DRUG-SUSCEPTIBILITY AND MOLECULAR CHARACTERIZATION OF EPIDEMIC AND ENDEMIC MALARIA IN KENYA

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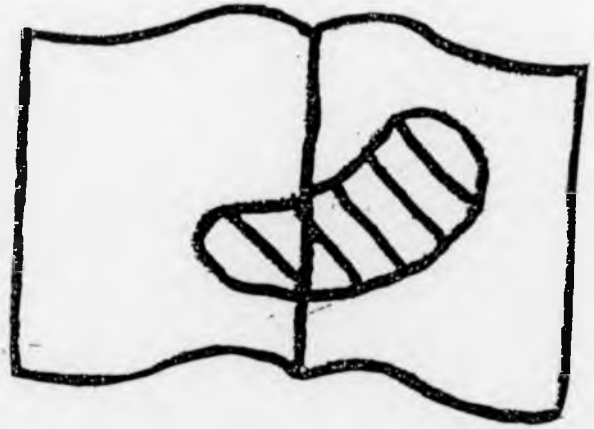

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ABSTRACT

Malaria in Kenya continues to pose a great threat to health. Trials with sulfadoxine/pyrimethamine (SP), currently recommended to replace chloroquine (CQ) in drug-resistant areas, were carried out between 1997 and 1999 in *Plasmodium falciparum* endemic and epidemic sites. *In vitro* assays showed that over 50% of isolates were resistant to CQ and SP and sensitive to both quinine and mefloquine. In Busia, an endemic site, efficacy of combination treatment with CQ and SP was compared with SP alone. The combination was more effective in fever resolution. SP and co-trimoxazole (sulfamethoxazole/trimethoprim) were compared in the Oyugis and Tiwi endemic sites and all outcomes were found to be equivalent. Codon changes in *dhps* and *dhfr* genes (on chromosomes 8 and 4) conferring *in vitro* resistance to sulfadoxine and pyrimethamine, predicted SP treatment failures in Mosoriot, an epidemic site, but age-related recovery seen in the endemic site Tiwi masked the influence of mutations in *dhps*. Resistance-related mutations in *dhfr* and *dhps* were mutually associated in both sites, which probably indicates drug selection acting on both gene-products. Mutations in *pfmdr1* and *pfcrt* genes (chromosomes 7 and 5) associated with *in vitro* CQ resistance, were examined in the Mwea endemic site. The *pfcrt*Thr76 mutation was found on presentation in all CQ-susceptible and resistant cases. The *pfmdr1* mutations, Tyr86 and Tyr1246, were similarly present in most of the pre-treatment infections, but an increase in prevalence was seen in samples from recrudescent infections. Genotyping of parasite strains revealed that parasites from both endemic and epidemic areas were genetically diverse suggesting that the source of epidemic outbreaks was endemic sites of high transmission. Quantitation of parasite-specific RNA by nucleic acid sequence-based amplification (QT-NASBA) was evaluated for the detection of low malaria parasitaemias. The test maybe useful in monitoring therapeutic response to drugs.

DEDICATION

This thesis is dedicated to my parents Ahmed Omar Jezan and Zena Said Bakor whose strength and commitment towards my education is priceless.
(May God Rest Their Souls In Eternal Peace).

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LIST OF ABBREVIATIONS

Ala	Alanine
*Af*lIII, *Alu*I, *Apo*I, *Bsr*I, *Bst*UI, *Dde*I, *Dra*I, *Eco*RV, *Fok*I, *Mnl*I, *Mwo*I, *Nla*III, *Tsp*5091, *Vsp*I, *Xmn*I	Restriction enzymes
ACR	Adequate clinical response
AQ	Amodiaquine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BM	Bratton Marshall
CG2	CG2 protein
CI (95%)	Confidence Interval (95%)
CQ	Chloroquine
CQR	Chloroquine resistance
DCQ	Desethylchloroquine
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
dNTP	Deoyribonucleoside tri-phosphate
EDTA	Ethylenediaminetetraacetic acid
EIPA	5-*N*-ethyl-*N*-isopropyl amiloride
EIR	Entomological inoculation rate
ETF	Early Treatment failure
GFM	Glass fibre membrane
Glu	Glutamic acid
GLURP	Glutamate rich protein
Gly	Glycine

Hb	Haemoglobin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High performance liquid chromatography
Ile	Isoleucine
Leu	Leucine
LSHTM	London School of Hygiene and Tropical Medicine
LTF	Late treatment failure
Lys	Lysine
Mdr	Multidrug resistance (gene)
MOH	Ministry of Health
MSP1	Merozoite Surface Protein 1
MSP2	Merozoite Surface Protein 2
MQ	Mefloquine
NMCP	National Malaria Control Programme
NASBA	Nucleic acid sequence-based amplification
QT-NASBA	Quantitative Nucleic acid sequence-based amplification
Na_2HCO_3	Sodium bicarbonate
NMCP	National Malaria Control Programme
NPV	Negative predictive value [Percentage of negative tests correctly predicting a successful treatment]
OR	Odds Ratio
PABA	Para amino benzoic acid
PCR	Polymerase chain reaction
3D7, 7G8, Dd2, KI, FC-27, MAD20, RO33, T9/996, TN-1, V1/S	*Plasmodium falciparum* clones or strains
*Pfmdr*1	*Plasmodium falciparum* multidrug resistance gene
Pfcrt	*Plasmodium falciparum* chloroquine resistancetransporter gene

Phe	Phenylalanine
Pyr	Pyrimethamine
PPV	Positive predictive value [Percentage of positive tests correctly predicting an unsuccessful treatment]
QN	Quinine
R	Resistance
RFLP	Restriction fragment length polymorphism
RI, II, III	Levels of parasitological resistance
RNA	Ribonucleic acid
R_0	Basic reproduction rate
S	Sensitive
SD	Standard deviation
SDX	Sulfadoxine
Ser	Serine
SP	Sulfadoxine-pyrimethamine
SN	Sensitivity [Percentage of drug failures correctly predicted by the positive test]
SF	Specificity [Percentage of successful treatments correctly predicted by the negative test]
TAE	Tris-acetate/EDTA (electrophoresis buffer)
TBE	Tris-borate/EDTA (electrophoresis buffer)
TBPEE	Tetrabromophenolphthalein ethyl ester
TNF	Tumour necrosis factor
Thr	Threonine
Tyr	Tyrosine
Val	Valine
WHO	World Health Organization

CHAPTER 1

Literature review

1.1 Global malaria situation

A recent review has concluded that every year about 1 million deaths (range 744,000-1,300,000) from the direct effects of malaria occur in Africa with more than 75% of them in children (Snow *et al.*, 1999). The impact of malarial disease on human suffering and economic loss remains unacceptably high with the direct economic costs of malaria weighing particularly heavily on poor countries with few resources. Recent research has shown a close correlation at the country level between the rate of economic development and the burden of malaria, indicating malaria is an important constraint on economic progress. The annual economic burden of malaria infection in 1995 was estimated at US$ 0.8 billion, for Africa alone (Foster and Phillips, 1998). This heavy toll can hinder economic and community development activities throughout the region, (see Figure 1.1.).

1.2 Plasmodium species

The protozoan genus *Plasmodium* belongs to the phylum Apicomplexa; class Sporozoa; subclass Coccidia; family: Plasmodiidae; order Eucoccidiorida; sub-order Haemosporina), (Levine *et al.*, 1980). There are more than 100 species of the genus found in the blood of infected birds, reptiles and mammals (Gilles, 1993). Of the 4 species of plasmodia affecting humans (Garnham, 1966) *Plasmodium falciparum*, is the most virulent. *P. vivax*, *P. ovale* and *P. malariae* are less virulent and related mortality is very low (Bruce-Chwatt and Peters, 1979). *P. falciparum* is prevalent in sub-saharan Africa, parts of India, Central and South America, South East Asia and the Pacific. *P. malariae* has a similar global distribution. *P. ovale* is mainly restricted to West Africa and the Western Pacific. *P. vivax* affects the tropical and temperate regions of S. America and Asia and parts of Eastern and Northern Africa

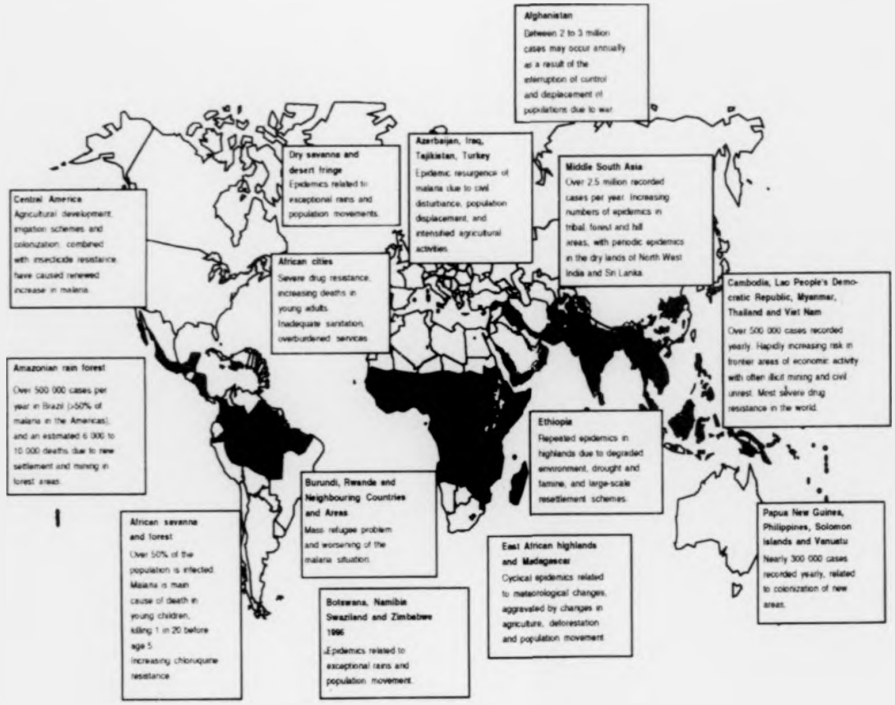


Figure 1.1 World Malaria distribution and problem areas (boxed). Shading indicates main malaria transmission areas (from WHO/Control of Tropical Diseases/ Health Map, 1997, In Trigg and Kondrachine, 1998).

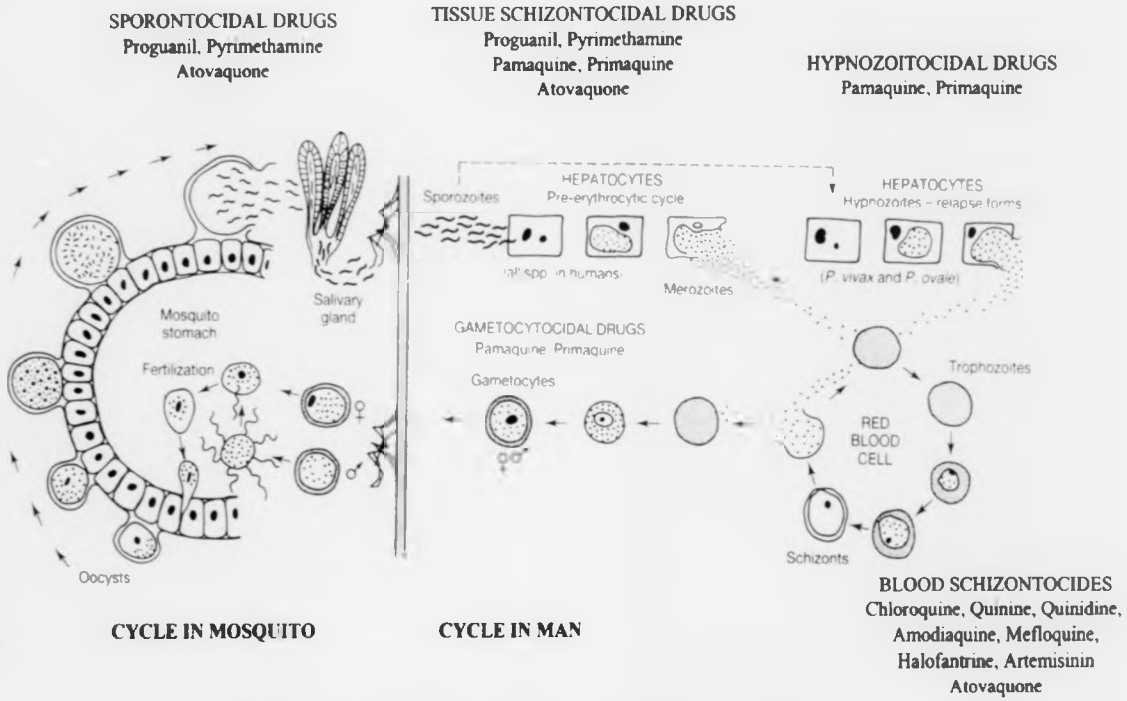


Figure 1.2 Malaria parasite life cycle showing stages where antimalarial drugs act, [slightly modified from Gilles and Warrell, 1993].

