 94:13944-13949
6. Nzila- Mounda *et al.*, 1990 Antimicrob. Agents Chemother. 42: 164-169	f. Wang *et al.*, 1995 Mol. Biochem. Parasitol. 71: 115-125
7. Peterson *et al.*, 1990 Proc. Natl. Acad. Sci. USA. 87: 3018-3022	g. Wang *et al.*, 1995 Mol. Biochem. Parasitol. 71: 115-125
8. Reeder *et al.*, 1990 Am. J. Trop. Med. Hyg 55: 209-213	h. Triglia *et al.*, 1998 EMBO J. 17: 3807-3815
9. Zindrou *et al.*, 1996 Exp. Parasitol. 84: 56-64	
10. Zolg *et al.*, Mol. Biochem. Parasitol. 36: 253-262	
11. Cowman *et al.*, 1998 Proc. Natl. Acad. Sci. USA. 85: 9109-9113	
12. Peterson *et al.*, 1998 Proc. Natl. Acad. Sci. USA 85: 9114-9118	
13. Basco *et al.*, 1998 Antimicrob. Agent Chemother. 42: 1811-1814	
14. Wilson *et al.*, 1993 Mol. Biochem. Parasitol. 57: 151-160	
15. Awadh-El-Karim *et al.*, 1992 Trans. R. Soc. Trop. Hyg. 52 (6): 565-568	

Table 2.9.4 (b)

DHFR/ DHPS mutations implicated in SP *in vivo* resistance

The listed references which provide evidence of a correlation between mutations in DHFR and /or DHPS and resistance to SP respectively

Paper and study region	Correlation of SP resistance to DHFR mutations	Correlation of SP resistance to DHPS mutations	Recommended combinations for genotyping tests
Curtis *et al.*, 1996 Trasc. R. Soc. Trop. Med. Hyg. 90: 678-680 (Tanzania)	Asn 108	Gly 437	Asn 108 and Ile51/Arg 59 **AND** Gly 437 and/or Gly 437/Glu 540
Basco *et al.*, 1998b Antimicrob. Agents Chemother. 42: 1811-1814 (Cameroon)	Asn 108	Gly 437	Asn 108 and Ile51/Arg 59
Kublin *et al.*, 1998 Lancet: 351: 1629-1630 (Peru)	Asn 108	Gly 437	Asn 108 and Ile51/Leu 164 **AND** Gly 437 and/or Glu540/Gly 581
Masimirembwa *et al.*, 1990 Int. J. Antimicrob. Agents. 12: 203-211 (Vietnam)	Asn 108	Gly 437	Asn 108/Ile51/Arg 59/Leu164 **AND** Gly 437 and/or Gly 581/613 Ser or Thr
Diourte *et al.*, 1999 Am. J. Trop. Med. Hyg. 60: 475-478 (Mali)	Asn 108 and Arg 59	No correlation observed with any mutation	Asn 108 and Arg 59
Basco *et al.*, 2000 J. Infect. Dis. 182: 624-628 (Cameroon)	Asn 108	Ala and Ser 436/Gly or Ala 437/Lys 540/Ala 581/Ala 613	Asn 108/Arg 59/Leu 51 **AND** Ala 436/Ala 437/Lys 540/Ala 581/Ala 613 or Ser 436/Gly 437/Lys540/Ala 581/Ala613

A technical consultation of experts of WHO, *Antimalarial Combination Therapy* Organised by WHO, Geneva in April 2001, identified the following options in priority order as currently available antimalarial combination therapies with a potential for introducing into the treatment policy or deploying in Africa if cost was not an issue:

1. Artemether-lumefantrine currently available only as Coartem
2. Artesunate (3 days) + amodiaquine
3. Artesunate (3 days) + SP in areas where SP efficacy remains high
4. SP + amodiaquine in areas where efficacy of both amodiaquine and SP monotherapy remain high; limited to countries in West Africa) (WHO, 2001).

Although safety and efficacy have been demonstrated with extensive use of artemisinin-based combinations in South-east Asia, there is limited experience with its use in Africa. There is, therefore, an urgent need to document efficacy and safety among young children, pregnant women, and breastfeeding mothers and their babies. Artemisinin-based combinations that include chloroquine are not recommended as policy options for Africa because of the unacceptably high level of resistance to chloroquine (WHO, 2001).

The efficacy of combination therapy with 4-aminoquinolines (CQ, Amodiaquine) with SP has been reviewed (McIntosh and Greenwood, 1998) which showed improved parasitological clearance as compared to SP alone.

2.11 Tests for drug resistance

There are four basic methods for testing malaria for drug resistance namely *in vivo* tests, *in vitro* tests, molecular characterisation, and animal models. Of these, only the first 3 are routinely done.

In vivo tests: In these tests, patients with clinical malaria are given a treatment dose of an antimalarial drug under observation and are monitored over time for either failure to clear parasites or for reappearance of parasites.

In vitro tests: In these tests, blood samples from malaria patients are obtained and the malaria parasites are exposed to different concentrations of antimalarial drugs in the

laboratory. Some methods call for adaptation of parasites to culture first, while others put blood directly from patients into the test system.

Molecular characterisation: For some drugs (chloroquine, SP and similar drugs, atovaquone), molecular markers have been identified that confer resistance. Molecular techniques, such as PCR or gene sequencing can identify these markers in blood taken from malaria-infected patients

Animal models: These are in essence *in vivo* tests conducted in non-human animal models and therefore influenced by the same extrinsic factors as *in vivo* tests. They allow testing parasites, which cannot be adapted to *in vitro* environments, provided a suitable host is available and the testing of experimental drugs is not yet approved for use in humans (WHO, 2001).

2.12 Use of radioisotopes in biomedical research

Apart from increasing sensitivity of detection over ethidium bromide staining and confirming the specificity of the amplicon, hybridization with specifically designed complementary probes, can be used to detect point mutations in its nucleotide sequence. The radioisotope Phosphorus –32 is used to label the probes. The atom phosphorus –32 (^{32}P) is an unstable isotope of phophorus-31 (^{31}P), with emissions that can be detected by methods including those that involve autoradiography. Nylon membranes can be reused repeatedly for dot blot hybridization. The probe can be stripped off the membrane after autoradiography, and re-probing carried out with another radiolabel- led probes. In addition the cost of radio isotopic hybridization is less expensive than non-radio isotopic probe based methods (Cockerill, 1999). Radioisotopes are also simple, quick and convenient for labeling probes (Sinclair, 2000). Even the short half-life of P-32, which is 14 days, has its advantages, as its disposal is less problematic.

2.12.1 Autoradiography

The photographic emulsion can be exposed by light photons, X-rays, gamma rays, or, in the case of autoradiography, by moving charged particles such as electrons or positrons (Knoche, 1991). According to the Gurney-Mott theory, light photons oxidise the bromine

atoms, causing them to release electrons (Carlton *et al.*, 2001). These electrons migrate toward imperfections in the lattice, such as the silver sulfide specks. The electrons attract the silver ions, which reduce to atomic silver and form clumps called latent image centers. When a single silver halide grain contains a threshold number of latent image centers, the grain becomes developable. The developable grains form the dark areas of the image when the emulsion is developed (Knoche, 1991; Carlton *et al.*, 2001) Intensifying screens are used to reduce the exposure time or increase the sensitivity in the detection of radiolabelled samples. Intensifying screens diminish the resolution of an image compared to a direct exposure where the decrease in resolution is due to the increased distance from the origin (sample) and the emulsion, where the image is formed. Intensifying screens work by generating photons through the interaction of the radiolabelled particles energy and the phosphor in the intensifying screen. The energy from the beta particles interacts with the phosphor to generate a large number of photons with optimal sensitivity in such a system being reached when the photons generated by the screen wavelength match the peak spectral response of the film. All intensifying screens perform optimally at -60 to -80 degrees Celsius. The reason for this simplistically is, the activation energy required to form a stable latent image where as chemicals are required to create a permanent image on the film is lowered. At room temperature larger activation energy is required to form the stable latent image. Therefore more energy is required to achieve an image at room temperature using an intensifying screen compared to using a screen at -60 degrees Celsius. The activation energy needs to be reduced because the photons though more numerous are less energetic than the radioisotope particles. Conventional intensifying screens work by placing a radiolabelled sample on a sheet of autoradiography film with the intensifying screen lying under the film where the film is sandwiched between the screen and the sample (Knoche, 1991; Carlton *et al.*, 2001).

The process of development transforms the latent image into a visible image by reducing silver ions into twisted strands of metallic silver. The reducing agent hydroquinone is used to react with silver ions to form silver atoms and quinone. The reaction is catalysed by the latent image centers, and can be accelerated by the agents' phenidone. Development is carried out until nearly all the silver contained within

developable grains is reduced. After development, the image is fixed with a thiosulfate solution, which forms complexes with undeveloped silver halide and hardens the gelatin within the photographic emulsion. The image is then ready for qualitative or quantitative analysis (Knoche, 1991; Carlton *et al.*, 2001)

2.13 Parasite diversity

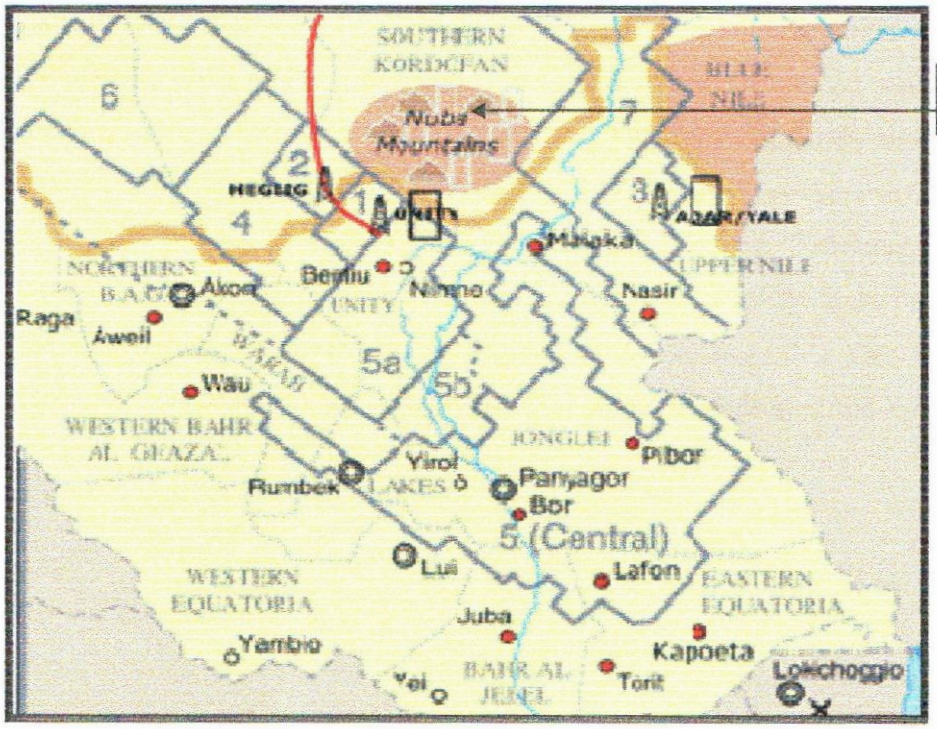
Differences in transmission intensity between endemic regions have been implicated as one of the factors leading to geographical variation and parasite diversity (Creasey *et al.*, 1990; Paul *et al.*, 1995; Babiker *et al.*, 1997). Studies have suggested a positive correlation between the mean number of genotypes per infected individual and transmission intensities (Paul *et al.*, 1995; Babiker *et al.*, 1997). Multiple parasite genotypes have been frequently observed in individuals living in endemic areas (Creasey *et al.*, 1990; Ntoumi *et al.*, 1995). High prevalence of drug resistant associated polymorphisms has been associated with severe malaria in Kenya (Omar *et al.*, 2001).The level of transmission and multiplicity of clones per infection, are among important factors that influence the speed at which resistance will emerge and spread. This study investigated parasite diversity in Ngorban country within Nuba Mountains, Southern Sudan, by determining drug resistant associated with mutations and multiplicity of infections in the region.

CHAPTER THREE

3: MATERIALS AND METHODS

3.1 Study site

The location was in Ngorban Country, Nuba Mountains, the MSF field station, Southern Sudan. The area has one rain season, mainly in June to July. Annual rainfall ranges from 650mm to 1100mm.The area experiences high temperatures throughout the year ranging from 28 to 35 degree Celsius. During the rain season, there are ample breeding sites for *A. gambiae*, *A. arabiensis* and *A. funestus*, the important malaria vectors in the area.