1.3 Malaria Life Cycle

The plasmodia of mammals are transmitted in nature by their appropriate species of mosquito belonging to the genus *Anopheles*. The life cycle of the four human malaria parasites are essentially similar. In the cycle the mammalian host (man) is considered as the intermediate host while the definitive host is the invertebrate (mosquito). The life cycle of *Plasmodium* (Gilles and Warrell, 1993) [Figure 1.2] begins with the inoculation of sporozoites with mosquito saliva into the subcutaneous tissue, bloodstream and lymph vessels of man by the bite of the female infected mosquito during its night-time blood meal. The sporozoites then invade the liver cells where they undergo the first stage of asexual development (liver schizogony: 6-20 days) producing merozoites which are infective to red blood cells. In the red blood cells (erythrocytes), the merozoite develops into a trophozoite stage which later divides to form the erythrocytic schizont. The schizonts burst open to release more merozoites which can again infect other erythrocytes to perpetuate the erythrocytic cycle. This growth cycle, in the case of *P. falciparum* takes 48 hours. Some merozoites entering erythrocytes will differentiate into the sexual male and female gametocytes. Transmission from the human host to the anopheline vector requires the production of these transmission stages. Developing gametocyte-infected RBCs sequester from the peripheral circulation to the blood spaces of the bone marrow and the spleen (Smalley *et al.*, 1980). This takes 8-10 days, after which "mature" gametocytes are released into the circulation and become infectious to the mosquito vector after a further 2-3 days. Anopheline mosquitoes can pick up infection when they feed on the blood of infected humans. Sexual fusion takes place within the stomach. Multiplication then takes place in oocysts on the mosquito stomach and eventually the invasive sporozoites escape, migrate and finally enter the salivary gland of the insect. (10-30 days). When the infected mosquito takes a blood-meal from human, it injects a dose of sporozoites marking the beginning of another cycle. *P. vivax* and *P. ovale* can form dormant stages from a proportion of the inoculated sporozoites which do not immediately develop into pre-erythrocytic schizonts. The dormant stages known as hypnozoites, (Krotoski *et al.*, 1982), may persist in the liver for several months, from 3 to 9 months or even 5 years before

undergoing schizogony and giving rise to blood stages and symptomatic malaria, (Warhurst, 2001a).

1.4 Clinical manifestations and pathogenesis

Clinically malaria begins as a flu-like illness 10 to 16 days after the infected mosquito bite. Typical cycles of fever, as a result of synchronous rupture of blood stage schizonts, are seen in untreated individuals every 48 to 72 hours depending on the *Plasmodium* species. Shaking chills and drenching sweats may then develop (Miller *et al.*, 1994). The frequency depends on the malaria species, coinciding with cycles of parasite multiplication and erythrocyte destruction leading to anaemia. Falciparum malaria may not always show this cyclic pattern but may have a daily fever and can be fatal if untreated or if treated with less effective drugs. In a proportion of cases, *P. falciparum* infection progresses from mild illness to severe or complicated disease. Some of the manifestations of severe and complicated falciparum malaria include, cerebral malaria, repeated generalized convulsions, hyperpyrexia, hypoglycaemia, pulmonary oedema, metabolic acidosis and renal failure, (Warrell, 1992). In areas of moderate to high malaria endemicity severe and complicated forms of malaria are mainly seen in children, while in low endemic areas, adults are also affected. Severe malarial anaemia is seen most frequently in areas of very intense transmission and mostly in young children, whereas cerebral malaria predominates in areas of moderate transmission affecting older children (Greenwood, 1997). The epidemics of clinical malaria usually develop in situations where many non-immune individuals have moved to malaria endemic areas or when malaria transmission suddenly emerges in a region with mostly non-immune individuals. Many aspects of *P. falciparum* pathology associated with organ failure, and which may lead to death, are apparently due to cytoadherence of late-stage parasitized erythrocytes to the endothelium of micro-vessels supplying the brain (cerebral malaria), kidney or other vital organs (Warrell, 1992). Malarial anaemia is the consequence of acute haemolysis, whose severity seems to correlate with parasitaemia, and dyserythropoietic changes in the bone marrow impairing proper red cell production (Harinasuta and Bunnag, 1988). Renal failure can be the

consequence of severe haemolytic anaemia, haemoglobinuria and hypovolemia (Miller, 1985).

1.5 The malaria vector

Although there are about 400 species of *Anopheles*, the vector that transmits malaria parasites, only 60 of them transmit malaria under natural conditions, and only 30 are of major importance, (Bruce-Chwatt, 1985). Of these the *Anopheles gambiae* complex and *Anopheles funestus* are the most efficient vectors for *P. falciparum* transmission. Of all factors related to malaria transmission, apart from host immunity, the number (density), human biting habits, and longevity of anopheline mosquito vectors are the most important, (Breman, 2001). Mosquito longevity is particularly important, because the portion of the parasite's life cycle that takes place within the mosquito, that is from gametocyte ingestion to subsequent inoculation, can take from 8-30 days, depending on the ambient temperature. In general, sporogony within the mosquito is not completed at temperatures below 16-18°C, and transmission does not occur, (Breman, 2001). The entomologic inoculation rate (EIR), defined as the number of sporozoite-positive mosquito bites per person per year, is the most common measure of malarial transmission. This reflects the transmission pattern varies in different geographic areas. It can be as low as less than one (<1) infective bite per person per year as is seen in some parts of South East Asia and Latin America to >300 in parts of tropical Africa. A high EIR results in stable and intense transmission, with young children and pregnant women, being the most vulnerable for severe illness, (Beier *et al.*, 1999; McElroy *et al.*, 1997). The lower the EIR, the greater the number of susceptible individuals who, on infection, can develop severe illness (McElroy *et al.*, 1997).

1.6 Malaria Endemicity

The pattern of transmission of malaria in a particular locality is graded as epidemic, hypoendemic, mesoendemic, hyperendemic or holoendemic (Marsh, 1993). These definitions correspond with increasing prevalence, decreasing time periods between periods of local transmission and increasing levels of infection

acquired immunity among the human host population (Mendis and Carter, 1993; Miller *et al.*, 1994).

1.6.1 Classification of endemicity

1.6.1.1 Holoendemic malaria

This can be defined as a locality experiencing an annual transmission intensity of at least 10 infective bites per person per year and acquired immunity develops within the population (Miller *et al.*, 1994; Sauerwein and Meuwissen, 1995; Snow and Marsh, 1995). The spleen rate (% of enlarged spleens) in age group 2-9 years is constantly over 75%, adult spleen rates are low, and the parasite prevalence rate in children 2-9 years is over 75%. Transmission is perennial with peak levels during the wet seasons. Age is an important factor, where the longer exposure of adults makes them less likely to develop malaria symptoms.

1.6.1.2 Hyperendemic malaria

Child spleen rate is constantly over 50% and adult spleen rate is high (over 25%). The parasite prevalence rate in children (2-9 years age group) is over 50%. Although transmission is hardly ever perennial, and varies with intensity with the season, it continues for the greater part of the year. High degree of immunity is acquired by the permanent inhabitants due to re-infection many times in the year.

1.6.1.3 Mesoendemic malaria

Child spleen rate is 11-50%. Parasite prevalence rate in children (2-9 years) is 11-50%. Seasonal transmission may result in outbreaks. Permanent residents acquire a substantial degree of immunity to clinical disease.

1.6.1.4 Hypoendemic

Child spleen rate in hypoendemic malaria area is about 0-10% per cent. Parasite prevalence rate is 0-10%. The inhabitants never acquire effective immunity. Sporadic epidemic outbreaks may occur.

1.7 Malaria Epidemicity

Malaria epidemics not only increase malaria-specific morbidity and mortality but also affect the general health of the population. Over the past 20 years, efforts to control malaria have met with less and less success. Mosquito vectors have become increasingly resistant to insecticides, and malaria parasites are developing resistance to drugs that should prevent or control this deadly disease. Projections on global warming also predict that there will be significant increases in the territory where malaria can occur. In addition to this changing geographic diversity and expanded potential for transmission, the ecologic and social factors that are influencing the resurgence of newly emerging disease all influence the reemergence of malaria as well (Najera *et al*, 1998).

1.8 Highland vectors

Although there is a great diversity of anopheline species in the highland areas of Africa, members of the *A. gambiae* complex are the principal vectors of malaria, as is true for most of the continent. *A. gambiae sensu stricto* have been reported in the Kenyan Highlands (White, 1972) and implicated as upland vectors. In the Africa highlands, malaria transmission is probably much more focal in its distribution than in many lowland areas.

1.9 Spread from lowlands to highlands

Chloroquine-resistant parasite strains are likely to pose a greater threat in the highlands, where immunity to malaria is low or absent, than in the lowlands, where exposure to the disease is generally greater. It seems likely that chloroquine resistant strains of falciparum malaria found in the hills (Khan *et al.*, 1992) may have spread there from the lowlands.

1.10 Kenya malaria situation

Projected population data for the year 2000 indicates that 41% (13.5million) of Kenya's population are aged between 5 and 20 years, with about 53% of school population living in areas where malaria transmission is said to be unstable, (Brooker, *et al* 2000). Assuming children in stable areas experience age-

specific risks, and those living in unstable areas experience attacks once every 3-4 years, first approximation suggest that between 0.7 and 5.3 million clinical attacks of malaria occur in stable areas, and between 0.8 and 1.2 million in unstable areas in school children in Kenya, (Brooker *et al.*, 2000).

Malaria attacks have an impact on school attendance and educational outcomes. Data suggest that malaria accounts for between 3% and 8% of all reasons for absenteeism (Brooker *et al.*, 2000).

1.10.1 Disease Burden

In Kenya malaria is responsible for approximately 30% of the total outpatient clinic visits and estimates of infant and child mortality on the Kenyan Coast show that at least 58 infants per 1000 live births and 12 children per 1000 children aged between 1 and 4 years die of malaria each year (Snow *et al.* 1994).

Cost to malaria includes loss of 15 days/man/year that is equivalent to 375 million working days. In addition, in rural areas agricultural output is reduced during epidemics with more than 55% of workers remaining absent. In tourism, 200 per 100,000 visitors are affected. Cost of antimalarials constitutes over 300,000 UK sterling pounds a year, (Ministry of Health [MOH] Report, 1996).

1.10.2 Epidemiological Stratification

Kenya has both seasonal and perennial malaria transmission, see Figure 2.1 In endemic regions (below 1,300 meters)-stable malaria is found with transmission occurring for more than 6 months of the year. These areas e.g., western and coastal regions experience stable and hyper- to holo-endemic malaria. Transmission becomes less stable and more seasonal (3-months), following the rainfall pattern, between altitudes 1,300-1,700 meters in the central region or districts of Kenya.

1.10.3 Epidemic malaria in Kenya

Malaria epidemics have been reported in the western highlands of Kenya since 1926, occurring once every two years in areas between the altitude 1,500-2,000 meters. In 1941, an outbreak was reported at an exceptionally high altitude (>2,250 meters). After several decades of quiescence, resurgence of malaria epidemics have occurred since 1980's in the western highlands and in the unstable malaria areas of the semi-arid north east and the Lake Turkana region.

The highlands were considered free of malaria through the 1960s, but since the 1980s malaria has been increasing (Oloo *et al.*, 1996). More than 90% of malaria in Kenya is caused by *P. falciparum* (Khaemba *et al.*, 1994) and transmitted most often by *A. gambiae*, with *A. funestus* as a secondary vector. It is generally assumed that malaria in the highlands of western Kenya is not due to local transmission but is imported from the nearly holoendemic-disease areas around Lake Victoria by the frequent travel of the tea plantation workers and their families.

1.11 Malaria control

1.11.1 Control strategies and constraints

The four basic elements of the global strategy for malaria control (WHO, 1993) are as follows:

1) To provide early diagnosis and prompt treatment.
2) To implement selective and sustainable preventive measures, including vector control.
3) To 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 a country's malaria situation, in particular the ecological, social and economic determinants of the disease.

1.11.2 Early diagnosis and prompt treatment

Case-management remains the basis of malaria morbidity and mortality control (WHO, 1996). Timely treatment for every clinical episode (especially in young children) with the correct dosage of an effective drug in order to avoid severe disease and mortality. Antimalarial drugs can be used to treat malaria infections and to prevent malaria infections when used prophylactically. Drugs could be used to kill gametocytes in attempts to prevent transmission. The primary aims of treatment in any situation are to prevent death from the disease and to reduce suffering.

1.11.3 Prevention and control of epidemics

Factors associated with epidemics in Africa have been broadly mentioned (see above). However, to control epidemics it is essential to establish a monitoring system that can detect early any undesirable trends in malaria morbidity and mortality, and environmental changes usually associated with epidemics. A number of factors appear to be contributing to the resurgence and epidemics of malaria. One of these may be due to the rapid spread of resistance to antimalarial drugs.

1.11.4 Chemotherapy

The role of chemotherapy in malaria control is to deal with malaria as a disease. Eradication is no longer considered an achievable aim by any means except the hoped for vaccine.

1.11.4.1 Blood schizontocides

Examples of this group of drugs include chloroquine, amodiaquine, mefloquine and quinine. They are characterized by their rapid action on the erythrocytic stages of malaria parasites and can be divided into 2 groups depending on their effect on morphology of malaria parasites, (Gilles and Warrell, 1993) [Figure 1.2]. In the first group, e.g., chloroquine (CQ), amodiaquine (AQ) and

quinacrine, these have 2 highly electronegative (easily protonatable) nitrogen atoms. These drugs act on the growing intraerythrocytic stages actively digesting haemoglobin. They have a rapid effect on the haemoglobin-containing digestive vesicles (pigment clumping) of the intraerythrocytic parasites causing fusion of adjacent vesicles followed by the sequestration of the fused vesicles into large autophagic vacuole, (Warhurst, 1986).

Type II blood schizontocides are typified by quinine-like compounds such as mefloquine, halofantrine and benflumetol (lumefantrine). Pigment clumping does not occur in this group and they have one highly electronegative nitrogen atom. Cross-resistance develops more or less readily among type I blood schizontocides, but infections moderately resistant are treatable using type II blood schizontocides. The selective toxicity of antimalarial blood schizontocides is based on the selective uptake by infected erythrocytes. Uptake is glucose dependent in sensitive strains of *P. falciparum*. CQ accumulation in resistant strains is glucose insensitive (Fitch *et al.*, 1974). The mechanism involved in the concentrative uptake of blood schizontocides is through sequestration of the base in the acid digestive vesicles and complexing with haemin ferriprotoporphyrin IX (FPIX) (Chou *et al.*, 1980). Artemisinin derivatives are also blood schizontocides which complex with haem released from haemoglobin in the digestive vesicles (lysosomes) of parasite and produce toxic free radicals.

1.11.4.1.1 Quinine

Quinine (QN) is used as a third-line drug in the treatment of uncomplicated malaria and as first-line in severe malaria. Resistance although rare, has been reported (Reacher *et al.*, 1981; Brandicourt *et al.*, 1986; Pukrittayakamee *et al.*, 1994; Adagu *et al.*, 1995). Oral quinine may also be combined with an antibiotic to treat uncomplicated malaria in areas of multi-drug resistance (Watt *et al.*, 1992). However, there is poor compliance with oral quinine because of its bitter taste and consistent adverse effects (cinchonism), comprising nausea, dysphoria, tinnitus, and high-tone deafness. The main dangerous adverse effect of quinine is hypoglycemia, especially in children and pregnant women.

1.11.4.1.2 Mefloquine

Mefloquine is structurally related to quinine and is used both in prophylaxis and oral treatment of uncomplicated malaria. Its long terminal half-life of 14-21 days probably contributed to the rapid evolution and spread of mefloquine resistance in Southeast Asia, (White, 1999). However, the combination of artesunate and mefloquine in Thailand has slowed the further development of mefloquine resistance (Price *et al.*, 1996). Mefloquine resistance is associated with amplification of the multi-drug resistance (*mdr*) genes that encode *Plasmodium* P-glycoprotein pumps, which reduce drug concentrations within parasites (Wilson *et al.*, 1993; Price *et al.*, 1997).

There has been considerable controversy over the true incidence of neuropsychiatric side effects of mefloquine used for both treatment and prophylaxis (see 1.11.4.6). Extensive therapeutic use of MQ has been implicated in rapid development of resistance. Some side effects have also been reported such as nausea, vomiting, dizziness, loss of balance, diarrhoea. More serious concerns have been identified after prophylactic and treatment use of MQ resulting in neuropsychiatric disturbances (e.g. hallucinations, mood changes and anxiety), (Harinasuta *et al.*, 1983).

1.11.4.1.3 Artemisinin and its derivatives

Artemisinin and its derivatives are sesquiterpene lactone peroxides derived from the leaves of sweet wormwood (*Artemisia annua L.*). Developed in China, they are used increasingly in Asia and Africa. They kill all growing blood stages of the malaria parasite, including "young rings" by interacting with heme to produce carbon-centered free radicals that alkylate protein and damage the parasites' microorganelles and membranes (Meshnick *et al.*, 1996).

Artesunate is water-soluble and is dispensed for intravenous, intramuscular, oral (tablets), or rectal administration. Artemether and arteether are oil-soluble and available in tablets, capsule, or intramuscular injection form (Meshnik *et al.*, 1996). The artemisinin derivatives presently show no clinical cross-resistance with

other known antimalarials and this makes them useful for treating severe malaria in areas of multi-drug resistant malaria (White, 1994; Meshnick et al., 1996).

1.11.4.1.4 Halofantrine

Halofantrine, a structural relative of mefloquine, is better tolerated and also effective in the treatment of chloroquine-resistant falciparum malaria, (Cosgriff *et al.*, 1982; Boudreau *et al.*, 1988). However, the development of cross-resistance with mefloquine and the finding of an association with sudden death, probably linked to prolongation of the electrocardiograph Qt_c. interval (Nosten *et al.*, 1993), have raised doubts about the safety and utility of halofantrine.

1.11.4.1.5 Amodiaquine

Amodiaquine AQ has been used for initial treatment of malaria infections in CQ resistant areas and in cases of CQ failure (Childs *et al.*, 1989). AQ is a more effective drug than CQ (Peters, 1987). However, reports of AQ resistance (Hall *et al.*, 1975; Childs *et al.*, 1989) and cross-resistance with CQ (Young and Johnson, 1972) have been made. Side effects of AQ include liver cell damage (hepatotoxicity) and blood disorders.

1.11.4.2 Antimetabolites [antifolates]

Antimetabolites are generally classified as type I (sulfonamides and sulfones) and type II antifolates (pyrimethamine, trimethoprim and cycloguanil, the active metabolite of proguanil). They mimic essential metabolites of the malaria parasite, and are active against all the growing stages in the liver (liver schizonts), erythrocytic stages in the blood (blood schizonts) and growing stages in the mosquito (sporogonic stages). These parasite stages are all affected to a greater or lesser extent by the antifolate drugs or their combinations. It has been demonstrated that *P. falciparum* can synthesize tetrahydrofolate cofactors from GTP, PABA, and L-glutamate, showing that the parasite possesses a *de novo* folate synthetic pathway (Krungkrai *et al.*, 1989). Additionally, the enzymes in the folate synthetic pathway, GTP cyclohydrolase (Krungkrai *et al.*, 1985), 6-hydroxymethy-7,8-dihydropterin pyrophosphokinase (PPPK), dihydropteroate synthase (DHPS),

and dihydrofolate reductase (DHFR), have been identified in different plasmodium species. (Ferone, 1977; Sherman, 1979). Recently, the gene encoding dihydrofolate synthase/folylpolyglutamate synthase DHFS/FPGS was isolated and characterized (Lee *et al.*, 2001). The enzymes DHFS and FPGS catalyse the synthesis and polyglutamation of folate derivatives, (Salcedo *et al.*, 2001).

Inhibition of parasite dihydrofolate reductase results in a reduction in the intracellular pool of tetrahydrofolate cofactors which are used in most cells for the *de novo* synthesis of purines, methionine and thymidylate and for the interconversion of glycine with serine. Serine is the only identified source of methyl groups of methionine and thymidylate. Serine hydroxymethyltransferase converts serine to glycine with the formation of N^5-N^{10}-methylene tetrahydrofolate, the cofactor used to provide the methyl group that converts deoxyuridine monophosphate to the deoxythymidine monophosphate required for DNA synthesis. This activity is conducted by the enzyme action of thymidylate synthase (TS) and results in regeneration of dihydrofolate (DHF), (see Figure 1.3). The major enzyme targets of malaria chemotherapeutics in the folate biosynthetic pathway are DHPS and DHFR. DHPS, not found in mammals is responsible for the synthesis of 7,8-dihydropteroate from 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine pyrophosphate and PABA. The drugs of the sulfa group are analogs of PABA and have been shown to be competitive inhibitors of the DHPS enzyme from the murine malarias *P. berghei* (Ferone, 1973, 1977; McCullough and Maren, 1974) and *P. chabaudi* (Ferone, 1977; Walter and Konigk, 1980), as well as in *P. falciparum* (Zhang and Meshnick, 1991; Triglia *et al.*, 1997). DHFR is two steps further down the folate biosynthetic pathway from DHPS and is responsible for the reduction of 7,8-dihydrofolate to tetrahydrofolate. Pyrimethamine and cycloguanil (DHFR inhibitors) are potent inhibitors of this enzyme (Ferone *et al.*, 1969; Ferone, 1970; Sirawaraporn and Yuthavong, 1984; 1987; Zolg *et al.*, 1989; Sirawaraporn *et al.*, 1990, 1997). Pyrimethamine is generally used in combination with the sulfonamide compound, sulfadoxine, as they show a marked synergism (Chulay *et al.*, 1984; Scott *et al.*, 1987), most likely due to the fact that they act on two different enzymes in the same biosynthetic pathway.

The selective action of the antimalarial type II antifolates (DHFR inhibitors) resides in the greater affinity of the drug for the parasite enzyme than the host enzyme. Pyrimethamine binds 10^2-10^3 times more tightly to the plasmodial enzyme than to the host enzyme, similarly for drugs like cycloguanil and trimethoprim (Gutteridge and Trigg, 1971).

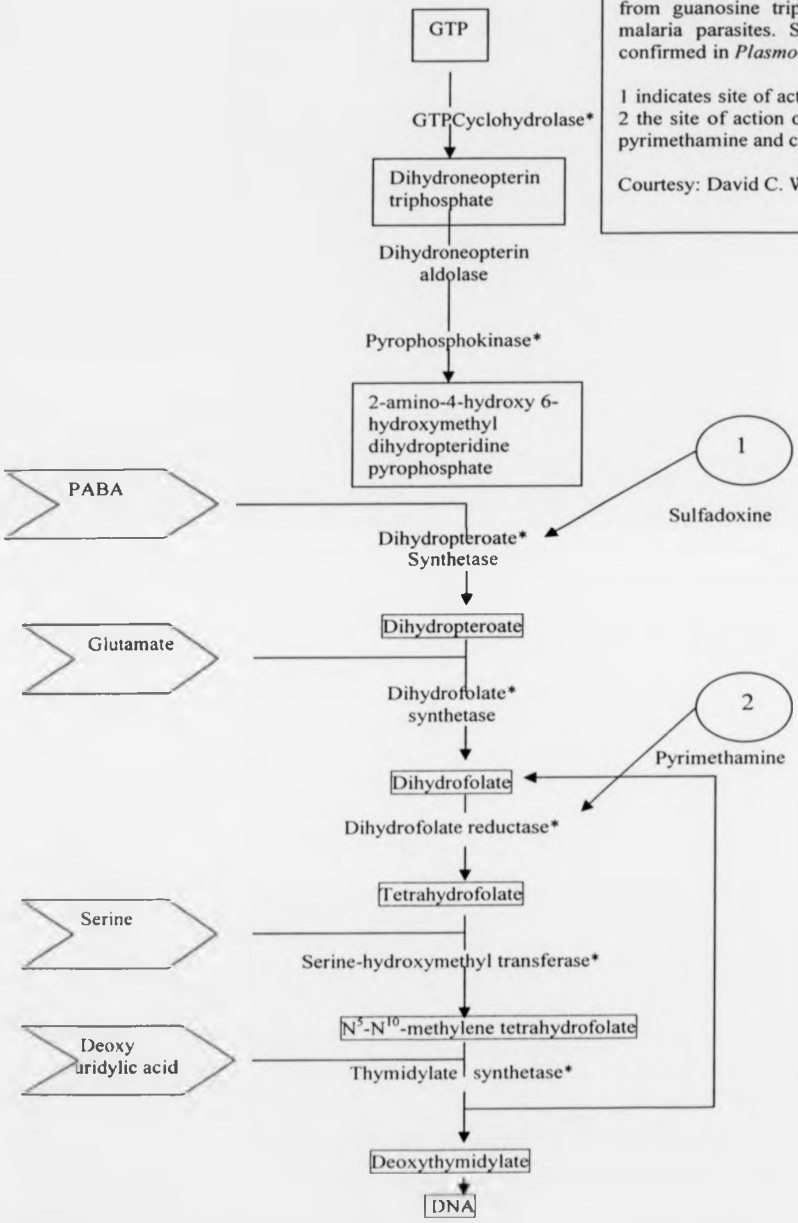


Figure 1.3 The folate pathway in *Plasmodium.*

Central role of the tetrahydrofolate synthetic pathway in synthesis of DNA from guanosine triphosphate (GTP) by malaria parasites. Starred enzymes are confirmed in *Plasmodium*.