USAID, © 1993 DeLome mapping donated by Response.Net

Figure 3.1 Study site, Nuba Mountains, Southern Sudan.

3.2 Ethical consideration, inclusion and exclusion criteria

Southern Sudan does not have an ethics review board. The study was approved by the health authority of the Sudan Relief and Rehabilitation Commission, and by the health secretariat of the Nuba Relief Rehabilitation and Development Association (NRRDA).

The study also received ethical approval by the ethics review board of *Medecins Sans Frontieres*. Informed consent was obtained from adults or parents of children aged 6-59 months old before they were enrolled in the study according to WHO (2003b) protocol for monitoring antimalarial drug resistance. Patients visiting the outpatient clinics were enrolled in the study if they met the following criteria including consent, mono-infection with *P. falciparum* and parasitemia between 2,000-200,000 parasites/ µl of blood, fever, weight ≥ 5 kg, auxiliary temperature ≥ 37.5 °C or with a history of fever and no history of antimalarial drug intake during the previous week as confirmed by urine tests, no signs of severe malaria, no concomitant febrile conditions apart from mild upper respiratory tract infection of presumed viral origin, and no reported hypersensitivity to study drugs. Patients were excluded from the study if there was administration of any additional antimalarial drugs. Arm allocation was randomized by sealed envelopes. Children received either three daily doses of AS (4mg/kg/day; Arsumax®, sanofi) plus a single dose of SP (1.25 mg/kg sulfadoxine and 25mg/kg pyrimethamine; Fansidar®, Roche), or AS as above plus three daily doses of AQ (10mg/kg/day; Camoquin®, Parke-Davis). Drug-intake was observed on days 0, 1, 2, and reassessed children both clinically and parasitologically on days 2, 3, 7, 14, 21 and 28, or any other days incase of illness. Children were assigned for therapeutics end points according to the WHO classification (Table 3.2) (WHO, 2003).

Table 3.2 Efficacy endpoint classification (WHO, 2003)

Endpoints	
Early treatment failure (ETF) (day 1-3)	Parasite density on day 2> of day 0 Parasite density on day 3≥ 25% of day 0 Fever in presence of parasites on day 3 Severe malaria in the presence of parasites on days 1-3
Late clinical failure (LCF) (days 4-28)	Fever in the presence of parasites on day 4-28
Late parasitological failure (day 28)	Severe malaria in the presence of parasites on day 4-28 Parasites without fever on day 28
Adequate clinical and response (ACPR) (day 28)	Follow-up completed without meeting any of the above criteria

3.3 Sample size

Patients of between 6 months and 10 years were recruited for the study according to the WHO (2001) protocol. Sample size of 160 patients was used for this study.

The sample size was estimated using the double lot quality assurance methodology (DLQAM), as described by WHO in the "assessment of therapeutic efficacy of antimalarial drugs for uncomplicated *falciparum* malaria in areas with intense transmission", protocol (WHO, 1996).

3.4 Sample collection

Peripheral blood collected by lancet prick from a fingertip was used for direct microscopic diagnosis. Some amount of blood was also spotted directly on 3mm Whatman filter paper (Whatman®) (Sambroook *et al.*, 1989). Blood spots were allowed

to air-dry, then placed individually in plastic bags and stored at 4°C with a silica gel (Sigma) to prevent dampness until required for DNA extraction and analysis.

3.5 *Plasmodium falciparum* clones and isolates

To optimize for the specificity and sensitivity of the technique, six *P. falciparum* clones, (3D7, 7G8, Dd2, K1, T9-96, W2 and HB3) from WHO/IAEA, which contain known *dhfr*, *Pfcrt* and *Pfmdr1* alleles were used. These were also used as controls in the scoring of the sample results on the autoradiograph, after radio probing.

3.5.1 Filter paper samples

The blood spot samples and clones collected using filter papers, were excised from the filter paper and the DNA extracted using Chelex-100® Bio- Rad method as described by Plowe *et al.* (1995). In this method, the scalpels, forceps and glass plate were cleaned, to get rid of any DNA contamination, by immersing for a few seconds into 5M HCl or by wiping with a tissue soaked in acid for the glass plate and tile followed by neutralisation with 5M NaOH, and a brief rinse with distilled autoclaved water. These were then dried using a clean tissue. The filter paper was then removed from the plastic bag using forceps. The area of filter paper with the blood spot was excised using a scalpel on a piece of glass without allowing the scalpel blade or the forceps to come into contact with the blood spot. The piece of filter paper was then transferred to a sterile 1.5ml microfuge tube (eppendorf®) using the sterile forceps, cleaning the scalpel, glass, and forceps between samples as above. About 1ml 0.5% saponin in 1 x FBS was then added to each tube. This was inverted several times and placed at 4°C overnight.

The brown solution was then removed and discarded into a beaker containing 10% bleach after microfuging the tubes for 5 seconds. This was then replaced with 1ml of PBS, the tube inverted again, and then placed at 4°C for 15-30 minutes. Most of the PBS was then removed and discarded into the 10% bleach after microfuging the tubes for 5 seconds. Then, 50µl of stock 20% (w/v) chelex- 100® Bio-Rad solution and the 100µl of DNase-free water (Sigma) were added directly to the tube containing the filter paper, after removal of the PBS wash. These were placed immediately into the heated block (Bio-Block), at 98 – 100 °C for 10 minutes with frequent vortex treatment at 2 minutes

intervals (Genie vortexer®) and once after incubation. After incubation, the tube was spun at 10,000 in a microfuge (Eppendorf-5414D®) for 2 minutes and the supernatant removed to a fresh tube. This was then spun for a further 2 minutes at 10,000, and then the supernatant transferred into another fresh tube. During the final transfer, care was taken not to transfer any of the chelex matrixes. The DNA solution was then stored at -20°C for the short term or at -70°C for long term in a freezer (Revco®).

3.6 DNA extraction, PCR amplification and product analysis

Extraction and purification of parasite DNA from blood samples on filter paper was performed using the Chelex-100 method (Plowe *et al.*, 1995). All of the blood from the single spot was used and the final volume of DNA solution obtained was approximately 200µl. Extracted DNA was used immediately or stored at -70°C for later PCR amplification of DNA.

3.6.1 PCR for RFLP

Parasites were examined for antifolate-resistance associated point mutations in the *dhfr* gene on chromosome 4 and *Pfcrt* gene on chromosome 7 using a nested PCR/RFLP method described by Duraisingh *et al.*, (1998). The nested PCR approach was used to amplify regions of *dhfr* and *Pfcrt* genes containing antifolate and 4-aminoquinoline associated point mutations. The nest I or outer amplification reaction volume was 20µl containing a sector of prepared 5 µl DNA from chelex extraction, 0.25µM of each nest 1 primer (See appendix II), 1× standard PCR buffer [1.5mM $MgCl_2$, 50mM KCl, 10mM Tris HCl (pH8.3), 0.5% DMSO], 200µM of each of the dNTPs, 1unit of Taq polymerase (Roche, Promega).

The reaction was allowed to proceed for 1 cycle at 94°C for 3 mins, then 40 cycles at 94 °C for 1 min, 50 °C for 2 min, 72 °C for 2 mins and finally 1 cycle at 72 °C for 10 mins. The nest II reaction consisted of 0.25µM of each nest II primer, standard PCR buffer, 200µM of each of the dNTPs, 1 unit of Taq polymerase, and 5µl of nest 1 PCR products as template. Tubes were briefly centrifuged and placed in a thermocycler (Bio-Rad / MJ Research). The reaction was cycled 40 times at 94°C for 1 min, 45°C for 1 min, 72°C for 2 mins and a final extension step of 72°C for 10 mins.

3.6.2 Restriction digests

These were done as described by Duraisinglm *et al.*, (1998). PCR amplification of the regions flanking the mutations was done using mutation specific primers as outlined above. Restriction digestion were carried overnight at optimum temperatures for each enzyme in the presence of 1× buffer, 1 BSA (where required) 1-2 units of the specific enzyme to a total volume of 15µl and at optimal conditions as indicated by the suppliers (New England Biolabs, UK).

Table 3.6.2 (a): Restriction digest premix

	Reagent	Final conc.	Volume per tube V=15µl
	10× NEB Buffer	1×	1.5
	100× NEB BSA	1×	0.15
	Restriction Enzyme eg.20, 000u/ml	1unit	0.05
	DNase free water		8.3

Restriction enzymes used for specific codons were as follows;

Table 3.6.2 (b) Restriction digest enzymes.

Codon	Amplification primers	Restriction enzyme	Incubation temp°C	Product fragment
Pfcrt 76	TCRD1/2	ApoI	50	126 & 73 bp for wild
dhfr 51	M3-F/	Tsp509I	65	154 & 64bp for wild
dhfr 59	F-M4	XmnI	37	163,137,26bp-Mutant 189,137 bp Wild
dhfr 108	F-M4 M3-F/ M3-F/	AluI BsrI BstNI	37 65 60	Ser Asn Thr

5µl of the PCR product was used without purification and when the incubation temperature was above 50°C, samples were overlaid with few drops of mineral oil.

3.6.2.1 Genotyping at the *Pfcrt* locus

Mutations in *P. falciparum* Chloroquine resistance transporter gene (*Pfcrt*) specifically those causing an amino acid substitution from lysine to threonine at position 76 (K76T), were detected by a two-step nested PCR reaction amplifying the target loci, followed by

mutation-specific RFLP analysis as previously described (Duraisingh *et al.*, 1998; Djimde *et al.*, 2001a; Djimde *et al.*, 2001b).

In the first round PCR oligonucleotide primer pairs TCRP1 and TCRP2 were included in a single reaction. Five microlitres of genomic DNA was used as template in the primary reaction. The reaction took place in a total volume of 25 µl consisting of the reagents as stated in table 3.6.2.1

Table 3.6.2.1: CRTP1/2 Nest I premix

	Reagent CRTP1/2 PCR premix	**Final conc. in PCR**	**Volume per 20µl tube**
	10× PCR Buffer (Roche)	1×	2.0
	2mM Mixed dNTPs (Promega)	200µM	0.2
	10µM CRTP1 (MWG Biotech)	100nM	0.2
	10µM CRTP2 (MWG Biotech)	100nM	0.2
	Taq DNA polymerase (5unit/µl) (Roche)	1unit/PCR	0.2
	DNase-free water (Sigma)		17.2

The samples were run on My Cycler® (Bio Rad) PCR machine under the following programme;

Primary denaturation	95°C	3 min	
Denaturation	94 °C	30 sec	Cycles 45
Annealing	56 °C	30 sec	
Extension	60 °C	1 min	
Final extension	60 °C	3 min	
Hold at	4 °C		

2-5 µl of the outer amplification products were used as the template in the nested amplification. The PCR pre-mix consisted of reagents in table 2.6.2.1

Table 3.6.2.1 (b) CRTD1/2 PCR Nest II premix

	Reagent	Final conc. in PCR	Volume per 20µl tube
	10× PCR Buffer (Roche)	1×	2.0
	2mM Mixed dNTPs (Promega)	200µM	0.2
	10µM CRTD1 (MWG Biotech)	100nM	0.2
	10µM CRTD2 (MWG Biotech)	100nM	0.2
	Taq DNA polymerase (5unit/µl) (Roche)	1unit/PCR	0.2
	DNase-free water (Sigma)		17.2
	Outer PCR product		5

The samples were also run on My Cycler® (Bio Rad) PCR machine under the following programme;

Primary denaturation	95°C	3 min
Denaturation	94 °C	30 sec
Annealing	47 °C	30 sec
Extension	64 °C	1 min
Final extension	60 °C	3 min
Hold at	4 °C	

Cycles 55

Restriction digestions were carried overnight at optimum temperatures of 50°C for ApoI enzyme.

3.6.2.2 Resistance mutation on *dhfr* by PCR/ RFLP

Oligonucleotides primers pairs M1 and M5 were included in a single outer PCR reaction. Using the PCR products generated from the primary reaction as templates, a nested PCR was then carried out using primers based on conserved sequences, so that the PCR product included codons 108, 51 and 59 in *dhfr*. M3-F/ were the nested primers used for codon 51 and 108 (Asparagine, Threonine), while F-M4 primers were used for codon 59

and 108 (Serine). Control amplification using DNA from *P. falciparum* parasites clones known to contain the possible different alleles at position 108, 51 and 59 of *dhfr* were used to ensure specificity and sensitivity of the technique (Table 3.6.2.2,a).