1 indicates site of action of sulfonamides, 2 the site of action of antifolates such as pyrimethamine and cycloguanil.

Courtesy: David C. Warhurst

1.11.4.3 Antimalarial drug combination

The Fansimef® (sulfadoxine, pyrimethamine, mefloquine) triple combination was used because resistance to MQ was found to develop more slowly when it was used in such a combination in a *P. berghei* model of malaria (Peters and Robinson, 1984). However, due to severe gastro-intestinal and dermatological side effects associated with the combination coupled with the pharmacokinetic incompatibility of Fansidar® and MQ, Fansimef® was withdrawn.

To prevent the high recrudescence rates that result when artemisinin or one of its derivatives is used alone, and to combat the development of resistance, they should always be combined with another unrelated antimalarial, such as mefloquine or doxycycline (Hien *et al.*, 1993; Meshnik *et al.*, 1996).

Oral tetracycline and doxycycline probably act by damaging *P. falciparum* mitochondria (Kiatfuengfoo *et al.*, 1989). They act slowly and should always be used in combination with another antimalarial, such as in a 7-day course to supplement the faster acting drugs quinine and artesunate (Watt *et al.*, 1992). Tetracyclines should not be used in children or pregnant women because of its calcium chelation property, but clindamycin is safe (Kremsner *et al.*, 1994). Oral fluoroquinolones though showing some activity *in vitro*, are not effective clinically (Watt *et al.*, 1991). Co-trimoxazole is effective in areas where sensitivity to pyrimethamine-sulfadoxine is retained.

A new hydroxynaphthoquinone antimetabolite (atovaquone) has been developed, based on the traditional antimalarial Lapacho heartwood. Atovaquone is used with proguanil, [a pro-drug metabolized to the antimalarial DHFR inhibitor cycloguanil] because of rapid resistance development when used alone. This combination attacks all growing stages in the malaria cycle (de Alencar *et al.*, 1997). It is effective in uncomplicated malaria, resistant to CQ and the antifolates.

1.11.4.4 Hypnozoitocidal drugs

P. vivax and *P. ovale*, but not *P. falciparum* or *P. malariae*, characteristically leave dormant parasites within hepatocytes (hypnozoites), which give rise to future relapses. Primaquine, an 8-aminoquinoline, kills these hypnozoites, (Warhurst, 1984). It is also used as a single dose to eradicate *P.*

falciparum gametocytes and this was demonstrated in Nicaragua but with limited effects (Garfield and Vermund, 1983). The limited success was attributed to timing of mass drug administration at a high-transmission period of the year. Treatment with primaquine carries the potential risk of haemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. Etaquine, a more active and slowly eliminated successor to primaquine, is currently undergoing clinical trials.

1.12 Drug resistance

Drug resistance is defined as the ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication (WHO, 1963). Such resistance may be relative, yielding to increased doses of the drug that can be tolerated by the host or complete, when the parasite withstands maximum doses tolerated by the host.

1.12.1 Types of drug resistance

A number of different mechanisms can account for resistance or changes in sensitivity of organisms to drugs, some inherent and others acquired (see Peters, 1987). There is a selection by drug treatment from a "wild" population where a mixture of sensitive and resistant parasite clones may already exist, and these may differ extensively in a variety of genes. Alternatively, random mutations enhancing drug resistance may be selected by drug treatment of a population derived from a single clone.

Different levels of drug resistance are encountered in malaria chemotherapy and criteria employed have been defined by WHO (1996) [see section 2.2.2.5].

1.12.2 Emergence and spread of quinoline resistance

Although the 4-aminoquinolines are still effective and widely used for treatment and prophylaxis, resistance has severely impaired their use in many malarious countries (Wernsdorfer and Payne, 1991). Resistance to CQ is prevalent

in most areas of the world where the drug has been used (Sansonetti *et al.*, 1985; Delfini, 1989; Rapouda *et al.*, 1997; Shretta *et al.*, 2000).

1.12.2.1 Biochemical mechanisms of resistance to Chloroquine

The emergence of CQ resistance has stimulated intensive investigations in understanding its molecular and biochemical basis. Proposed mechanisms of resistance to CQ have been based on the observations that CQ resistant (CQR) parasites accumulate much less drug than CQ susceptible parasites (Fitch, 1969; Verdier *et al.*, 1985; Krogstad *et al.*, 1987; Bray *et al.*, 1992b). This hypothesis was confirmed when isolated vacuoles from CQR parasites were observed to have reduced CQ accumulation compared to CQ sensitive CQS parasites (Saliba *et al.*, 1998). The conflicting hypotheses are discussed below.

1.12.2.1.1 CQ resistant parasites have increased vacuolar pH

One biochemical explanation for CQ resistance in *P.falciparum* is that the pH in the digestive vacuole is elevated in the CQ resistant parasites and because CQ is a weak base, an increase in the vacuolar pH would result in decreased levels of CQ in the parasite digestive vacuole. Decreased vacuolar acidification in resistant isolates has been linked to a weakened proton pump ATPase or a reduced activity of a vacuolar chloride channel (Ginsburg and Stein, 1991; Bray *et al*, 1992a). These workers showed that the discrepancy between steady-state drug levels seen in CQR and CQS strains could be explained by a weakened proton pump in resistant strains. Further support was provided by Bray *et al.*, 1992a, using the vacuolar ATPase inhibitor bafilomycin A1; and CQ in combination with bafilomycin A1 produced an apparent reduction in sensitivity of CQS and CQR strains. Cloning and characterisation of vacuolar ATPase from *P.falciparum* have identified 2 subunits with sequence homology to the A and B subunits found in a variety of other organisms. However, no differences were observed between CQR and CQS parasites in their A and B subunits composition to help explain CQR phenotype (Karcz *et al.*, 1993; Karcz *et al.*, 1994).

1.12.2.1.2 Enhanced drug efflux of chloroquine

Earlier Krogstad *et al*, (1987) found that resistant strains of *P. falciparum* released pre-accumulated [^{3}H]-chloroquine 40 to 50 times faster than in sensitive strains. The magnitude of this difference was found to be consistent with the difference in the IC_{50} for both strain types. This led the authors to conclude that the lower steady-state levels of CQ in resistant parasites could result from export as in resistant tumour cells. This proposal was supported by the demonstration that enhanced efflux of CQ was partially reversed by verapamil (Martin *et al*, 1987). Other studies illustrated that CQ resistance could also be reversed by tri-cyclic antihistamines such as cypro-heptadine (Peters *et al*, 1989). CQ-induced ultrastructural changes seen in CQ sensitive *P. falciparum* and *P. chabaudi* become evident in CQ resistant parasites only after the co-administration of verapamil (Jacobs *et al*, 1988; Ohsawa *et al*, 1991). The reversal mechanism is assumed to result from competition between verapamil and CQ for efflux protein drug translocation sites, thus causing an increase in steady-state accumulation of CQ and partial return to sensitivity. However, Bray *et al*., (1992b) demonstrated an apparent equivalent efflux capacity for both sensitive and resistant parasites. This led them to suggest that changes in CQ accumulation force rather than efflux rate are responsible for CQ resistance. The observations that differences in drug resistance correlated more favourably with rates of drug uptake rather than drug efflux have been confirmed in other studies (Bray *et al*., 1994; Martiney *et al*., 1995; Bray *et al*., 1996).

1.12.2.1.3 The reduced drug binding hypothesis

It was suggested that CQR may be due to a reduced affinity of the intra-parasitic binding site for CQ in resistant strains (Fitch, 1973). The proposed receptor for the 4-aminoquinolines in *P. falciparum* is haemin, ferriprotoporphyrin-IX [FPIX] (Chou *et al*., 1980). It has been suggested that resistant parasites may have a reduced amount of FPIX complex (Fitch, 1983). It

has been suggested that both the activity and saturable uptake of CQ is due to the binding of drug to ferriprotoporphyrin-IX (Bray *et al.*, 1999). CQ sensitivity could be determined by the amount of this free haemin available within the vacuole. A parasite could become CQR by reducing the amount of high-affinity drug binding sites (Fitch, 1983), or by reducing the accessibility of the drug to ferriprotoporphyrin IX (Bray *et al.*, 1998).

1.12.2.1.4 Chloroquine resistance due to loss or relocation of CQ transporter

It was postulated by Warhurst (1986) that CQ accumulation in malaria parasites could be in part due to the presence of a specific drug importer or permease, with the permease situated on either the plasma membrane or, in resistance, on the food vacuole membrane. It was suggested that the permease could export drug from the vacuole, into the cytosol and thus out of the parasite in resistance, a viewpoint partially supported by the mathematical model of Ferrari and Cutler, (1991). The hypothesis that an outward drug export could be responsible for CQR is consistent with the observations of enhanced drug efflux from resistant strains presented by Krogstad *et al.*, (1987). However, this argument goes against the idea that change in inward drug transport is responsible for CQ resistance. Recently Sanchez *et al.*, (1997) demonstrated that CQ-uptake was temperature-dependent, saturable and could be inhibited by EIPA [5-*N*-ethyl-*N*-isopropyl amiloride], a specific inhibitor of Na^+/H^+ exchanger (NHE). Furthermore, they suggest differences in the kinetics of CQ uptake between CQS and CQR parasites is due to an import mechanism with reduced affinity for CQ. Wunsch *et al.*, (1998) added further evidence demonstrating inhibition of CQ uptake by NHE inhibitors and suggested CQR parasites have an elevated cytoplasmic pH over CQS parasites due to a constitutively activated NHE. They suggested that CQ activates the NHE of CQS parasites, resulting in the CQ uptake during a Na^+/H^+ exchange. In CQR parasites, the NHE is incapable of being activated as it is already constitutively activated. This differential stimulation of the NHE would result in lower accumulation of CQ within the digestive vacuole. Bray *et al.*, (1999) showed that CQ uptake into isolated parasites occurs at normal

rates in the absence of sodium, and under these conditions the NHE should not function.

1.12.2.2 Molecular basis of chloroquine resistance

Drug resistance in cancer cells is characterized by amplification of multi-drug resistance *mdr* gene (see below for details on mdr genes) and overexpression of its protein product (Endicott and Ling, 1989). It was therefore reasoned that if mdr phenotype of tumour cells and CQ resistance have common mechanism, then it is possible that amplification and overexpression of mdr homologues in *P. falciparum* may be involved in CQ resistance. Subsequent work identified two homologues designated *pfmdrl* and *pfmdr2* located in chromosome 5 and 14 respectively (Foote *et al.*, 1989). The *pfmdrl* gene is amplified and its protein product over- expressed in some, but not all CQ-resistant isolates. The presence of *pfmdr1* gene in some CQ resistant isolates at a copy number equivalent to that of CQ sensitive parasites led Foote *et al.*, (1989) to suggest that perhaps the *pfmdr1* gene of CQ resistant parasites was mutated at one or more positions within the primary sequence. They suggested that mutation might lead to functional alteration manifested at the level of the gene product which might thus render a parasite competent for CQ resistance.

1.12.2.2.1 *Plasmodium falciparum* multi drug resistance genes

The *mdr* genes are members of a superfamily of genes that are similar in sequence, predicted secondary structure and functions, referred to as the ATP binding cassette (ABC) proteins (Higgins *et al*, 1992). The *mdr* gene encoded proteins, P-glycoproteins (Pgps), were first identified in mammalian tumour cells as causing multidrug resistance against anticancer drugs by active drug export (Juliano and Ling, 1976). The Pgp in mammalian cells are located in the plasma membrane and consist of two similar halves each with six transmembrane domains and an intracellular ATP binding site (Endicott and Ling, 1989). The putative transmembrane regions may together form pores through which chemotherapeutic drugs as well as endogenous substrates can pass. Carbohydrate residues are attached to the extracellular portion of the protein. The ATP binding domains are

necessary for energy dependent efflux. The internal portion also contains appropriate configuration for initial binding and subsequent transport of a variety of compounds of different structure. *P. falciparum* contains two *mdr* homologs-*pfmdrl* and *pfmdr2*. The proteins encoded by these two genes are designated P.glycoprotein homologue-l (*Pgh-1*) and P.glycoprotein homologue-2 (*Pgh-2*). Some studies have shown association between Tyr-86 allele of the *pfmdr1* gene with CQR for both *in vivo* and *in vitro* drug tests (Foote *et al.*, 1990b; Basco *et al.*, 1995b; Adagu *et al.*, 1995; Adagu *et al.*, 1996; von Seidlein *et al.*, 1997; Duraisingh *et al.*, 1997). However, others (Awad-el-Kariem *et al.*, 1992; Wilson *et al.*, 1993; Basco *et al.*, 1998) failed to observe any association. Association between CQR and mutation on *pfmdr1* is not complete and this has raised the thought that CQR might involve a multi-genic mechanism. Although other genes might be involved, the CQR phenotype may still be affected by *pfmdr1* gene changes. The *cg2* gene and/or a neighbouring gene on chromosome 7 (Su *et al.*, 1997) which had been linked to CQR in a laboratory genetic cross (Wellems *et al.*, 1991) are currently of most interest.

1.12.2.2.2 *Cg2*

Wellems *et al.*, (1990, 1991) provided evidence that rapid efflux of chloroquine from the digestive vacuole of the malaria parasite is governed by a single locus on chromosome 7. Subsequently, Su *et al.*, (1997) mapped the chloroquine resistance locus within a 36-kb region of chromosome 7 and identified several open reading frames in this region. The author suggested that *cg2*, a gene encoding a highly polymorphic protein, could be the chloroquine resistance mediator. The CG2 protein of approximately 330kDa is expressed in erythrocytic asexuals and is localised to the parasitophorous vacuole (Wellems *et al.*1998). Wellems *et al.*, (1998) reported that CG2 protein is not an integral membrane protein although it is associated with the membrane. The CG2 protein was suggested to be a sodium/hydrogen ion exchanger (NHE) (Wunsch *et al.*, 1998) as they claimed it had the common binding motifs common with other NHE proteins The polymorphisms in the *cg2* gene included variations in three repetitive sequences (κ, γ, ω) changes in the length of a central poly-Asn tract, and 12

individual point mutations outside the repetitive sequences (Su *et al.*, 1997). *Cg2* had complex polymorphisms which appeared to be associated with CQR in a series of laboratory isolates from Africa (Su *et al.*, 1997; Basco and Ringwald, 1999; Adagu and Warhurst, 1999b) and S.E Asia (Su *et al.*, 1997). Indeed from this selection it appears that this polymorphism is highly associated with CQR but not sufficient, as there is a single Sudanese isolate which has the complex *cg2* polymorphism associated with CQR but is CQ sensitive.

1.12.2.2.3 *Plasmodium falciparum* chloroquine resistance transporter

More recently, a sharper focus has been gained on the parasite's means of subverting CQ chemotherapy. *In vitro* studies involving transfection analysis conducted by Fidock (Fidock *et al.*, 2000a, 2000b) in *P. falciparum* have validated the candidate CQR gene, *pfcrt* (*P. falciparum* chloroquine-resistance transporter), whose locus was first identified in the 1990 genetic cross of CQR and CQS parasite clones. The *pfcrt* product is a predicted integral membrane protein with 10 transmembrane segments (Fidock *et al.*, 2000b) and CQR forms of this protein carry multiple mutations, at residues 74, 75, 220, 271, 326, 356 and 371 supplementary to an essential 76 mutation from lysine to threonine. Patterns of mutations have been detected that are characteristic of laboratory lines of *P. falciparum* strains from Southeast Asian/African or South American origin. The Lys76Thr (K76T) mutation has been invariably found in all *in vitro* CQR strains and no sensitive strains, regardless of origin. This is in the first transmembrane segment of the predicted *pfcrt* product (Fidock *et al.*, 2000b). Transfections by Reed *et al.*, (2000) have also clarified the long-controversial role of polymorphisms in *pfmdr1* (*Plasmodium falciparum* multidrug resistance gene 1) in the parasite response to quinolines, including CQ. Alterations in the primary sequence of *pfmdr1* increased the levels of CQ resistance of a *P. falciparum* strain already slightly resistant (i.e., possessing mutant *pfcrt*), but not that of a strain with a sensitive allele. Taken together, these studies indicate the primacy of *pfcrt*, and the accessory role of *pfmdr1*, as loci for genetic mutations conferring the chloroquine-resistant phenotype in *P. falciparum* malaria. The first prospective field study designed to directly evaluate the *pfcrt* in the field was conducted in

Mali, West Africa in 1998 (Djimde *et al.*, 2001), where CQR remains low (3% RII and RIII). The *pfcrt* K76T mutation, identified as a definitive marker for *in vitro* CQR by Fidock *et al.*, (2000b) and the *pfmdr1* N86Y polymorphism were used by Djimde *et al* (2001) to evaluate the contributions of mutant *pfcrt* and *pfmdr1* to CQ failure. Consistent with the *in vitro* studies of established and transfected parasite strains, all blood isolates with *P. falciparum* persisting post-treatment harboured the K76T *pfcrt* mutation.

The function of the Pfcrt protein is still unknown, although it has been suggested that it normally operates in facilitated diffusion of positively charged amino acids (such as histidine) or similar peptides from the digestive vacuole into the cytoplasm. When mutated, the protein may enhance CQ export capability, (Warhurst, 2001b).

1.13 Resistance to antimetabolites

1.13.1 Resistance to type II antifolates

1.13.1.1 Biochemical mechanism

The evidence suggesting that the major mechanism of resistance to pyrimethamine in *Plasmodium* spp, was due to altered drug binding to DHFR is consistent with most of the work that has analyzed the enzyme kinetics of DHFR in the rodent malaria parasites, *P. berghei*, *P. vinckei*, and *P. chabaudi*, as well as studies in *P. falciparum*. The most striking feature of this work is the almost universal finding that the inhibition constant (K_i) for pyrimethamine of the DHFR enzyme had increased in pyrimethamine-resistant strains and isolates (Ferone, 1970; McCutchan *et al.*, 1984; Walter, 1986; Sirawaraporn and Yuthavong, 1984; Chen *et al.*, 1987; Zolg *et al.*, 1989). One exception to this finding was the observation that the DHFR activity from a pyrimethamine-resistant *P. falciparum* isolates had the same K_i as the DHFR from a sensitive isolate; however, it was suggested that the enzyme expression was increased 30-to 80-fold (Kan and Siddiqui, 1979). More recent analysis of equivalent field isolates has not shown any altered levels of enzyme and confirms that the DHFR enzyme in field isolates is structurally altered in resistance (Cowman *et al.*, 1988; Peterson *et al.*, 1988).

The cloning of the gene encoding the *dhfr* gene from *P. falciparum* (Bzik *et al.*, 1987; Cowman *et al.*, 1988) and *P. chabaudi* (Cowman and Lew, 1989) allowed a detailed analysis of alterations in this enzyme and their role in the mechanism of resistance. The *dhfr* enzyme had previously been shown to co-purify with thymidylate synthase (*ts*) in *Crithidia fasciculata* (Ferone and Roland, 1980), and the association of the *dhfr* and *ts* genes was confirmed for *P. falciparum* when it was shown that a single open reading frame (Bzik *et al.*, 1987; Cowman *et al.*, 1988) encoded both activities (Sirawaraporn *et al.*, 1990), a property that is shared by most protozoan organisms (Garrett *et al.*, 1984; Grumont *et al.*, 1986).

Analysis of alterations in the *dhfr* gene of experimentally induced pyrimethamine-resistant *P. chabaudi* and *P. falciparum* strains has shown that both amplification and mutation of the gene can occur (Inselburg *et al.*, 1987; Cowman and Lew, 1989, 1990; Tanaka *et al.*, 1990a, 1990b; Watanabe and Inselburg, 1994). Induction of pyrimethamine-resistant *P. chabaudi* (Cowman and Lew, 1989, 1990) and *P. falciparum* (Inselburg *et al.*, 1987; Tanaka *et al.*, 1990a, 1990b), by slowly increasing the level of drug, resulted in increased expression of *dhfr-ts*. This was due to a duplication of part of the chromosome containing the gene, which resulted in an increase in karyotype from 14 chromosomes to 15 chromosomes. Increased selection for high levels of pyrimethamine resulted in a mutated *dhfr* gene from a serine to asparagine at position 108 (Cowman and Lew, 1990). This is the equivalent mutation that has been found to be important in resistance to pyrimethamine in field isolates of *P. falciparum* (see below).

1.13.1.2 Molecular mechanism

Analysis of a genetic cross between pyrimethamine-resistant and pyrimethamine-sensitive *P. falciparum* parents demonstrated that the DHFR containing the Asn-108 segregated with the resistance phenotype (Peterson *et al.*, 1988), proving that this alteration was the determinant of the drug-resistant phenotype. The availability of the *dhfr-ts* gene sequence allowed the comparison of the gene from a large number of pyrimethamine-resistant and -sensitive field isolates of *P. falciparum* and the identification of important amino acid changes that appeared to be involved in the mechanism of resistance (Cowman *et al.*, 1988; Peterson *et al.*,

1988; Zolg *et al.*, 1989; Snewin *et al.*, 1989; Tanaka et al., 1990a; Thaithong *et al.*, 1992). This analysis suggested that the amino acid at position 108 in *dhfr* was critically important in the development of resistance to pyrimethamine. Pyrimethamine-sensitive field isolates have Ser-108, while resistant isolates have Asn-108. The *dhfr* gene from isolates that were highly resistant to pyrimethamine had additional mutations which suggested that pyrimethamine resistance arose as a single point mutation and subsequent drug pressure selected for additional mutations that conferred higher levels of drug resistance (Sirawarporn *et al.*, 1997). These studies provide strong suggestive evidence that the amino acid differences found between pyrimethamine-sensitive and pyrimethamine-resistant *P. falciparum* were responsible for the mechanism of resistance. A serine in position 108 of the *dhfr* gene is linked to *in vitro* sensitivity to both pyrimethamine and cycloguanil. A mutation to asparagine at position 108 seems to be the key mutation for conferring *in vitro* pyrimethamine resistance (de Pecoulas *et al.*, 1996), although a genotype without this mutation has been recently described (Wang *et al.*, 1997b). An asparagine to isoleucine change at position 51 and a cysteine to arginine at position 59 appear to modulate higher levels of *in vitro* pyrimethamine resistance when they occur with the asparagine 108 mutation, and an isoleucine to leucine mutation at position 164 in combination with the asparagine-108 and arginine -59 mutations has been found in *P. falciparum* lines that are highly resistant to both pyrimethamine and cycloguanil (Basco *et al.* 1995a, Reeder *et al.*, 1996). Ala-16 to Val and 108 Ser to Thr have also been found together giving resistance to cycloguanil. It is clear that cross-resistance to both pyrimethamine and cycloguanil does occur and has been well documented (Schapira., 1984; Watkins *et al.*, 1984).

Combinations of type I and type II antifolates (like SP) have generally been effective in the presence of pyrimethamine or cycloguanil resistance. Medium level cross-resistance between these two drugs occurs when the DHFR gene has Asn-108, and much higher levels of cross-resistance are seen with accumulation of mutations (Foote *et al.*, 1990a; Peterson *et al.*, 1990; Sirawaraporn *et al.*, 1997). For example, in southeast Asia SP is no longer useful, (Peterson *et al.*, 1990) and many isolates of *P. falciparum* have mutations in *dhfr*, Ala16Val with Ser108Thr and Ile164Leu with Ser108Asn are seen.

1.13.2 Mechanism of resistance to type I antifolates

1.13.2.1 Biochemical mechanism

The sulfa drugs are an important group of antimalarial compounds and have generally been used in combination with DHFR inhibitors such as pyrimethamine. Sulfadoxine, the most commonly used sulfa drug, has been used extensively in combination with pyrimethamine. A sulfa-based drug dapsone (a sulfone) is used in combination with chlorproguanil and registered as LapDap and is currently undergoing clinical trials, (Winstanley, 2001; Mutabingwa *et al.*, 2001). The sulfa drugs are known to inhibit the enzyme DHPS from murine malaria parasites *P. berghei* (Ferone, 1973), and the enzyme from *P. falciparum* (Zhang and Meshnick, 1991;Triglia *et al.*, 1997). They are structural analogues of PABA and act as competitive inhibitors of DHPS (Triglia *et al.*, 1997). Additionally, DHPS may convert the drug to its sulfa analogue, which would inhibit dihydrofolate synthase, the next enzyme in the folate biosynthetic pathway. The gene encoding the enzyme *dhps* has been cloned, and is on chromosome 8 and codes for a bifunctional enyzme with 6-hydroxymethyl-7, 8-dihydropterin pyrophosphokinase (PPPK) at the N terminus of the protein (Triglia and Cowman, 1994; Brooks *et al.*, 1994). This is consistent with previous data from mouse malaria species showing that both enzyme activities co-purify (Ferone, 1973, 1977).

1.13.2.2 Genetic mechanism of drug resistance to sulfadoxine

Point mutations have also been described in *dhps* gene, but its contribution to sulfadoxine resistance by *P. falciparum* is less clear (Wang *et al.*, 1997b). Polymorphisms at 5 highly conserved positions within the *dhps* gene (codons 436, 437, 540, 581 and 613) have been found in sulfadoxine-resistant parasites (Triglia *et al.*, 1994; Brooks *et al.*, 1994; Wang *et al.*, 1997a; Jelinek *et al.*, 1998). The most sulfadoxine-resistant parasites reported to have been assayed *in vitro* carried the Phe-436, Gly-437 and Ser-613 alleles (Wang *et al.*, 1997a). Using the recently developed transfection method in *P. falciparum*, Triglia *et al.*, (1998) introduced specific mutations in the *dhps* gene that corresponded to the ones identified in

sulfadoxine-resistant field isolates. They found that the change from Ala- to Gly-437 gave the parasite a 5-fold increase in sulfadoxine-resistance and accumulation of further mutations raised the level of resistance to 24-fold.