Table 3.6.2.2 (a): Positive and negative control parasite clones for the DHFR locus.

Parasite clones	*dhfr*						
	Codon 108			Codon 51		Codon 59	
	Ser (W)	Asn(M)	Thr (M)	Asn (W)	Ile (M)	Cys (W)	Arg (M)
3D7	**positive**	negative	Negative	**Positive**	Negative	**Positive**	negative
Dd2	negative	**positive**	Negative	Negative	**Positive**	Negative	**positive**
HB3	negative	positive	Negative	Positive	Negative	Positive	negative
V1/S	negative	negative	Negative	negative	Positive	Negative	positive

W= wild type, M= mutant
Controls labelled in bold were the major ones used for the experiments.

The amplification programme for M1/M5 PCR was as follows

Primary denaturation	95°C	3 min	
Denaturation	94 °C	1 min	Cycles 40
Annealing	50°C	2 min	
Extension	72 °C	2 min	
Final extension	72 °C	10 min	
Hold at	4 °C		

Five microlitres of genomic DNA was used as template in the primary reaction. The reaction took place in a total volume of 25 µl consisting of the following.

Table 3.6.2.2 (b): M1/5 PCR premix

	Reagent	Final conc. in PCR	Volume per 20µl tube
	10× PCR Buffer (Roche)	1×	2.5
	2mM Mixed dNTPs (Promega)	200µM	2.5
	25mM $MgCl_2$ (Roche)	1.5mM	0.75
	10µM Sp1 (MWG Biotech)	100nM	0.125
	10µM Sp2 (MWG Biotech)	100nM	0.125
	Taq DNA polymerase (5units/µl) (Roche)	1unit/PCR	0.2
	DNase-free water (Sigma)		13.8
	Outer PCR product		5

The amplification programme for M3, F/ and M4, F primers PCR was as follows

Primary denaturation	95°C	3 Min	
Denaturation	94 °C	1 min	Cycles 40
Annealing	45°C	1 min	
Extension	72 °C	2 min	
Final extension	72 °C	10 min	
Hold at	4 °C		

Restriction digestions were carried out overnight at incubation temperatures which differ for different enzymes such as Tsp5091 at 65°C, XmnI at 37°C and AluI at 37°C for *dhfr* codon 51, 59 and 108, respectively.

3.6.3 Distinguish recrudescence from new infection

To distinguish genuine recrudescence of resistant parasites from the appearance of parasite arising from reinfection during the follow-up period, day 0 (pre-treatment) and the day of recrudescence-R (post-treatment) isolates were genotyped at two highly

polymorphic gene loci using merozoite surface protein-1 (*MSP 1*) and merozoite surface protein-2 (*MSP 2*) (Ranford-Cartwright *et al.*, 1993)

3.6.3.1 Genotyping at the MSP1 locus

In the primary PCR, oligonucleotide primers pairs 01 and 02, corresponding to the conserved sequence on either side of the repetitive region of *MSP1* (block 2), were included in a single reaction. Using the PCR product generated from the primary reaction as template, the polymorphic repetitive regions of *MSP1* was amplified in a nested PCR using specific primers N1 and N2.

In the primary and nested MSP1 PCR amplification, the reaction took place in a total volume of 25µl containing the following as in table 3.6.3.1

Table 3.6.3.1: MSP 1 PCR premix

	Reagent 01/2 PCR premix	**Final conc. in PCR**	**Volume per 20µl tube**
	10× PCR Buffer (Roche)	1×	2.0
	2mM Mixed dNTPs (Promega)	200µM	0.2
	10µM primer 1	100nM	0.2
	10µM primer 2	100nM	0.2
	Taq DNA polymerase (5units/µl) (Roche)	1unit/PCR	0.2
	DNase-free water (Sigma)		17.2

Five microlitres of the parasite DNA was used as template in the primary reaction, and 2µl of the primary PCR product was used as template in the nested PCR reaction.

The amplification for *MSP 1* was identical for both reactions.

Amplification were performed using a PTC-100^{TM} PCR machine (MJ Research, Inc., Waltham, MA, USA) under the following programme;

Primary denaturation	95°C	3 min	
Denaturation	94°C	25Sec	Cycles 30
Annealing	50°C	35Sec	
Extension	68°C	2 min 30 sec	

Final extension	68 °C	8min
Hold at	4°C	

Five microlitres of nested PCR were mixed with a 1-2 µl of 10× loading dye and analyzed by agarose gel electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.8 µg/ml) in Tris-acetate EDTA buffer. Each day 0 (pretreatment) isolate was run alongside its day of recrudescence (R) isolate. DNA was visualized by ultraviolet transillumination and band sizes determined by comparison with a standard 100-base pair DNA ladder.

3.6.3.2 Genotyping at the MSP2 locus

Oligonucleotide primers pairs S2 and S3, corresponding to the conserved sequence surrounding the central repetitive and polymorphic regions of *MSP1* of block 2 and 3 of *MSP 2* were amplified by nested PCR. In this case specific primers S1 and S4 were used. In the primary and nested *MSP2* PCR amplification, the reaction took place in a total volume of 25µl containing the following as in table 2.6.3.2

Table 3.6.3.2: MSP 2 PCR reagents; S2/3 PCR premix

	Reagent	Final conc. in PCR	Volume per 20µl tube
	10X PCR Buffer (Roche)	1×	2.0
	2mM Mixed dNTPs (Promega)	200µM	0.2
	10µM primer 1	100nM	0.2
	10µM primer 2	100nM	0.2
	Taq DNA polymerase (5units/µl)	1unit/PCR	0.2
	DNase-free water (Sigma)		17.2

Five microlitres of parasites DNA was used as template in the nested PCR reaction, and 2µl of the primary PCR product was used as template in the nested PCR reaction. Amplification were performed using a PTC-100™ PCR machine (MJ Research, Inc., Waltham, MA, USA) under the following programme;

Primary denaturation	95°C 3 min	
Denaturation	94 °C 60 sec	Cycles 30
Annealing	42 °C 35 sec	
Extension	65°C 2 min	
Final Extension	65 °C 8 min	
Hold at	4 °C	

In nested PCR, the programme was different with annealing for 60 seconds at 50 °C, extension for 60 seconds at 70 °C and final extension for 8 minutes at 70 °C, and the rest was the same as in the primary amplification. Five microlitres of nested PCR were mixed with a 1-2 µl of 10× loading dye and analysed by agarose gel electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.8 µg/ml) in Tris-acetate buffer-EDTA buffer. Each day (0) isolate was run alongside its day of recrudescence (R) isolate. DNA was visualized by ultraviolet transillumination and band sizes determined by comparison with a standard 100-base pair DNA ladder.

Three categories of recrudescent infections were identified, namely genuine recrudescence infections, infections with evidence of recrudescent and also of new infections and entirely new infections.

3.6.4 PCR and Dot blotting at the MDR86 locus

Oligonucleotides primers pairs MDR/A1 and MDR/A3 were included in a single outer PCR reaction. Using the PCR products generated from the primary reaction as templates, a nested PCR was then carried out using primers MDR/A4 and MDR/A2. Control amplification using DNA from *P. falciparum* parasites clones known to contain the possible different alleles at position 86 of *mdr1* were used to ensure specificity and sensitivity of the technique. 3D7 and 7G8 were used as wild type while K1 and Dd2 as mutant type.

3.6.4.1 A1/A3 PCR amplification

Amplification were performed using a PTC-100™ PCR machine (MJ Research, Inc., Waltham, MA, USA). In the primary PCR amplification, the reaction took place in a total

volume of 20µl containing 10mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200µM of each dNTPs (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 0.1 µM each and one unit of Taq DNA polymerase. Five microlitre of parasite DNA was used as a template in the primary reaction. Two microlitre of DNA extracted from *P. falciparum* clones 3D7 and 7G8, which has *mdr1* encoding Asn at position 86 were used as the wild type, while K1 and Dd2 which have *mdr1* encoding Tyr at position 86, was used as mutant types.

Amplification programme for A1/ A2 was as follows

Primary denaturation	95°C	3 min	
Denaturation	94 °C	30 sec	Cycles 30
Annealing	50 °C	1 min	
Extension	65°C	2 min	
Final Extension	65 °C	8 min	
Hold at	4 °C		

3.6.4.2 A4/A2 PCR amplification

In the nested PCR amplification, the reaction took place in a total volume of 30µl containing 10mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200µM of each dNTPs (dGTP, dATP, dTTP, dCTP), primers at a final concentration of 0.1 µM each and one unit of Taq DNA polymerase. Two microlitre of primary PCR was used as template in the nested PCR reaction.

Amplification programme for A1/ A2 was as follows

Primary denaturation	95°C	3 min	
Denaturation	94 °C	30 sec	Cycles 30
Annealing	50 °C	1 min	
Extension	65°C	2 min	
Final Extension	65 °C	8 min	
Hold at	4 °C		

5 microlitre of nested PCR were mixed with a 1-2 µl of 10× loading dye and analysed by agarose gel electrophoresis on 1.5% agarose gel stained with ethidium bromide (0.8 µg/ml) in Tris-acetate buffer-EDTA buffer. DNA was visualized by ultraviolet transillumination and the expected nested PCR product band sizes determined by comparison with a standard 100-base pair DNA ladder.

3.6.4.3 Preparation of dot blotting

This was as described by Abdel Muhsin (2002). Twenty microlitres of each PCR product were denatured in 10 mM EDTA and 0.4M NaOH at 100°C for 10 minutes to a final volume of 30µl and then neutralized in an equal volume of 2M ammonium acetate, pH 7.

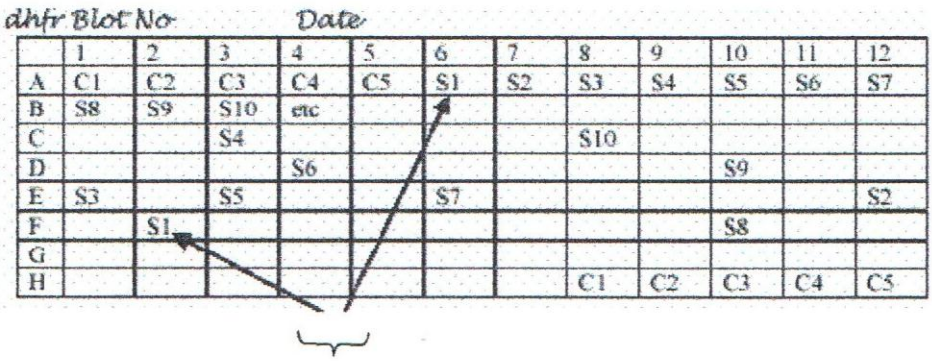
Table 3.6.4.3: Denaturation mix

Reagent	Final Conc.	Volume per 30µl tube
PCR product		20
0.1M EDTA	10mM	3
4M NaOH	0.4M	3
DNase Free water		4

A piece of nitrocellulose membrane (Millipore) was cut to the exact size to fit the manifold. This was then pre-wetted by soaking in TE buffer (10mM Tris-Cl: 1mM EDTA) or 2× SSC for 10 minutes. The nitrocellulose membrane was fitted on to the dot blotting apparatus (Bio Rad) by layering it on the apparatus avoiding bubbles in between the layers, and the apparatus reassembled as per the manufacturers instructions then the screws tightened in a diagonal crossing. The membrane was then re-hydrated by pippeting 200µl of TE buffer into each well and this was then drained. Each denatured sample was then loaded into two non-adjacent positions on the blot by loading 30µl in the two positions as illustrated below (Figure 2.6.4.3)

The solution was then allowed to stand for 30 minutes before releasing the vacuum. The membrane was then removed and neutralised by washing it in 2× SSC for 30-60 seconds followed by a 30-60 seconds wash in 0.4M NaOH to ensure complete denaturation of immobilised DNA and a final rinse in the neutralising solution (1M Tris-HCL, 1.5M NaCl, and pH 8) for 30 seconds. UV cross-linking in an UV cross linker (Vilber Lourmat) for 5 minutes was then done to fix the PCR products onto the membrane. This

was done by placing the membrane on a clean glass plate; this was then placed in the cross linking chamber set at 0.120 joules for 5 minutes. The membrane was then wrapped using cling film and stored at -20°C until it was needed.