Different genotypes of *dhfr* and *dhps* have been found in different geographical areas where malaria is endemic. Broadly, the prevalence of mutated genes seems to correlate with the amount of SP usage in a determined region (Wang *et al.*, 1997a). From comparison of isolates taken before and after treatment with SP it was concluded that *in vivo* selection of both pyrimethamine and sulfadoxine-resistant parasites occurs, while the most common number of mutations in the *dhps* gene was two, while *dhfr* was often triply mutated (Wang *et al.*, 1997a; Curtis *et al.*, 1998).

1.14 Genetic diversity

The genetic diversity among *P. falciparum* from different malaria endemic regions may be the result of differences in intensities of infection, transmission patterns, human/ vector distribution, and the population dynamics of the parasite (Creasey *et al.*, 1990). In Kenya, the 5-8 yearly cycles of malaria epidemics in the highlands, (Khan *et al.*, 1992, MOH, 1994-2001), have become a yearly occurrence since 1992 (MOH 1994-2001). A recent report (Shanks *et al.*, 2000) suggest that drug resistance may be contributing to malaria epidemics in Kenya. It is possible that drug resistant strains have gradually spread from nearby endemic regions to the epidemic areas. Population movement between epidemic and endemic areas is likely to be a major vehicle for the spread. The transfer of infective anopheline mosquitos, trapped in luggage, cars or lorries may possibly also take place. An epidemic in S. America has been reported to be due to clonal infection (Laserson *et al.*, 1999) with individuals being infected with a single parasite clone. However, in areas where malaria transmission is stable, *P. falciparum* parasite populations are genetically diverse (Babiker and Walliker, 1997). Polyclonality and extensive diversity are thought to be more likely in endemic infections than in epidemic outbreaks.

Polyclonality in field isolates has been reported (Snewin *et al.*, 1991; Ntoumi *et al.*, 1995; Viriyakosol *et al.*, 1995). Parasites examined in this thesis (for

drug susceptibility/ and sequence variations in resistance associated genes) were typed to assess their clonality/ genetic diversity (see chapters 4 and 5, respectively). Analysis of genetic variants of *P. falciparum* is facilitated because the parasite is haploid except for the zygote stage occuring briefly in the mosquito. This avoids the use of complex analyses to interpret diploid patterns of inheritance. The repetitive regions in 3 markers encoding merozoite surface proteins 1 and 2 (*msp1* and *msp2*) and glutamate rich protein (*glurp*) are used to investigate genetic diversity/ clonality among isolates of *P. falciparum*. (Viryakosol *et al.*, 1995).

1.15 Quantitative nucleic acid sequence-based amplification (QT-NASBA)

Rapid diagnosis and prompt treatment is a key step in the control of malaria infection; more so in the phase of continuous spread and persistence of antimalarial drug resistance. Traditionally, diagnosis of infection or treatment failures/ re-infection is by microscopic examination of Giemsa stained thick and thin blood smears. Although it is cheap, it requires expert microscopists to detect low level parasitaemia. Microscopic diagnosis of malaria is time consuming when dealing with large number of samples and sensitivity is low with a limit of detection of about 20 parasites/ μl (Bruce-Chwatt, 1984). Antigen detection methods such as Parasight-F®, ICT MalariaPf® are rapid manual tests incorporating a dual-antibody immunoassay against the histidine-rich protein II antigen (HRP-2) of *P. falciparum* (Pieroni *et al.*, 1998). The OptiMAL® test detects a species-specific enzyme, parasite lactate dehydrogenase (pLDH), produced by live parasites (Palmer *et al.*, 1998). The techniques are fast and simple to perform, but at low parasitaemias (of approximately 100 parasites/μl) the sensitivity decreases drastically (Makler *et al.*, 1998). Polymerase chain reaction (PCR) assays have been developed and evaluated (Barker *et al.*, 1994; Smits *et al.*, 1997), however these techniques have been shown to allow only for semi-quantification of parasites (Hommel *et al.*, 1998). A quantitative method capable of detecting very low level parasitaemia (less than 20 parasites/ μl) will be a useful tool in the control, treatment and prevention of malaria infection.

In this thesis, a quantitative nucleic acid sequence-based amplification [QT-NASBA] (van Gemen, *et al.*, 1995) was developed and evaluated as reported in

chapter 6. The technique could utilize for example, a ribonucleic acid (RNA) as target in the amplification and detection of malaria parasite which would be more likely to distinguish between live and dead parasites. It is envisaged that this method could detect the low parasitaemias present in early recrudescences after treatment. This would enhance the *in vivo* drug evaluation schemes used to follow the spread of resistance. Although the technique is still under development the results of the initial evaluation described in chapter 6 suggest that it is a promising method. Further optimization and evaluation will be needed to meet the much needed sensitivity, specificity, cost-effectiveness and speed that are characteristics of an effective diagnostic procedure.

1.16 Objectives of the study

Documented reports of malaria control in Kenya consisted of anti-larval activities such as oiling water pools and surface drainage. Due to the abundance of breeding sites this approach was not effective, thus leaving drug treatment the only useful measure. In spite of many efforts *Plasmodium falciparum* malaria remains the foremost cause of serious morbidity and mortality in Kenya and has undergone a dramatic resurgence in the last decade apparently fuelled by resistance to the drug chloroquine [CQ] (WHO Epidemiological Record, 1993). Historically the first case of indigenous CQ resistant malaria was reported in Kenya in 1982 in a child from Kisumu (Spencer *et al.*, 1983). Since then resistance has spread countrywide and failure rates between 60% and 80% has been reported (MOH, 1997). By end of 1997 CQ use in the treatment of uncomplicated malaria was halted by a government decree, (MOH Report, 1997). Sulfadoxine-pyrimethamine (SP Fansidar®) was subsequently introduced as the first-line drug for uncomplicated malaria.

As case finding and effective disease management is the cornerstone of the Kenyan National Malaria Control Programme (National Malaria Control, 1993) and rapid treatment of acute malaria with an effective drug is regarded as lifesaving, it is vital to monitor these changes in resistance. Only then will it be possible for drug provision and use to be guided by up to date information.

1.16.1 Primary Objectives

A. To assess the *in vivo* resistance profiles of *Plasmodium falciparum* malaria to chloroquine and sulfadoxine/ pyrimethamine (SP, Fansidar®) in study sites chosen to comply with the Kenya National sentinel posts for monitoring drug treatment efficacy.

B. To characterize *P. falciparum* drug resistant determinants associated with antifolate and chloroquine resistance.

C. To study the allelic variability of the major merozoite surface protein gene-1 and -2, (*msp1, msp2,*) glutamate rich protein gene, *glurp* and to establish genetic diversity profiles in endemic and epidemic malaria in Kenya.

1.16.1.1 Specific Objectives

1. To assess *in vivo* efficacy of sulfadoxine-pyrimethamine in sentinel sites in Kenya.

2. To assess *in vitro* drug resistance profile to quinine, chloroquine, sulfadoxine-pyrimethamine, amodiaquine and mefloquine.

3. To determine the association of particular drug resistant determinants with treatment outcome to antifolates and chloroquine.

4. To evaluate predictors of antifolate resistance using molecular markers in malaria endemic and epidemic areas.

5. To determine allele frequencies of drug resistant determinants in epidemic and endemic malaria.

6. To establish the genetic diversity profiles in endemic and epidemic malaria regions of Kenya by using the variable markers of the major merozoite surface protein gene-1 and -2, *msp1, msp2*, and glutamate rich protein gene, *glurp*.

7. To assist in developing and evaluating a quantitative nucleic acid sequence-based amplification method (NASBA) and to evaluate the accuracy of the method for the determination of malaria parasite levels in blood samples. This will probably be a valuable tool for monitoring *in vivo* drug trials.

CHAPTER 2

Antimalarial drug susceptibility in Endemic and Epidemic areas of Kenya

2.1 Introduction

All studies reported in this thesis were ethically approved by the Kenya Medical Research Institute Ethical Steering Committee. Subjects were recruited with informed consent or parental/guardian permission (see Appendix A: for information on study sites Appendix B: consent form).

Antimalarial drug resistance is a large and growing problem. Several East African studies have reported incidences of parasitological resistance of *Plasmodium falciparum* to chloroquine (CQ) ranging from 50% to 80% (Wolday *et al.*, 1995; Rapouda *et al.*, 1997). The emergence of CQ resistance has been associated with increased malaria mortality, especially in children (Trape *et al.*, 1998). However most African countries continue to use CQ as the first-line agent to treat uncomplicated malaria (Bloland *et al.*, 1998) and has been reported to be at least partially effective in semi-immune African populations (Koella *et al.*, 1990; Cravo *et al.*, 2001).

SP was introduced in Kenya in 1983 as a second-line treatment for malaria. Initial studies showed little or no resistance to SP combination, but later studies reported cure rates ranging from 54% to 92% (Nguyen-Dinh *et al.*, 1982; Spencer *et al.*, 1986; Hagos *et al.*, 1993; Anabwani *et al.*, 1996; Clarke *et al.*, 1996). In 1997, the Kenyan National Malaria Control Programme, (NMCP) replaced CQ with SP as a first-line antimalarial treatment owing to the increasing number of CQ-treatment failures. However, this change was effected in August 2001, information obtained from the East Africa Network for Monitoring Antimalarial Treatment (EANMAT Scientific day meeting, June 2001).

Malaria endemicity in Kenya is influenced by both climate and topographic conditions. The altitude above sea level provides approximate criteria to stratify endemicity. Stable and hyper- to holoendemic malaria is found in the regions below 1300m on the coast and in Nyanza and Western provinces where the prevalence of infection in children between ages 1-9 years can be as high as 70-90 per cent during the long rains (MOH, 1987). Seasonal, but usually stable malaria is found between 1,300-1,700 meters, in areas such as Mwea (1,300 meters) where the disease incidence is 30-40 percent (MOH, 1997). In the higher altitudes (between 1,700-2,300 meters) such as the Kisii Highlands and Kericho District, there is unstable

transmission, and epidemics normally occur especially after unusually heavy rains (MOH, 1994-2001).

Malaria transmission in Kenya is mainly by the *A. gambiae* complex, notably *A. gambiae, A. arabiensis*, and *A. funestus*. *A. gambiae sensu stricto* have been reported in the highlands of Kisii and Kericho as well as the Nandi foothills from where the vector spreads up to the shores of Lake Victoria, north-west of Kisumu (Service *et al.*, 1978). The proportion of this species increases towards the cool highlands as described by White (White, 1972). *A. gambiae* has also been identified as the main vector in the coastal region (Mosha *et al.*, 1982). Low frequencies of this species have been found in the coastal region (Mosha *et al.*, 1982). It has also been documented in the Mwea irrigation scheme. *A. merus* is mainly restricted to the coastal strip (Mosha *et al.*, 1982).

To assess the current extent of SP resistance among patients infected with *P. falciparum* in Kenya, cross-sectional and longitudinal *in vivo* studies were conducted. The studies were conducted in sentinel sites established by the NMCP (see Figure 2.1a-b). In addition, CQ efficacy studies were carried out in one site at least before CQ was replaced as first-line drug in the treatment of uncomplicated malaria in Kenya. The efficacy of CQ, SP, co-trimoxazole and CQ plus SP (CQSP) combinations was assessed.

The *in vivo* and *in vitro* resistance profile of Kenyan *P. falciparum* was investigated as reported in this chapter.

2.2 Materials and Methods

2.2.1 Description of study sites

2.2.1.1 Endemic sites

2.2.1.1.1 Mwea

Mwea is situated in the Central Province of Kenya at an altitude of 1,500 meters above sea level. Mwea is a rice irrigation scheme and an urban area which is moderately populated (0.25-0.32 million); 39% of which are children under 5 years. Malaria transmission is seasonal accounting for between 30-40% paediatric and adult outpatient attendances, (MOH 1994). *P. falciparum* resistant to CQ is the most important public health problem, (MOH, 1999). For information on temperature and rainfall for all sites described here, see Table 2.2.1. Information supplied by University of Nairobi Weather Department.

2.2.1.1.2 Tiwi

Malaria is hyperendemic in Tiwi, a lowland area in Kwale District in Coast Province of Kenya. Tiwi is an urban area and moderately populated (0.75-1.05 million); 20-27% of which are children under five years. There are two rainy seasons, viz "long rains" (February-June) and "short rains" (October-December) with mean annual rainfall of 1,000 millimeters [mm]. Malaria transmission is all year around, peaking in May, June and July months. In Tiwi, malaria is the major cause of morbidity, accounting for more than 30% of paediatric and adult outpatient attendances.