	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C2	C3	C4	C5	S1	S2	S3	S4	S5	S6	S7
B	S8	S9	S10	etc								
C			S4					S10				
D				S6						S9		
E	S3		S5			S7						S2
F		S1								S8		
G												
H								C1	C2	C3	C4	C5

Figure 3.6.4.3(c): Duplicate loading of sample and controls

3.6.4.4 Labeling of oligonucleotide probes

Probes for all the possible alleles in each of the codon were labeled, such as Asn and Tyr probes (MWGBiotech) for codon 86 of the *Pfmdr1* gene. Ten picomoles of each probe were labeled with 10μCi of [γ-^{32}P] dATP using 10 units of polynucleotide kinase. This was prepared by adding 25μl water to the tube containing the Ready-To-Go T4 PNK, (Ready To-Go T4 Polynucleotide kinase) (Amersham Biosciences, UK, Cat 27-0737-01) then the tube was incubated at room temperature for 2 to 5 minutes, as recommended by the manufacturer. The contents of tube were then mixed by pippeting up and down briefly. One μl of each 10μM 5'-ends oligonucleotide was added and the reaction volume made to 49μl using sterile water (23μl). From this stage all procedures were carried out in a radiation containment room/area, using beta-shields such as 1cm acrylic for protection from the radiation, and wearing appropriate personal radiation monitors such as film badges. Solid and liquid wastes were disposed off according to the advice of the local Atomic Energy Agency/radiation protection advisors. To the reaction mix of 1μl of [γ-^{32}P] dATP (Amersham Biosciences, UK: Redivue [γ-^{32}P] ATP, 3000Ci/mmol: Cat No. AA00068) was added and mixed gently. These were then spun briefly in a microfuge to collect the contents at the bottom of the tube followed by incubation at 37°C for 30

minutes in a programmable heating block (Eppendorf). Adding 5µl of 250mM EDTA, into the tube, stopped the reaction.

3.6.4.5 Removal of unincorporated [γ-^{32}P] ATP

Unincorporated [γ-^{32}P] dATP was removed using G-25 Micro spin columns (Amersham Pharmacia Biotech, UK. Cat 27-5325-01). These were prepared by re-suspending the resin in the column by vortexing gently. The column was then placed in 1.5 ml screw cap microfuge tube for support, and pre-spun for 1 minute at 3000 rpm, in an eppendorf microfuge, to pack the sephadex resin. The column was placed in a new 1.5ml tube and all of the labeling mixture applied to the center of the angled surface of the compacted resin bed, being careful not to disturb the resin. The column was spun for 2 minutes at 3000 rpm. The purified sample was collected at the bottom of the support tube. The column was discarded accordingly, as radioactive solid waste. The labeled purified probe was stored at –20 °C shielded until required. Labeled probes were used within 2-4 weeks. Unused probes were disposed off as per radioactive items.

3.6.4.6 Hybridization and stringent washes

The prepared blot was unwrapped and placed into a rotor bottle, making sure that there were no overlapping areas, into which 20 mls hybridization buffer were added (0.25-0.125ml per cm² of membrane). The hybridization buffer consisted of 5× SSPE, 5x Denhardt's reagent, 0.5% SDS, 0.02mg/ml sonicated salmon sperm DNA (Gibco) in DNase-free Water. This was then incubated at the appropriate temperature in a hybridization oven for 30 minutes with agitation making sure that the bottle was closed properly and the buffer does not leak. 20µl of the labeled oligonucleotide probe (1µl for every 1ml of the hybridization buffer) was then added into the bottle contents. Hybridization at appropriate temperature for at least 5 hours with agitation followed. Overnight hybridization was preferred especially when the labeled probe was 2-3 weeks old.

Table 3.6.4.6: Hybridization and washing conditions for *mdr1-86* probes

Locus	Probe name	First wash	Stringent washes
Pfmdr1	mdr1Tyr-specific	2×SSC, 20min @ 43°C	[1×SSC/0.1%SDS) 10min] x 2 @ 43°C
	mdr1Asn-specific	2×SSC, 20 min @ 41°C	[1×SSC/0.1%SDS) 10min] x 2 @ 41°C

The hybridization solution was poured off and disposed of accordingly. An excess (at least 1ml/cm² blot) of wash buffer 1 (2 × SSC) was added at the corresponding temperature and incubated with agitation in the oven for 20 minutes at the same temperature. The washes were repeated once or twice using excess (at least 1ml/cm² blot) wash buffer 2 (1× SSC/0.1% SDS) at the same temperature and incubated with agitation for 10 minutes each. The washing solutions were poured off and disposed of accordingly then the blot sealed by wrapping in cling film without allowing the blot to dry out.

3.6.4.7 Autoradiography

The sealed blot was taped right side up (DNA-side up) into an autoradiography cassette (Kodak) with intensifying screens. To avoid problems with autorad orientation, a marker strip was used or the film folded at bottom right corner and this allowed for accurate positioning of the autorad after developing. Blots were exposed on Kodak® (Rochester, NY) X-Omat film for 12-24 hours at –70°C in the freezer (Revco®).

The films were then removed from the cassette and developed to score the sample against the controls. A second piece of film could also be exposed to the membranes for longer period, to assist in the detection of weak bands. If any of the controls showed non-specific hybridization, an extra stringent wash was carried out. The autorad was obtained by developing the image in a developer solution (Kodak) for 5 minutes followed by a brief rinse in clean water and finally fixing the developed image in a fixative solution (Kodak) for 5 minutes and the fixative rinsed off with clean tap water. These processes were done in a dark room as the films are light sensitive. The films were then air dried then scored.

3.6.4.8 Stripping the probe from the membrane

The membranes were then stripped off the probe using excess of 0.1 M NaOH, for 15 minutes, while incubated at room temperature with agitation, repeating the process followed by a brief wash with 5× SSC and the blot was then probed again or stored, after sealing the blot by wrapping in a cling film, at -20°C or dried and store at room temperature sandwiched and taped between two pieces of clean filter paper.

3.7 Data recording, processing and analysis

After restriction digest, the restriction fragments that resulted were used to score the isolates based on the controls used. After radio probing, parasite clones and isolates were scored either as wild type or mutants compared to the specific probe used in a given blot or as indicated by the controls. Data recording was done on MS Excel sheets, MS Access database and also on the laboratory workbook. Epi-Info computer software was used to calculate the P values of mutations in codon 51, 59 and 108 of *dhfr* gene before treatment.

CHAPTER FOUR

4.0 RESULTS AND DATA ANALYSIS

4.1 Sample collection

A total of 160 samples were collected from the study site. These were all pre-treatment blood samples collected on Whatman filter paper. These were all positive cases according to the microscopists at the study site. AS + AQ arm completed follow-up; in the AS + SP arm one was lost to follow-up and one withdrawn due to repeated dose vomiting. No significant adverse events were reported.

4.2 Restriction digests (PCR/RFLP)

4.2.1. Pfcrt

A PCR-RFLP strategy was used to demonstrate the presence or absence of the T76 mutation in *Pfcrt* that was recently associated with chloroquine resistance (Djimde *et al.*, 1999; Fidock *et al.*, 2000). The 134-bp nested PCR amplification followed by restriction enzyme analysis indicates that *ApoI* digestion cuts the (K76) wild-type allele but not the (T76) mutant one.

PCR products with the wild-type codon were cleaved by *ApoI* into two fragments (126 and 73 bp). Following enzymatic digestion, the PCR products were separated by 2% ethidium bromide-stained agarose gel.

Agarose gel electrophoresis

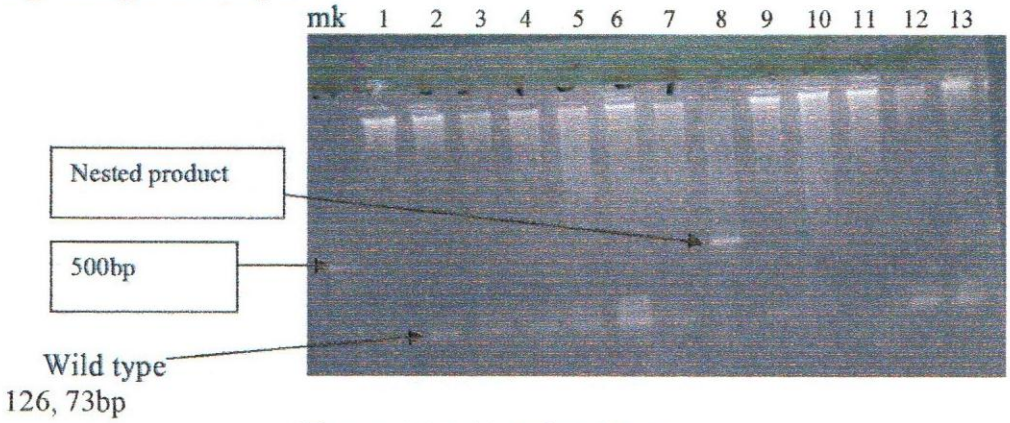


Figure 4.2.1 (a): Pfcrt-RFLP

4.2.1. (a) Schematic representation of agarose gel electrophoresis; PCR-RFLP products of the *Plasmodium falciparum* chloroquine resistant transporter (*Pfcrt)* gene using TCRP/TCRD primers. Mk is the 100 base pair molecular weight marker (Roche). The intense band is at 500 base pair fragment. Lane 1 is water negative PCR control; lane 3 to 7 consists of the field isolates. Lane 8; nest II product and lane 2 is W2 wild control.

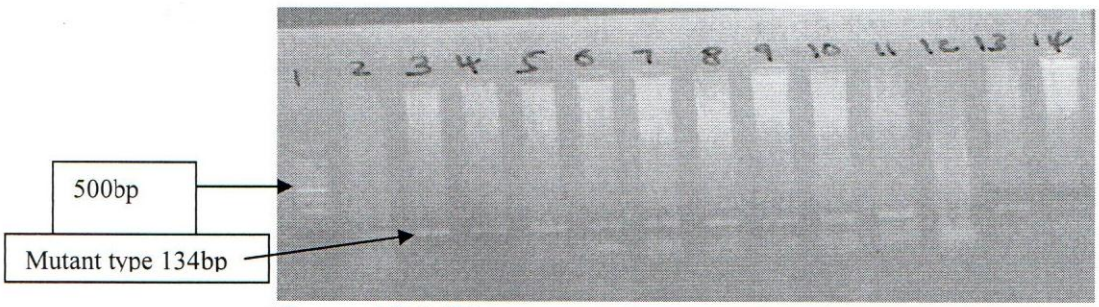


Figure 4.2.1 (b)

4.2.1. (b) Schematic representation of agarose gel electrophoresis; PCR-restriction fragments length polymorphism products of the *Plasmodium falciparum* chloroquine resistant transporter (*Pfcrt)* gene using *ApoI* enzyme. Lanes 1 is the100 base pair molecular weight marker, lane 2 to 14 are field isolates, all having mutant type.

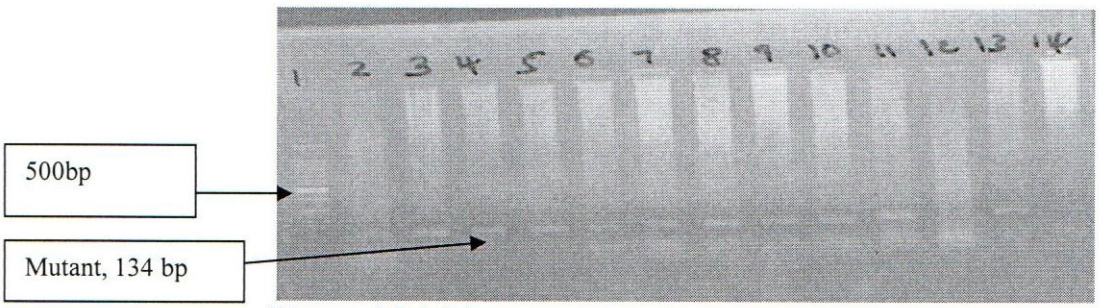


Figure 4.2.1 (c)

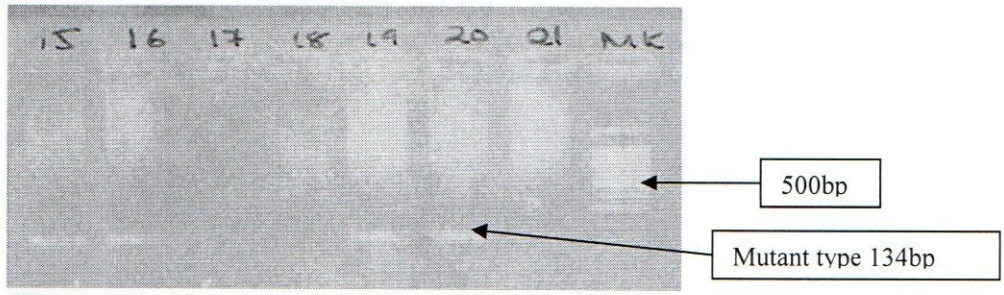


Figure 4.2.1(c).cont

4.2.1(c) Schematic representation of agarose gel electrophoresis; PCR-RFLP products of the *Plasmodium falciparum* chloroquine resistant transporter (*Pfcrt)* gene using TCRP/TCRD primers. Lane 1 is the 100 base pair molecular weight marker (Roche). Lanes 2 to 16 and 19-21 consists of the clinical specimens showing positive amplifications. Wells 17 and 18 had no detectable amplification. Restriction fragments of 126 and 73 bp are consistent with sensitive K76 (AAA) allele. Uncut 134-bp amplicons are indicative of resistant T76 (ACA) allele.

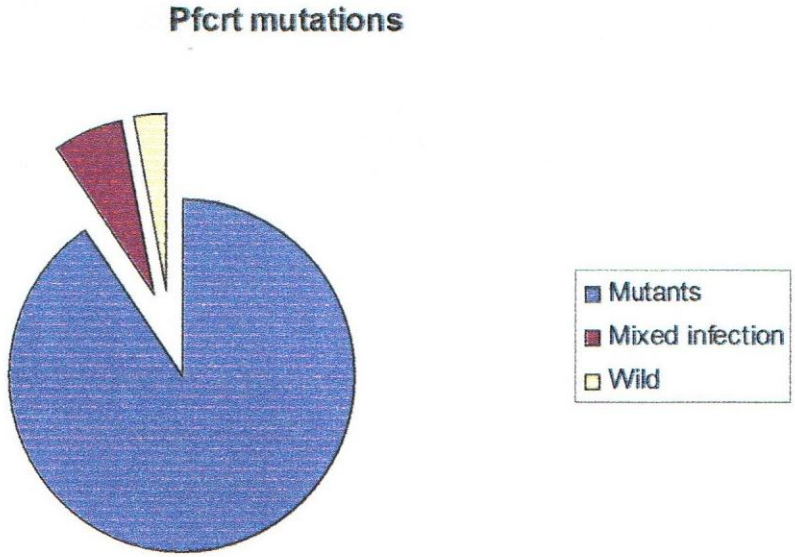


Figure 4.2.1 (d) *Pfcrt* mutation results

Overall, 90.0% (144/160, 95% CI 84.3-94.2) of pre-treatment infections carried Pfcrt K76T mutations, although 16 of these were mixed with a wild-type genotype.

4.2.2: Dhfr codon 108, 59 and 51.

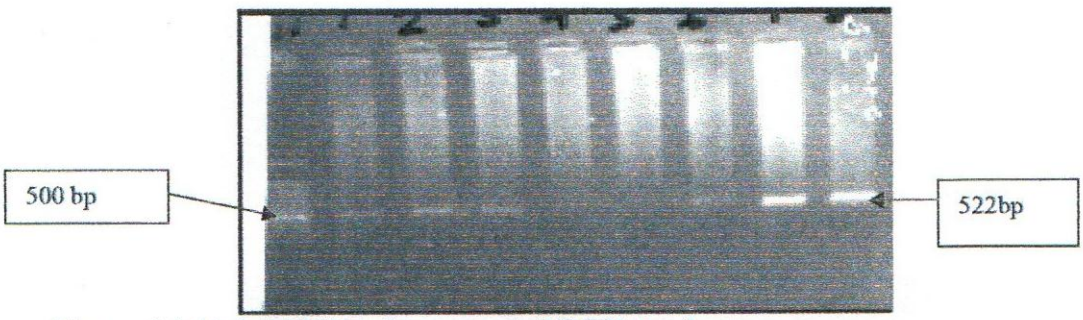


Figure 4.2.2(a): M3 F/ primers nested PCR products



. Figure 4.2.2(b) *dhfr* nest II products

Schematic representation of agarose gel electrophoresis of nest-II PCR products of the dihydrofolate reductase (*dhfr)* gene, for RFLP.

Mk is the 100 base pair molecular weight marker (Roche). The intense band is at 500 base pair fragment. These were run on 1.8% agarose gel on a potential difference of 80V for 30 min. Figure **a** and **b** has the 522bp product of the M3 F/ primers.



Figure 4.2.2(c): The F M4 primers nested PCR products

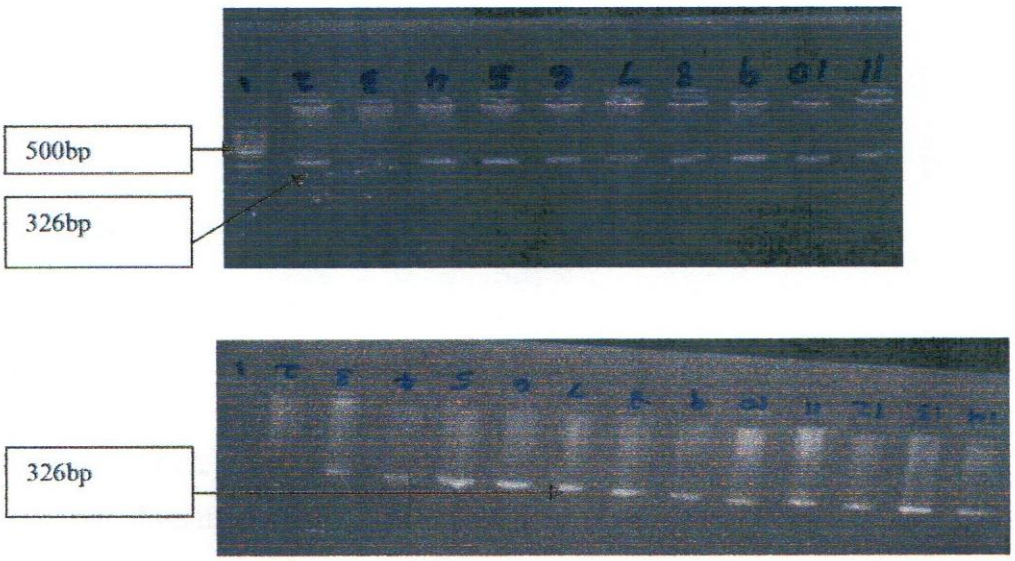


Figure 4.2.2 (d)

4.2.2. (c, d) Agarose gel electrophoresis; nest-II PCR products of the dihydrofolate reductase (*dhfr)* gene, from field isolates for RFLP using F M4 primers giving a 326bp product. These were run on 2% agarose gel on a potential difference of 80volts for 30 minutes.

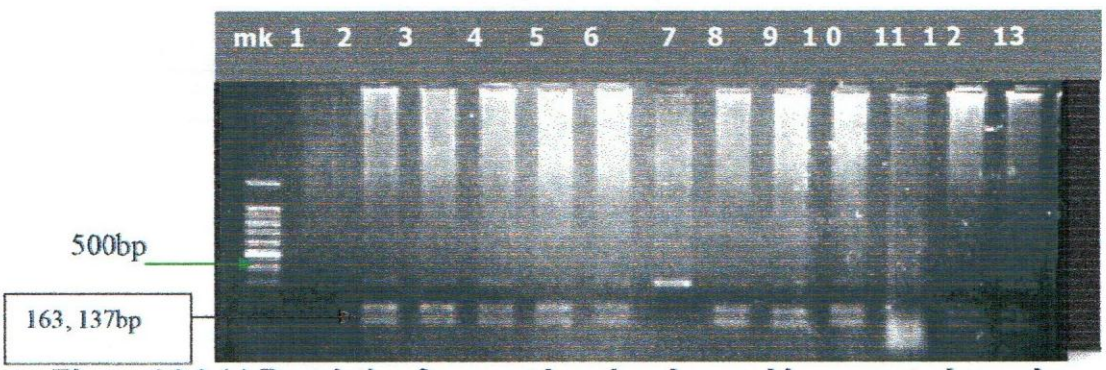


Figure 4.2.2 (e) Restriction fragment length polymorphisms- control samples

Restriction digest of the 326bp fragment of the F M4 primers, using *Xmn*1 restriction enzyme, to detect mutation in the *dhfr* 59 on the quality control samples from IAEA/Glasgow. Well 1 had a negative control for the digest, Wells 6 had a mutant type control (Dd2) with restriction fragments sized at 189 and 137bp while well 7 contains an

un-cut (non restricted nest II product) control. These were run on 2% agarose gel on a potential difference of 80volts for 30 minutes.

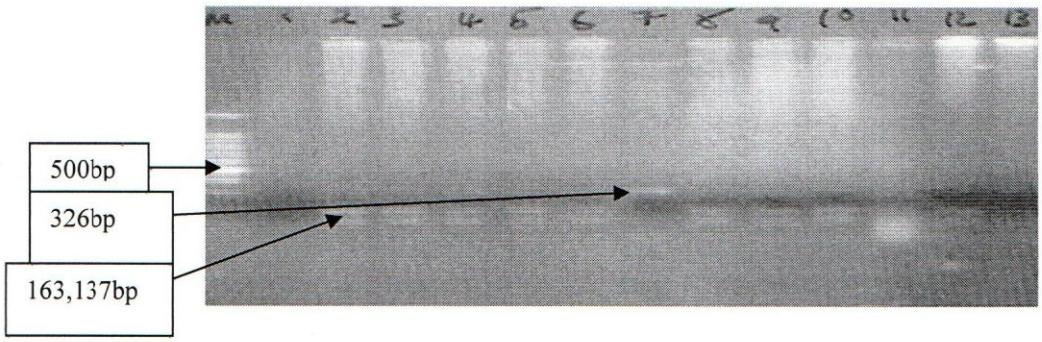


Figure 4.2.2 (f): *dhfr* codon 59

Restriction digest of the 326bp fragment of the F M4 primers, using *Xmn*1 restriction enzyme, to detect mutation in the *dhfr* 59 on the clinical samples. Wells 1 had a negative control (water) and well 2-6 and 8-13 are clinical isolates with mutant type restriction fragments sized at 163 and 137bp, while lane 7 is unrestricted nest II product control. The gel was run for 30 minutes before visualisation.

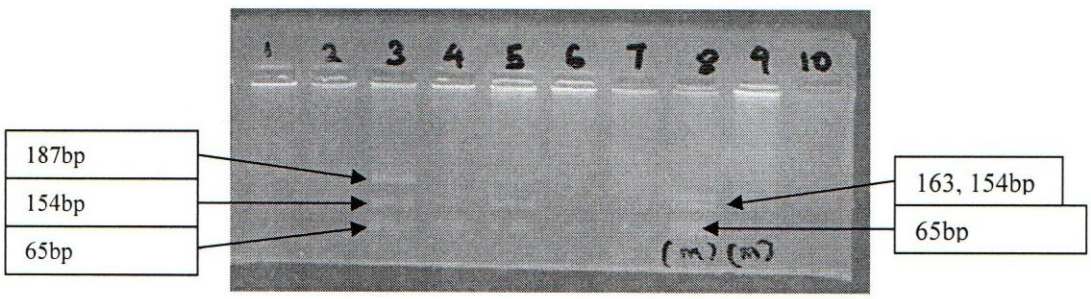


Figure 4.2.2(g): *dhfr* codon 51

Restrictions digest of the F M4 primers, using *Tsp5091* restriction enzyme, to detect mutation in the *dhfr* 51 on the clinical samples. Wells 3-7 and 10 had a mutant type control with restriction fragments sized at 187, 137bp and 65bp while well 8 and 9 had a wild control with fragments sized at 163, 154 and 65bp. Well 1 and 2 contains an un-cut non restricted nest II product control. The gel was run for 30 minutes before visualization.