2.2.1.1.3-5 Busia (Nambale), and Oyugis and Kendu Bay

These 3 sites are endemic for malaria and are situated in the western region of Kenya at 1,300 meters above sea level. Malaria transmission is very high and all year around, peaking in May, June and July months. Children aged 1-9 years are the most vulnerable group accounting for between 70 and 90% of malarial illness in the outpatients during long rains (MOH 1994). Malaria morbidity accounts for 40% of paediatric and adult outpatient attendance (MOH, 1997). SP is therefore now routinely used for the treatment of uncomplicated malaria. There are two rainy seasons, with an average annual rainfall ranging between 1,000-1,300 mm.

2.2.1.2 Epidemic sites

2.2.1.2.1 Mosoriot

Mosoriot is in the Nandi District of Western Kenya, a highland area (of altitude ranging from 1,700-2,500 meters above sea level) where transmission is seasonal and unstable. Epidemic malaria affects all age groups and when samples were collected, malaria was responsible for 40% of hospital attendance, (MOH, 1997).

2.2.1.2.2 Chogoria

Chogoria is located on the eastern slopes of mount Kenya. It is a geographically diverse catchment area at the boundary of Mt. Kenya National Forest at an altitude of 1,710 meters. The area experiences an annual rainfall of 2,000 mm. The land is very fertile and cash crops such as tea and coffee serve as the main source of income. Chogoria is close, about 60-70 kilometers [km] to the mesoendemic region of Meru district of Kenya.

2.2.1.2.3 Mt. Elgon

2.2.1.2.4 Kisii

Mt. Elgon and Kisii are located at higher altitudes between 1,700-2,300m. Malaria transmission is unstable and epidemics occur especially after unusually heavy rains.

Clinical malaria is responsible for 45-50% of outpatient cases and for 45% of inpatient admissions during epidemics. Case fatality rate for inpatients is high, at 5% (MOH, 1999). See Figure 2.1 for study sites.

Table 2.2.1 Rainfall and temperature parameters of the study sites

Study sites	Year	Temperature (°C)		Mean Annual Rainfall (mm)
		Minimum	Maximum	
Mwea[Meso]	1997	19	33	1,000
Tiwi[Hyper]	1997	22	32	1,000
Busia[Holo]	1998	24	33	1,100
Kendu Bay[Holo]	1999	23	32	1,000
Oyugis[Holo]	1998	24	34	1,300
Mosoriot[Epi]	1997	9	24	1,200
Chogoria[Epi]	1997	12	25	2,000
Kisii[Epi]	1999	9	23	1,500
Mt. Elgon[Epi]	1999	9	23	1,500

Key to table 2.2.1: Meso = mesoendemic; Hyper = hyperendemic; Holo = holoendemic; Epi = epidemic

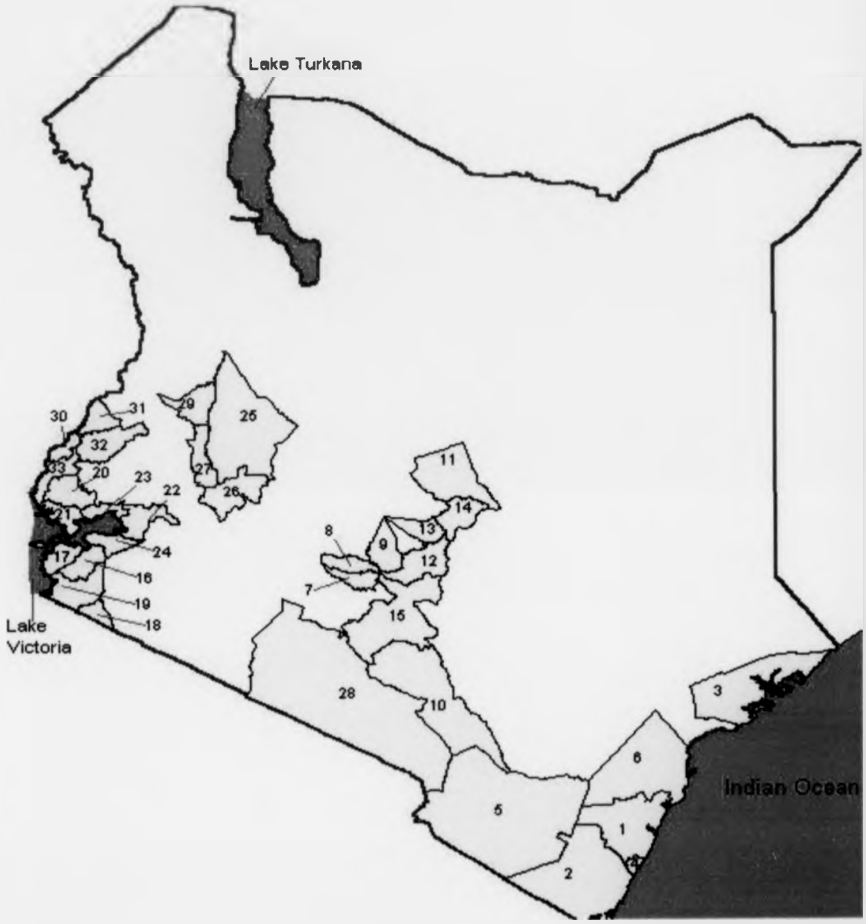


Figure 2.1a A representative map of endemic regions of Kenya showing study sites (numbers = Districts). 2 = Tiwi; 9 = Mwea; 24 = Oyugis and Kendu Bay [both in the same district]; 33 = Busia

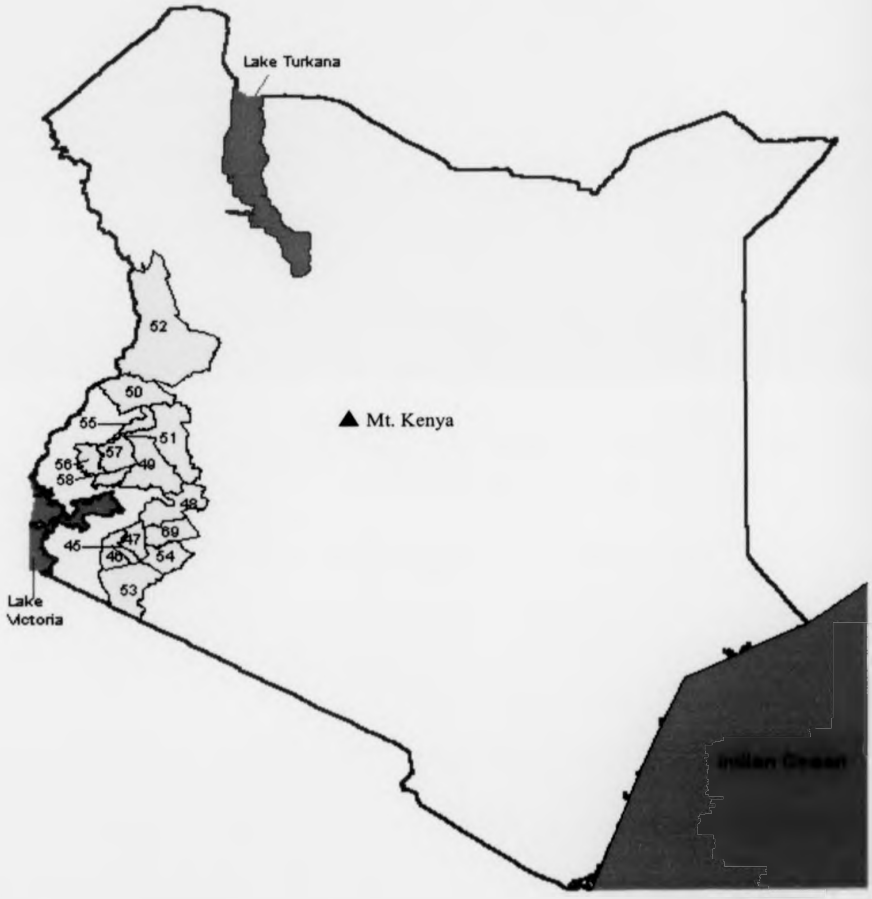


Figure 2.1b A representative map of epidemic regions of Kenya showing study sites and ▲ = (Chogoria); (numbers = Districts). Mt.Kenya = Chogoria; 50 = Mt.Elgon; 49 =Mosoriot; 47 = Kisii

2.2.2 *In vivo* antimalarial drug sensitivity studies

2.2.2.1 Patients

Patients presenting to the outpatient clinics were enrolled in the study if they met the following criteria: informed consent (see Appendix B): monoinfection of *P. falciparum* with parasitaemia between 1,000 and 200,000 parasites /μL of blood, axillary temperature $\geq$ 37.5°C or with a history of fever, no history of antimalarial drug intake during the previous week as confirmed by urine tests. Blood was obtained by finger prick for thin and thick smears. If patients did not return for scheduled follow-up, they were visited and assessed at home. If patients could not be located by the home health visitor, (demographer) they were classified as lost to follow-up. Patients were excluded from the study for the following reasons: (1) administration of any additional antimalarial drugs, (2) emergence of any non-malarial febrile illness that would interfere with the classification of malaria-treatment outcome, (3) patient relocation outside study area, or (4) withdrawal from the study.

The *in-vivo* studies on antimalarial drugs were chosen to comply with the sentinel sites identified by the Kenyan National Malaria Control Programme (Kenyan NMCP). In order to maximize a good representative of the sites, the WHO (1996) protocol of malaria enrolment procedure was followed.

2.2.2.1.1 Sample size

The sample size estimation was calculated using the Double Lot Quality Assurance methodology (DLQAS), (described in the "Assessement of Therapeutic Efficacy of Antimalarial Drugs for Uncomplicated Falciparum Malaria in Areas with Intense Transmission" [WHO protocol, 1996]). P0 [defined as the upper threshold level of clinical failure beyond which replacement of the drug under the study is deemed necessary], was assumed to be 25 % and P1 [defined as the lower threshold level of clinical failure below which it would be acceptable to continue the utilisation of the drug], was assumed to be between 10-12.5 %. With the above assumptions, (WHO, 1996), the minimal sample size derived according to the lot quality assurance method with a confidence level of 95 % and power of 80 % was 63 patients per study site. The goal of patient recruitment was therefore to have

≥63 subjects on the appropriate drug and followed up until 14, 21 or 28 days later. Due to the dispersed population and geographical constraints, we expected the proportion of people 'lost to follow up' to be high, despite our efforts. Therefore allowing for a 20% loss to follow up we required a final sample size of 80 persons for each drug. The decision on sample size was made this way but was largely influenced by dominant considerations borne from practical decisions on the availability of time, staff and money. The number of patients satisfying the inclusion criteria (section 2.2.2.1) who were enrolled into the study in the initial 5 days was an important factor.

2.2.2.2 Sample collection

Pre- and post-treatment blood samples were collected from the patients by fingerprick as dried blood spots on 3MM Whatman filter paper (Sambrook *et al.*, 1989) or glass fibre membranes (GFM), (Warhurst *et al*, 1991). Samples were allowed to air dry. The serially labeled samples were then transferred to matching labeled self-seal bags. Samples were stored at 4°C with desiccant to prevent dampness until required for DNA extraction/ polymerase chain reaction.

2.2.2.3 Treatment and follow-up

Chloroquine resistance became widespread in Kenya posing serious limitations to malaria chemotherapy. This led to the withdrawal of the drug with partial replacement with SP by late 1997. In this study therefore, SP was the main drug used in all sites. Drugs or drug combinations were tested in other sites chosen at random. In Mwea, CQ and SP were used. Similarly, SP was used in a study conducted in Tiwi in 1997. SP alone and in combination with CQ (CQSP) were used in Busia, while in Kendu Bay, SP was the only drug used. In the 1998 Tiwi and Oyugis study, SP was used alone and compared with trimethoprim plus sulphamethoxazole combination (co-trimoxazole). The oral regimens prescribed are as detailed below: CQ (25 mg/ kg base body weight (Avloclor®, ZENECA), giving 10 mg /kg per body weight on days 0 and 1, and 5 mg /kg on day2. SP was administered as a single dose of 1.25 mg / kg pyrimethamine and 25 mg / kg sulfadoxine (Fansidar®, Roche) CQ and SP were given as (25 mg/ kg of CQ

(Avloclor®, ZENECA): 10 mg /kg on days 0 and 1 and 5 mg /kg on day 2 followed by a single dose of 1.25 mg / kg pyrimethamine and 25 mg / kg SP. Co-trimoxazole (Septrin®, Glaxo Wellcome, UK) paediatric suspension 2.5mls 12 hourly for 5 days was administered. The dose is equivalent to 6mg of trimethoprim and 30mg of sulfamethoxazole per kilogramme body weight. The WHO (WHO, 1996) *in vivo* test procedure was employed in all study sites. Patients enrolled into the studies were randomized into groups A or B for treatment as outlined below.