Table 4.2.2: Mutations in codon 51, 59 and 108 of *dhfr* gene before treatment

Dhfr genotype	Mutation			Frequency	
	N51I	C59R	S108N	n	%
No mutations	No	No	No	28	17.5
Single mutations	Yes	No	No	0	0.0
	No	Yes	No	0	0.0
	No	No	Yes	90	56.3
Double mutations	Yes	Yes	No	0	0.0
	Yes	No	Yes	6	3.8
	No	Yes	Yes	31	19.3
Triple mutations	Yes	Yes	Yes	5	3.1
Total				160	100.0

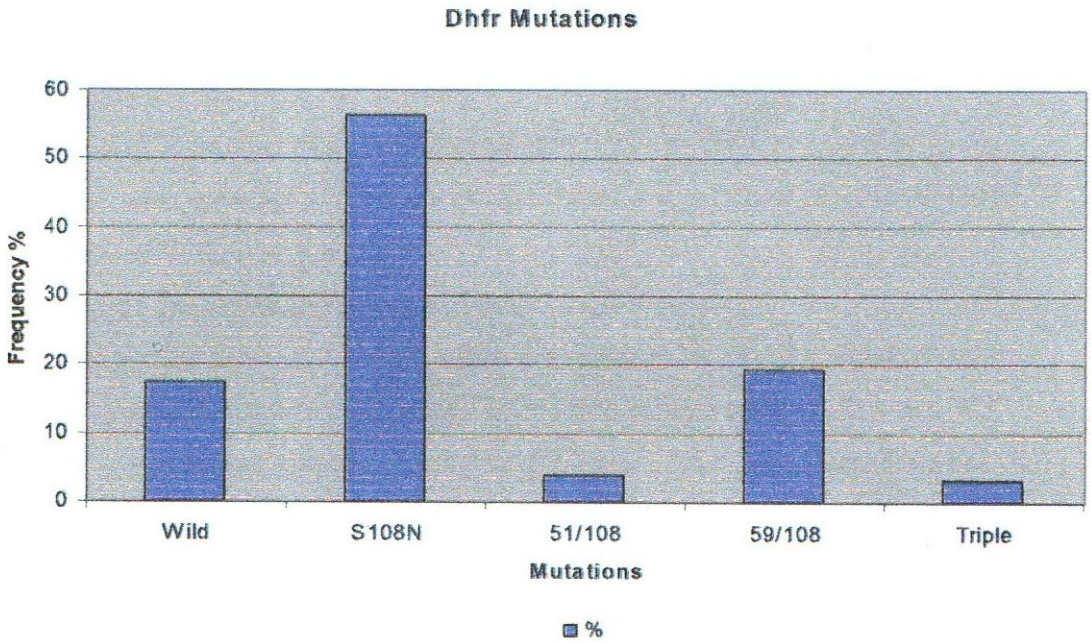


Figure 4.2.2 (h) *dhfr* mutation graph

Mutations in *dhfr*, 82.5% (132/160, 95% CI 75.7—88.0) of patients carried one or more mutations at *dhfr*, but none of these were I164L. N51I and C59R mutations occurred exclusively in the presence of S108N (Table 3.2.2), but this association was significant only for C59R ($P = 0.214$ for N51I; $P = 0.002$ for C59R). Triple *dhfr* mutations were infrequent.

4.3 Genotyping *MSP 1*/ *MSP 2* loci

Amplification of DNA from 50 pairs of isolates (day 0 and R) for the recrudescence or treatment failure was performed for the *MSP 1* and *MSP 2* loci. The sample pairs were classified as follows, based on the combined results from both loci typed:

a) If all the PCR products in the day 0 sample were also present in the R sample and there were additional fragments for both loci, this was considered a genuine recrudescence with all initials parasites recrudescing (all resistant) (Figure 4.3, a)

b) If PCR products in the day 0 sample were all completely different to those in the R sample this was considered evidence of reinfection during follow-up (Fig 4.3,b)

c) If PCR products in the day 0 sample were present in the R sample but the R sample also had new PCR products this was considered a 'mixed' results in the R sample with mixtures of drug resistant and new parasites (Figure 4.3, c)

d) If the day 0 sample had some PCR products not in the R sample but the R sample had PCR product that were all present in the day 0, this was considered a recrudescence but with mixtures of resistant and sensitive parasites in the day 0 sample (Figure 4.3, d)

Figure 4.3(a): Representation of gels of *MSP1* PCR products.

Each box represents a gel, Lane 1 on each gel shows PCR products obtained after amplification of the *MSP 1* locus of the day 0 DNA sample and lane 2 shows PCR products obtained after amplification of *MSP 1* locus of the day of recrudescence (R) DNA sample. (a)- (d) represents different possible scenarios, where (a) and (d) are genuine recrudescence, (b) represents a reinfection and (c) a combination of recrudescence and reinfection. Results for *MSP 2* products were similarly classified.

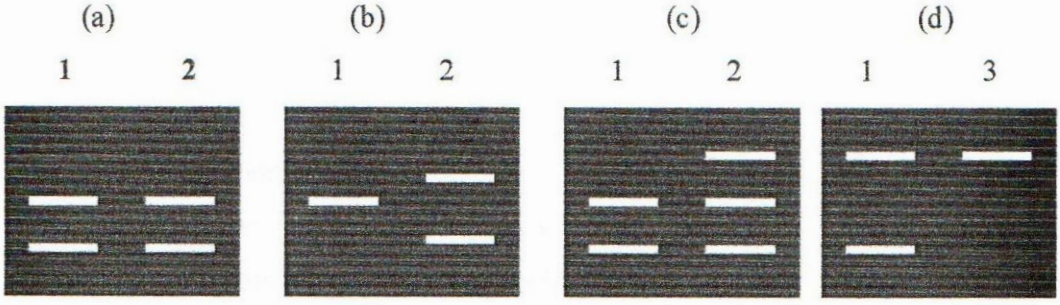


Figure 4.3 (a) Representation of gels of *MSP1* PCR products.

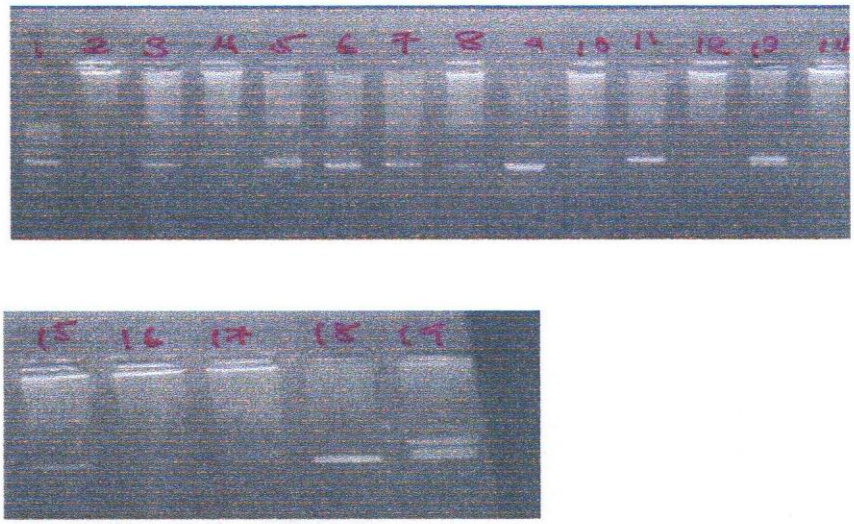


Figure4.3 (b) MSP 1 results

Agarose gel photos of nest 2 products of the *MSP 1* gene using O1/O2 and N1/N2 primers. The expected product size was 400-600bp. These were run on a 1.5% agarose gel for 30 minutes. The samples were from field isolates from day 0, before treatment run along follow-up days. For example, lane 1 is the DNA marker, lane 2 and 3 are from the same patient but day 0 and 7 respectively. The loading was the same for *MSP 2*.

Lane 1: DNA marker
Lane 2: SP1. day 0
Lane 3: SP1. day 7
Lane 4: SP2. day 0
Lane 5: SP2. day 7
Lane 6: SP2. day 14
Lane 7: SP5. day 0
Lane 8: SP5. day 7
Lane 9: SP5. day 14

Lane 10: SP7. day 0
Lane 11: SP7. day 7
Lane 12: SP7. day 14
Lane 13: SP7. day 28
Lane 14: SP8. day 0
Lane 15: SP8. day 7
Lane 18: SP 10. day 0
Lane 19: SP 10. day 7
Lane 16: SP9. day 0
Lane 17: SP9. day 14

Day 0 is before treatment.
Day 7, 14 and 28 are recurring (follow-up) days after treatment
SP are codes for patients treated with Fansidar (SP).

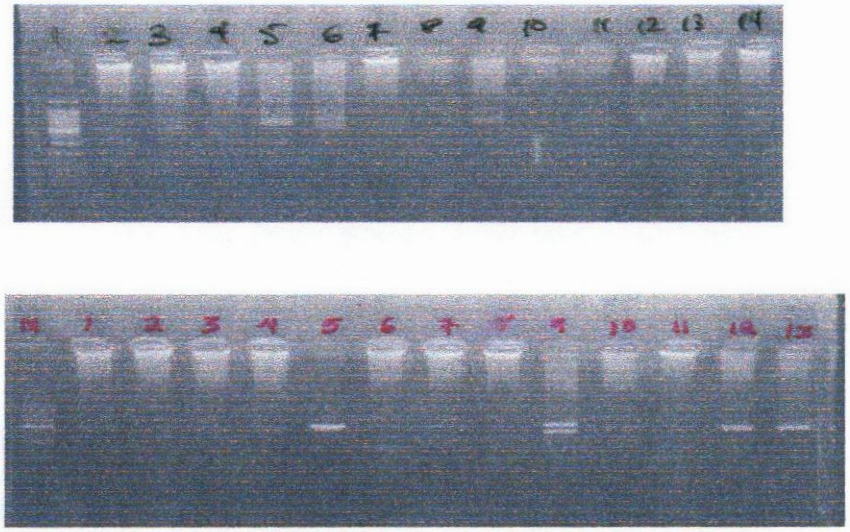


Figure 4.3 (c) MSP 2

Agarose gel photos of nest 2 products of the *MSP 2* gene using S3/S2 and S1/S4 primers. These were run on a 1.5% agarose gel for 30 minutes.

Of the 50 pairs of isolates analyzed, 41 (84%) had genuine recrudescent parasites as classified in Figure (a) and (d), 5 (10%) were entirely new infection, as shown in figure (b), and 3 (6%) had both recrudescence and re-infection in the R sample as classified in Figure (c). One pair of isolates did not amplify for *MSP1* locus.

4. 4 Dot blotting/ Radio probing for mdr1-86

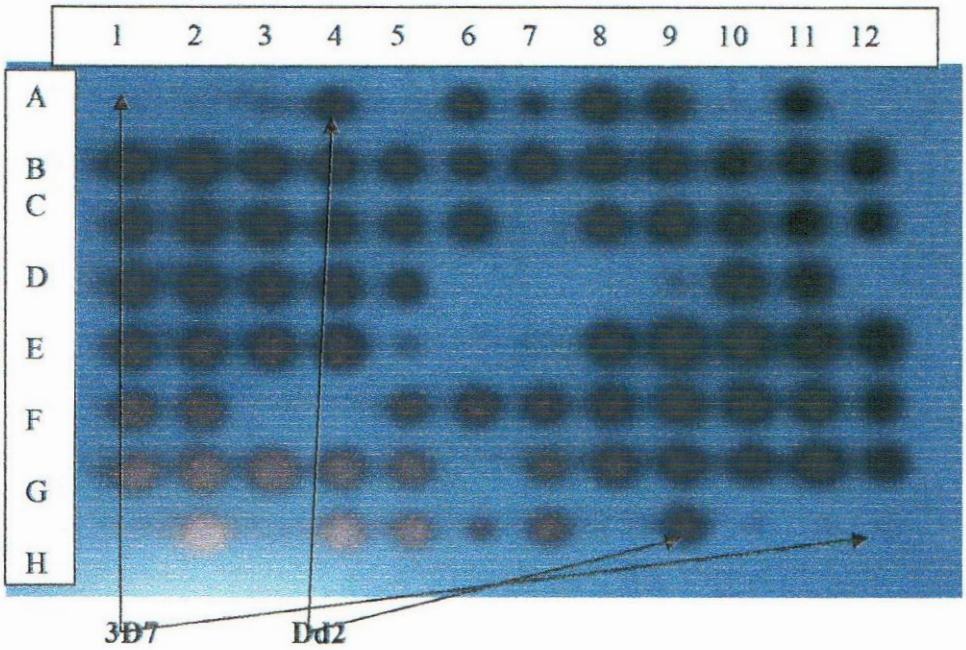


Figure 4.4 (a) Autoradiograph showing optimized conditions (Tyr- specific)

Autoradiograph showing optimized conditions giving specific hybridization with the Tyr-86 probe (mdr1-86Tyr: GAAATATTTTCGTGCAGTTAC), (associated with amodiaquine and chloroquine resistance).

Sample position A1, A2, A3, A4 and A5 consist of *Plasmodium falciparum* clones 3D7, 7G8,K1, Dd2 and water (negative control) respectively. The samples were loaded in duplicate, therefore sample position 12H, 11H, 10H, 9H and 8H are the controls, 3D7, 7G8, K1, Dd2 and water. The blot was hybridized for 10 hours at 43°C stringent washes were as described, and the blot was exposed to X-ray film for 14 hours.

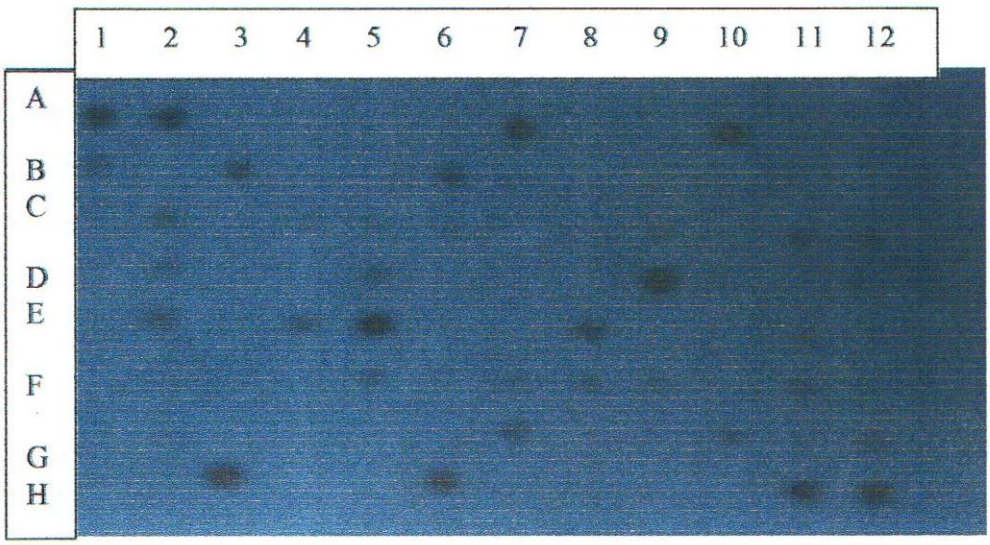


Figure 4.4 (b) Autoradiograph showing hybridization using Asn 86 specific probes

Autoradiograph showing optimized conditions giving specific hybridization with the Asn- 86 probe (mdr1-86Asn: GAAATATTTTCGTGCAGTTAC), associated with amodiaquine and chloroquine sensitivity.

Sample position A1, A2, A3, A4 and A5 consist of *Plasmodium falciparum* clones 3D7, 7G8,K1, Dd2 and water (negative control) respectively. The samples were loaded in duplicate, therefore sample position 12H, 11H, 10H, 9H and 8H are the controls. The blot was hybridized for 10 hours at 41°C stringent washes were as described and exposed for 14 hours.

Table 4.4 Dot blotting results

Sample ID	Position in dot blot	*mdr1*-86 Asn	*mdr1*-86 Tyr
7G8	A1, H12	+ (positive)	- (minus)
3D7	A2, H11	+	-
K1	A4, H9	-	+
Water (negative control)	A5, H8	-	-
F61	A6, H7	-	+
C66	A7, H6	+	+

C113	A8, H5	-	+
C89	A9, H4	-	+
C107	A10, H3	+	-
F74	A11, H2	-	+
C89	B1, G12	-	+
C92	B2, G11	-	+
C102	B3, G10	-	+
C101	B4, G9	-	+
F65	B5, F8	-	+
C69	B6, G7	-	+
C76	B7, F6	-	+
C110	B8, G5	-	+
C97	B9, G4	-	+
C95	B10, G3	-	+
F70	B11, G2	-	+
F67	B12, G1	-	+
F73	C1, F12	-	+
F71	C2, F11	-	+
F77	C3, F10	-	+
C103	C4, F9	-	+
F78	C5, F8	-	+
C105	C6, F7	-	+
C91	F5, C12	-	+
F97	E2, C11	-	+
C29	E1, C8	-	+
F62	E3, C10	-	+
C61	E4, C9	-	+
C85	E5, D9	+	-
C54	F1, D10	-	+
F63	F2, D11	-	+

C73	D1, E12	-	+
C77	D2, E11	-	+
F69	D3, E10	-	+
F1-70	D4, E9	-	+
C74	D5, E8	-	+

+/- indicates the presence or absence of an allele.

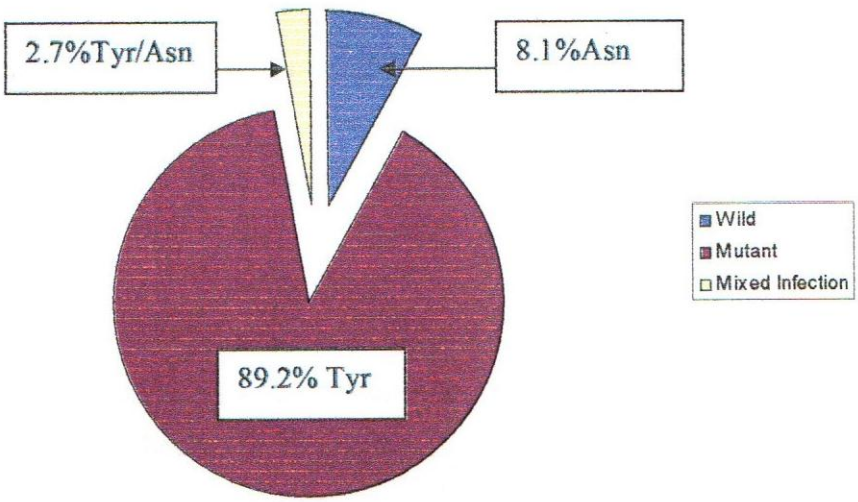


Figure 4.4(c): Dot blot chart result

Baseline dot-blot hybridization of the *mdr1* 86 locus was successful for 37 isolates: 33 (89.19%) carried parasites harboring the *mdr1*-86 Tyr resistance allele, while 3 (8.11%) carried *mdr1*-86 Asn sensitivity allele and 1 (2.7%) were of mixed infection, having both resistance and wild type allele. Mutations associated with CQ and AQ resistance are surprisingly frequent in Southern Sudan. This implies that, CQ would work very poorly here. AQ resistance mutations already present and could rapidly become dominant, especially if AQ monotherapy is more widely used. It is nevertheless clear that any future use of AQ, in Sudan as elsewhere, should occur strictly in a combination regimen.

CHAPTER FIVE

5.0 DISCUSSION

Several studies associating mutations in *dhfr*, *dhps*, *pfcrt* and *mdr1* genes with treatment outcome following the treatment of patients with SP, AQ and CQ respectively have been done. However, no data on *in vitro* antimalarial efficacy were available for the Nuba mountains and policy shift was done on an *in vivo* outcome. This is the first study of malaria in Nuba mountains region and the first to determine its molecular assessment in southern Sudanese territory.

War-affected Sudanese populations have poor access to health care, and malaria control has been particularly neglected. As elsewhere in Sudan, *P. falciparum* malaria is the main health problem for nearly half the year. Mutations associated with CQ and SP resistance are surprisingly frequent in the Nuba mountains, despite isolation and apparently limited drug use apart from 3 years of MSF intervention with CQ, SP and then AS + SP. This finding may have explanations aside from mere drug pressure. Firstly, a recent influx of returnees from the Khartoum area may have introduced previously absent mutant strains. Secondly, in such a markedly seasonal transmission setting, the ecological balance of strains could be heavily modulated by genetic drift (Anderson *et al.*, 2003). As shown elsewhere in Sudan (Abdel-Muhsin *et al.*, 2004), resistant mutants may survive more easily in the dry season, and could therefore be amplified in the subsequent seasonal peak.

The pre-treatment samples from those whom SP/AS treatment failed to clear parasitaemia were genotyped at the *Pfcrt* 76 locus. All (100%) of the pre-treatment sample posses parasites with the mutant allele *Pfcrt* 76, which confer resistance to CQ *in vitro*. One possible explanation for this could be poor drug absorption and /or fast metabolism of CQ in these children. It is also possible some clinical resistance to CQ could be the result of mutations in genes other than *Pfcrt*. Typing of the pre-treatment samples of individuals in who SP/AS was successful in clearing parasitaemia showed that *Pfcrt* is also present of 100% in samples, 23% of these being multiple clone infections where the K76 is also present. The *Pfcrt* allele therefore appears to be at very high prevalence in this region of Southern Sudan. Despite this, treatment failure rates with CQ

alone, the previous 1st line drug in southern Sudan until 2003, when it was replaced by SP were estimated at 40%[http://www.health. go.sn/ malaria.htm]. It is obvious that in this area, the *Pfcrt* mutations is not predictive of CQ treatment failure, and it is likely that mutations in other genes, such as *Pfmdr1* (Babiker *et al.*, 2001; Dorsey *et al.*, 2001; Mockenhaupt *et al.*, 2001) are necessary for clinical treatment failure. Host factors such as immunity may also contribute to clearance of parasites resistant to CQ. The level of the anti-parasite immunity is difficult to measure, but age can be used as an indicator (Djimde *et al.*, 2001; Omar *et al.*, 2001; Djimde *et al.*, 2003). Children in the age group of 25-49 months are more likely to clear resistant parasites than younger children of between 6-12 months. However, it was observed that children of age 6-12 months old are better at clearing resistant parasites than children age 13-24 month old though this was not statistically significant. This could be because younger children still retain some anti-parasite protective immunity from maternal antibodies. The maternal antibodies could have waned in children of age 13-24 months old and they have not had time to develop a significant and effective anti-parasite immunity themselves. Since malaria transmission in the study area is holoendemic, children were highly exposed. This could account for the increased rate of clearance of resistant parasites in the older group of between 25-49 months old due to age acquired immunity.

The spread of resistance of *Plasmodium falciparum* to chloroquine has pointed the need for several African countries to identify alternative drugs as first-line antimalarial treatment, taking into account their activity against local parasite strains but also their tolerability, ease of administration, and cost (Foster, 1994; Fevre *et al.*, 1999). Many African countries currently use a sulfadoxine- pyrimethamine combination (SP) or amodiaquine (AQ) to treat uncomplicated *Plasmodium falciparum* malaria. Both drugs represent the last inexpensive alternative to chloroquine. Several studies have indicated that AQ is effective in treating chloroquine-resistant *P. falciparum* malaria parasites (Deloron *et al.*, 1988; Olliaro *et al.*, 1996) and recent studies report high cure rates in Africa, (Staedke *et al.*, 2001; Adjuik *et al.*, 2002; Basco *et al.*, 2002) despite concerns about the haematological and hepatological side effects reported during prophylactic use of this drug (Phillip-Howard *et al.*, 1990). Our results support the finding that the *Pfcrt*

gene is an important determinant of chloroquine resistance in *P. falciparum* (Fidock *et al.*, 2000) and that *Pfmdr1* is involved in modulating the level of resistance (Reed *et al.*, 2000; Djimde *et al.*, 2001). Two allele of *Pfmdr1* previously were claimed to be associated with amodiaquine resistance; one was characterized by Y86, and the other by 1034C, 1042D and 1246Y. Most field studies have investigated the Y86 allele, which was associated with chloroquine and amodiaquine resistance in some studies (Foote *et al.*, 1990; Basco *et al.*, 1995) but not in others (Awad-El-kariem *et al.*, 1992; Haruki *et al.*, 1994). The current study found an association between *in vitro* amodiaquine resistance and the presence of *Pfmdr1*-Y86 on its own among the isolates; however, recent transfection work has provided evidence that *Pfmdr1* is involved in modulating *P. falciparum* amodiaquine resistance. It is likely that the AQ component of the drug combination is responsible for treatment outcome or success. In support of this hypothesis prior to the introduction of AQ/AS as the 1st line drug, CQ treatment failure rates were in excess of 30% (Talisuna *et al.*, 2002), whereas the combination drug gave an adequate clinical response of 92%.

Of the two combinations, AS + AQ is likely to have a longer therapeutic lifespan given global SP resistance trends (Roper *et al.*, 2004). AQ resistance seems to develop more slowly according to *in vivo* observations (East Africa Network for Monitoring Antimalarial Treatment, 2003). The genotyping basis of AQ resistance, however, is yet to be elucidated, representing a crucial missing element in decisions about which artemisinin-based combinations can be deployed in any given setting. The *Pfcrt* K76 mutations have previously been associated with AQ failure *in vivo* (Ochong *et al.*, 2003) but, in the present trial, even a 90% prevalence of K76T mutants failed to significantly affect AS + AQ efficacy. It is nevertheless clear that any future use of AQ, in Sudan as elsewhere, should occur strictly in a combination regimen, preferably prescribed in age-specific blister packs. For the Nuba mountains region, we recommend an urgent policy shift from CQ, to AS + AQ.

Mutations in *dhfr* are clearly linked to resistance to pyrimethamine and to SP. The key mutation at position 108 (Serine to asparagines) was present in all patients in whom treatment was unsuccessful, both pre- and post treatment with SP/ AS. None of the patient had parasite with the wild type allele *dhfr* 108Ser.

Two other mutations in *dhfr* in codon 51 and 59 have been linked to SP resistance. For codon 51, the majority of parasites typed had the wild form encoding 51Asn. Infections with purely the mutant form of codon 51 (51Ile) were found in 8% of pre-treatment samples from patients in whom SP/AS failed to clear parasitaemia. Mixtures of wild type (51 Asn) and mutant (51 Ile) parasites were found in two samples, one pre-treatment isolate in the SP/AS arm, and one isolate in the AQ/AS arm. A pure wild infection was found in 80% patients with AQ/AS post-treatment and 50% in SP/AS treatment failure. Because the majority of infections carried only the wild allele, there was significant association between the presence of different pre-treatment *dhfr* 51 allele and the treatment combination failure in AQ/AS and SP/AS treated patients

For codon 59, the frequencies of wild type (59Cys) and mutant (59Arg) alleles were more evenly balanced. Infections with only the wild type allele or only the mutant allele were found in a small number of pre-treatment patients in both treatment regimes. There was evidence for selection of parasites carrying the mutant genotype *dhfr* 59Arg following treatment with SP/AS. Seventeen patients with both parasites with wild type (59Cys) and mutant (59Arg) allele prior to treatment, half of them (8/17) had only parasites with the mutant allele at recrudescence, with the reminder unchanged as mixtures. It is also noteworthy that twenty five of thirty samples with apparently only parasites carrying *dhfr* 59Cys at admission were found with parasites carrying only *dhfr* 59Arg at recrudescence. These samples appeared to be genuinely recrudescent by *MSP1* and *MSP2* genotyping. One explanation for this is that parasites carrying 59Arg were present in the patient at admission, but were either at too low parasitaemia to detect, or were not in periphery at time of sampling. Selection following SP treatment favoured 59Arg parasites, so that their number increased to detectable levels by the time of clinical or parasitological treatment failure.

Analysis of combination of drugs to recrudescence showed that patients with parasites carrying pure *dhfr* 59Arg in D0 sample recrudesced significantly earlier than those with a mixture of *dhfr* 59Cys + 59Arg or those with pure *dhfr* 59Cys. This was true only for the SP arm of the study; it was not observed with the AQ/AS arm of the study, possibly because of the low numbers of parasites recrudescing early (< 14 days) in the latter arm.

It has been reported that combinations of mutations in *dhfr* are associated with SP treatment failure *in vivo*. Patients carrying infections with pure 108Asn/51Ile did not have wild type and mutant parasites following treatment with SP/AS or with AQ/AS. Patients carrying infections of pure *dhfr* 108Asn/59Arg double mutant parasites had significantly earlier recrudescence following SP treatment than those with other alleles. There was significant difference in the patients treated with AS/AQ, possibly because very few patients in this drug treatment arm had early treatment failure with recrudescence before day 14 post treatment. Patients carrying infections of pure triple mutant (dhfr108asn/ 51Ile/59Arg) recrudesced significantly earlier than patients with other infections in the SP/AS drug treatment arm, but not AQ/AS treated patients, for the same reasons discussed above. For the SP/AS-true recrudescence subset, even with the low numbers, there was still an association with the triple mutant and early recrudescence.

Treatment failures were studied by molecular analysis of the *MSP-1* and *MSP-2* genes to distinguish recrudescences from reinfection. Molecular genotyping is a suitable method for this purpose, since *in vitro* drug sensitivity data may not be sufficient to predict *in vivo* failures. Indeed, *in vivo* and *in vitro* data from high-transmission areas may not correlate, especially in the case of SP (Ringwald *et al.*, 1999; Basco *et al.*, 2000; Ringwald *et al.*, 2000). Since in areas of intense transmission of malaria, the majority of infections are due to multiple parasite lines, these mean values may hide minor clones with which recrudescence will originate. Furthermore, host immunity certainly plays an important role in adding its activity to that of the drug. The identification of molecular markers of resistance in parasite lines may be a useful alternative to *in vitro* tests. Currently the relevance of such markers still needs to be confirmed, in particular for 4-amino-quinoline compounds (Su *et al.*, 1997). In the present study, the amplification of two markers, *MSP-1* and *MSP-2*, was sufficient to distinguish reinfection from recrudescence. Both markers were useful since, if *MSP-2*, the most polymorphic marker was tested alone, results would have differed in 10 of the 47 instances: 9 cases of recrudescence plus reinfection would have been determined as recrudescence, and 1 case of recrudescence plus reinfection would have been established as pure reinfection.

Although the study was carried out in an area of low transmission, this may not indicate a high level of re- infection during the follow- up period. Only one sample was taken at admission and therefore parasites that were sequested, or in the liver stage, at the same time of sampling could have been missed. Therapeutic blood levels of CQ persist for 6-10 days (White *et al.*, 1998) and those of SP following the usual dose persist for 15- 52 days (Watkins and Masobo, 1993). Synergistic activity between sulfadoxine and pyrimethamine is lost after approximately 15 days and after this period, parasites respond only to pyrimethamine (Watkins and Masobo, 1993). Based on this, early recrudescence are therefore likely to be of drug resistant parasites as both drugs (SP/AQ) will still be at therapeutic levels, however shortly after 10 days for AQ and 15 days for SP, recrudescence could be of parasites with lower levels of resistance to AQ and Pyrimethamine (PY). Very late recrudescence could be of parasites sensitive to SP but probably resistant to PY. It is also likely that the novel parasite appearing in early recrudescence will be resistant to SP, whereas those appearing later on could be sensitive to SP. Three children were recrudescent at 16, 21 and 28 days post- treatment. Since the synergistic activity between sulfadoxine and PY lasts for approximately 15 days after which the reappearing parasites respond only to PY, then the reinfecting parasites in the first child are most probably resistant to SP. The reinfecting parasites in the other two children could have been SP sensitive but most probably PY resistant. Although this difference between the two drug regimens may be due to chance, it is possible that the AQ remains at levels suppressive for sensitive parasite for 1-2 months (Krishna and White, 1996) which is much longer than for the synergistic effect of SP at 15 days (Watkins and Masobo, 1993) and therefore this could prevent the growth of reinfection, drug sensitive parasites. In the SP/AS arm of the study once the levels of SP had dropped to sub- therapeutic levels, this could allow reinfection parasites to grow in treated children. In the AQ/AS arm, such parasites could have encountered therapeutic levels of both AQ and AS.

Our method of analysis is likely to overestimate both recrudescences and reinfections, but this constitutes the limits of genotyping as an adjunct tool in treatment efficacy studies. The study of additional genetic polymorphic markers such as *GLURP*, might have allowed the distinguishing of isolates that did not differ as regards to *MSP-1* and *MSP-2* and would have even reduced the number of pure reinfections.

5.1 CONCLUSIONS AND RECOMMENDATION

This study has attempted to investigate the role of double and triple mutant forms of *dhfr* in clinical treatment failure. The results show that parasite infections of pure double mutant (*dhfr* 108Asn/59Arg) are associated with significantly earlier recrudescence following treatment with SP. Infections with parasites carrying the triple mutant form alone (*dhfr* 108Asn/51Ile/59Arg) are even more significantly associated with early treatment failure.

The research presented suggests that the number of mutations present in *dhfr* has a significant effect on the type of treatment failure following SP chemotherapy. This has been previously assumed to be the case, but had not been formally demonstrated, although the association between any treatment failure and mutations in *dhfr* is well known. Mutations in *dhps* have also been shown to be implicated in resistance to SP. In this study we did not analyse sulfadoxine resistance mutations at the dihydropteroate synthetase (*dhps*) gene. These seem less strongly associated with SP failure (Kublin *et al.*, 2002), although some studies have suggested that they may play an important role, especially in individuals with low immunity (Khalil *et al.*, 2002). As the case may be, such an analysis would have provided a better overall prediction of SP resistance in Nuba.

The finding as discussed above will help in laying strategies that will lead to the delay of the spread of antimalarial drug resistance in Sudan. Operationally, our findings imply that: (1) CQ would work very poorly here and should be withdrawn as soon as possible and (2) SP resistance mutations, already present, could rapidly become dominant, especially if SP monotherapy is more widely used Multiple and particularly triple mutations at *dhfr* are reasonably predictive of *in vivo* SP failure. Presently these are rare, implying that the drug remains useful in Nuba.

Of the two combinations, AS + AQ is likely to have a longer therapeutic lifespan given global SP resistance trends. AQ resistance seems to develop more slowly according to *in vivo* observations (East African Network for Monitoring Antimalarial Treatment, 2003). The genotypic basis of AQ resistance, however, is yet

to be elucidated, representing a crucial missing element in decisions about which artemisinin-based combinations can be deployed in any given setting. The *Pfcrt* K76T mutation has previously been associated with AQ failure *in vivo* (Ochong *et al.*, 2003) but, in the present trial, even a 90% prevalence of K76T mutants failed to significantly affect AS + AQ efficacy. It is nevertheless clear that any future use of AQ, in Sudan as elsewhere, should occur strictly in a combination regimen, preferably prescribed in age-specific blister packs.

In vivo clearance of resistant parasites may involve host factors. Identifying these factors will improve our understanding of the immunological events and will have great implication for predicting resistance. WHO *in vivo* and *in vitro* tests are not well suited for countrywide resistance surveillance; therefore, there is a need to develop new and better methods for measuring drug resistance.

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APPENDEX III

Restriction Sites and Pattern of Restriction Digests

Restriction Enzyme	Point mutation	Restriction Site	Restriction Pattern
TSP5091	$AAT_{51}ATT$	5'.....AATT…3' 3'…..TTAA…5'	218, 120
AluI	$AGC_{108}AAC$	5'..ACTGGN…3' 3…..TGA_rCN…5'	326, 196
XmnI	$TGT_{59}CGT$	5'…GAANNNNTTC…3' 3'…CTTNNNNAAr…..5'	163, 137
BsrI	$AGC_{108}AAC$	5'…..CC GG….3'	332, 190
BstNI	$AGC_{108}ACC$	3…GG A_i CC….5'	326, 196
DraI	$ATT_{164}TTA$	5'…..TTTAAA…3' 3'…..AA_rTTT….5'	245, 143, 107

APPENDEX II

RADIOLABELLING PROBES for dhfr and mdr1

Pfmdr1-Asp86 probes: GAAATATTTTCGTGCAGTTAC

Pfmdr1- Tyr86 probes: GAAATATTTTCGTGCAGTTAC

dhfr108Ser: AACAAGCTGCGAAAGCATTCCAA

dhfr108Asn: AACAAACTGGGAAAACATTCCAA

dhfr108Thr: AACAACCTGCGAAAGCATTCCAA

dhfr51Ile: CCATGGAAATGTATTTCGCTAG

dhfr51Asn: CCATGGAAATGTAATTCGCTAG

dhfr59Arg: GAAATATTTTCGTGCAGTTAC

dhfr59Cys: GAAATATTTTTGTGCAGTTAC

APPENDEX IV

Restriction Sites and Pattern of Restriction Digests

Restriction Enzyme	Point mutation	Restriction Site	Restriction Pattern
TSP5091	$AAT_{51}ATT$	5'.....AATT...3' 3'.....TTAA...5'	218, 120
AluI	$AGC_{108}AAC$	5'..ACTGGN...3' 3.....TGA$_r$CN...5'	326, 196
XmnI	$TGT_{59}CGT$	5'...GAANNNNTTC...3' 3'...CTTNNNNAAr.....5'	163, 137
BsrI	$AGC_{108}AAC$	5'.....CC GG....3'	332, 190
BstNI	$AGC_{108}ACC$	3...GG A$_i$ CC....5'	326, 196
DraI	$ATT_{164}TTA$	5'.....TTTAAA...3' 3'.....AA$_r$TTT....5'	245, 143, 107

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