Endemic sites: In studies conducted in Mwea (year 1997), Busia, Oyugis and Tiwi- (year 1998) patients were randomized into 2 groups, A and B. In Mwea, group A received CQ and B were treated with SP. In Busia, patients in group A were treated with SP and B with CQSP combination. In Oyugis and Tiwi, patients in group A received SP and group B co-trimoxazole. Repeated doses for those patients treated with co-trimoxazole, CQSP or CQ was conducted as explained above. In 2 sites, Tiwi 1997 and Kendu Bay, SP was used as the only treatment regimen and for all the enroled patients.

Epidemic sites: In the epidemic sites of Mosoriot, Mt. Elgon and Kisii, patients were treated with SP. In Chogoria patients had been treated initially as outpatients with CQ, SP, or AQ. The Chogoria study was conducted by Professor Dieter Gump and Mr. Peter Ndaru (see Appendix C).

Shortly after enrolment drug intake was monitored and patients observed for 30 minutes to rule out vomiting. All patients received the antipyretic paracetamol. A follow-up protocol was adopted [14-days in endemic sites of Mwea and Kendu Bay and in the epidemic sites of Chogoria, Kisii and Mt. Elgon]; [21 days in endemic sites of Tiwi and epidemic sites of Mosoriot]; [28 days in endemic site of Busia].

2.2.2.4 Screening

Patients were initially screened to select those who met the enrolment criteria. Thick and thin blood smear were prepared for microscopic determination of *P. falciparum* monoinfections. The thick smear was used for screening for an initial rapid count of the parasites and thin smear for definitive count and for species identification. Thin smear was fixed in methanol before staining along with thick smear in 10% Giemsa stain. Parasite density was determined by counting the number of asexual parasites per 200 white blood cells (WBC) assuming a WBC count of 8,000/μl of blood. Body temperature was estimated in the axilla.

Urine samples were obtained from each patient and tested for drug intake prior to presentation at the clinic. The Saker-Solomon test (Mount *et al.*, 1989) was used for the detection of CQ and (desethylCQ) while a modified Bratton-Marshall test (De Almeida and De Souza, 1983) for sulfonamides was performed to detect use of SP or co-trimoxazole.

2.2.2.5 *In vivo* test

2.2.2.5.1 Clinical and parasitological criteria

The *in vivo* test was carried out following the revised WHO protocol (WHO, 1996) and classifying therapeutic response as outlined below. The first day of treatment corresponds to day 0; the days of follow-up were then counted from this date.

Early treatment failure (ETF):

-development of danger signs or severe malaria (see below for definition) on day 1, day 2 or 3;

-axillary temperature $\geq$ 37.5°C on day 2 with parasitaemia > that of day 0 [recruitment day];

-axillary temperature $\geq$ 37.5°C on day 3 in the presence of parasitaemia;

-parasitaemia on day 3 $\geq$ 25% of count on day 0.

Presence of one or more of the following criteria in the presence of asexual parasitaemia as reported by WHO (1994) define severe falciparum malaria: cerebral malaria, severe normocytic anaemia, renal failure, pulmonary oedema, hypoglycaemia, circulatory collapse, repeated generalized convulsion, acidosis, macroscopic haemoglobinuria.

Therapeutic response was classified as **late treatment failure (LTF):**

if the patients developed:

-danger signs or severe malaria in the presence of parasitaemia on any day from day 4 to day 14, [or 21 or 28 depending on the study period] without previously meeting any of the criteria of early treatment failure;

-axillary temperature $\geq$ 37.5°C in the presence of parasitaemia on any day from day 4 to day 14/ 21 or 28 without previously meeting any of the criteria of early treatment failure.

The response to treatment was classified as adequate clinical response (ACR) if the patients showed one of the following conditions during the follow-up period (upto day 14, 21 or 28).

Adequate clinical response (ACR):

-absence of parasitaemia on day 7, 14, 21 or 28 irrespective of axillary temperature, without previously meeting any of the criteria of early or late treatment failure

-axillary temperature < 37.5°C irrespective of the presence of parasitaemia, without previously meeting any of the criteria of early or late treatment failure.

Parasitological response (S, RI-RIII) was determined using the WHO criteria (WHO, 1994). If the day-3 parasite density was $\geq$ 75% of the day 0 density, the response was classified as RIII resistance. If the day-3 density was 25% of the day 0 density and the patient was parasitaemic on day 4-7, the response was classified as RII. RI parasitological responses were defined as day-7 parasite densities <25% of day-0 density, [following either an initial parasite clearance on day 3 or presence of parasite density on day 3 at <25% of day-0]. Patients with day

3 parasitaemia (<25%) of that on day 0 [or absence of parasitaemia], a negative blood smear on day 7 and negative smears for the remainder of the follow-up period were classified as sensitive (S).

As stated above and as defined by the WHO criteria for classification of therapeutic response, (WHO, 1996), the indication for successful or unsuccessful treatment at any time between day 0 and end of study period [either day 14, 21 or day 28], should be based on clinical and parasitological criteria. The main aim is to avoid an aggravation of the clinical condition and risk to the patient and to evaluate parasite clearance correctly. As stated by the WHO protocol, (WHO, 1996) and adopted in this thesis, evaluation of patient's clinical condition was not limited to fever, since danger signs may also develop in afebrile patients. The guidelines aim at maximizing sensitivity to ensure that most cases with a true malaria attack receive antimalarial treatment. The clinical criteria were also supported by parasitological evidence. Parasitological evidence is desirable for diagnosing treatment failures and severe disease in all situations, as well as for identifying the disease in areas of unstable or low transmission.

Additionally, the criteria for assessment of parasitological and clinical outcome during treatment and subsequent to that, are aimed at evaluating the proportion of treatment failures in the sampled population. The latter will provide the level of treatment failures in a particular study area to a given antimalarial drug regimen used. Persistence of parasitaemia and/ fever following treatment, therefore, will suggest that the drug is not efficacious in alleviating symptoms or clearing parasitaemia.

2.2.2.5.2 Parasite and fever clearance times

Parasite clearance time (PCT) and fever clearance time (FCT) was used to measure clearance of parasitaemia and fever, respectively, within the first week in treated patients. Parasite clearance time (PCT) was defined as the time from beginning of drug administration until there was no patent parasitaemia. For SP the earliest end-point measured was 48 hours. For CQSP, CQ, co-trimoxazole, the end-points measured after the first drug administration was 12, 24 and or 48 hours for each of the drug regimen. This is because treatment was given daily for 3 days

for CQ and CQSP and 5 days at 12 hourly intervals for co-trimoxazole and only once for patients on SP alone, see section 2.2.2.3. The time taken to clear parasites at the given time points was then calculated. For comparative analysis of SP with CQSP, CQ or co-trimoxazole, for PCT and FCT, the earliest end-point measured was 12 hours from commencement of respective treatment regimen.

The fever clearance time (FCT) was defined as the time from start of drug administration until the axillary temperature fell to 37.2°C or below and remained so for at least 48 hours [SP]. With regard to antimalarial drugs like co-trimoxazole, CQ and CQSP combination the temperature was monitored at 12, 24 and 48 hours. For comparative measurements with CQSP, CQ or co-trimoxazole, FCT was measured similarly to the SP treatment groups. Patients complaining of having had persistent fever without direct evidence (presumptive) were encouraged to visit the clinic for further assessment.

2.2.2.5.3 Haematological assessment

Improvement in haemoglobin levels could suggest effective treatment and in this study, Hb concentrations were obtained in patients during pre- and post-treatment period. Haematological response, on a population basis, gives an assessment of haematological effect of the drug used and can be compared over time or between different regimens or drugs. The usefulness of the test initial is to identify patients with severe anaemia prior to inclusion in the study [enrolment requires a Hb value of above 5.0 g/dL, (WHO, 1996)]. Patients with a Hb concentration of 5.0 g/dL or less, were not included in the study and were referred to the main hospital for appropriate treatment. Haematological response was compared between treatment groups (mean improvement by group) and between treatment successes and treatment failures. The haemoglobin level or concentration was measured on days 0, 3, 7 and 14 (and 21 or 28 wherever applicable). This was done using a haematometer (Compur 1000 Bayer Diagnostics and Electronic Gmbh, Munchen Germany) and performed on fingerprick blood obtained from patients.

2.2.2.5.4 Side effects monitored following treatment with antimalarial drugs

Upon treatment, the side effects were monitored. Vomiting, nausea, anorexia, diarrhoea, abdominal pain and fits were looked for. Where side effects

become severe, treatment is withdrawn and patient is excluded from the study and referred to outpatient department for adequate care and treatment.

2.2.2.6 Statistical analysis

Data were analysed using the Epi-info version 6 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Clinical success was defined as ACR and clinical failure defined as ETF or LTF. Parasitological responses were also included. A 2-tailed *t*-test analysis (not assuming equal variance) was performed when calculating for level of significance (P value less than or equal to 0.05).

2.2.2.7 Workers

A team of field workers or technicians recruited into the study carried out activities such as blood smear preparation, urine test, completing enrolment forms. These included demographers or follow-up officers and microscopists. A clinical officer or physician who looked after the patients was also involved in intravenous blood sample collection. Other activities include haemoglobin analysis, blood spotting on filter paper or glass fibre membrane, sample collection for nucleic acid sequence-based amplification analysis (NASBA). See Appendix C for list of field workers involved in the study.

2.2.3 *In vitro*

2.2.3.1 Introduction

In this section the *in vitro* efficacy of clinically useful blood schizontocides and antifolates against Kenyan *P. falciparum* were assessed. This is done with the aim of comparing *in vitro* drug efficacy with the *in vivo* findings. Therefore blood samples from infections examined in some of the endemic sites described in sections 2.2.1.1.1 to 2.2.1.1.4 were assessed using WHO *in vitro* Mark II micro test system (WHO, 1990). The endemic sites included Mwea, Tiwi, Oyugis and Busia where sample size of 48, 38, 69 and 65 from each site respectively, was collected.

2.2.3.2 Sample collection

Two millilitres [ml] of venous blood was obtained by venipuncture prior to treatment and using a heparinised 3ml sterile syringe with a 18 G needle. This was

performed by experienced technicians and paediatricians. Blood was transferred into sterile heparinized vacutainers (surface sterilized with 70% ethanol). Each tube was labeled with date and serial number. The tubes were inverted to mix the blood with the heparin. Samples were kept at 4°C in a cool box (with cool packs) or were kept in the fridge if available at the health units, until subjected to *in vitro* tests. The duration from the time the sample was obtained to the time the *in vitro* assay was carried out was a minimum of 2 hours and a maximum of 5 hours.

2.2.3.3 Media preparation

A one litre medium was prepared by dissolving a sachet of 10.4 gram (g) RPMI 1640 and 6g of HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] in 500ml ddH_2O (double distilled water). 2g of glucose was added and solution made up to 960 ml. 40mg of gentamicin was added and the medium was filter-sterilized through a 22 μm filter unit (Nalgene). 250ml aliquots were made and kept frozen at –20°C until needed. Medium preparation was carried out aseptically in a class II microbiological cabinet. Sodium bicarbonate [$NaHCO_3$] was prepared separately by dissolving 5g of the compound in 100ml of ddH_2O. This was filter-sterilized and kept at 4°C to be used within 2 weeks. $NaHCO_3$ solution was added to the growth medium at 4.2% (v/v) before use. This complete medium (CM) was used in the *in vitro* assay at pH 7.2-7.4.

In preparing the assay 100μl of infected blood sample was added to 900μl of CM. This was gently agitated to mix. Each test well of a sterile microtitre plate received 50μl of blood/ medium mixture: for media calculations, see Appendix D.

2.2.3.4 WHO Mark II 30 hour *in vitro* test

Standard test kits were obtained from the World Health Organization, Manila Philippines. Micro *in vitro* tests (WHO, 1990) [See appendix E], were performed to assess the sensitivity of *P. falciparum* to CQ, MQ, QN and SP. All procedures were carried out under aseptic conditions. Portable non-flow hood (MJ Research model number P-048-202) transported to the study sites was sterilised with 70% ethanol and by ultraviolet radiation prior to use. A stand-by petrol-powered generator (Suzuki model number SX 1000) was used when power failed.

Tissue culture plate, 12 x 8 wells, predosed with:

Chloroquine:	1-64 pmol/ well	
Mefloquine:	2-128 pmol/ well	
Quinine:	4-256 pmol/ well	
Sulfadoxine/ Pyrimethamine	Sulfadoxine:	10-10,000 pmol/ well
	Pyrimethamine:	0.125-125.0 pmol/ well

2.2.3.5 Aliquoting of test plates

The plastic sealing strips on each test plate was removed according to the number of isolates to be tested. Serial number for each isolate was written on the cover-lids of the plates, against the test row on the lid of each plate. The blood/ medium mixture was gently shaken to prevent cell deposit. Then starting with well A (control) through to H, each well was aliquoted with 50 μL of the blood:medium mixture. This procedure was repeated until the required test rows of each plate were dosed with 50μL of the mixture. The lids were replaced and each plate was gently shaken so that drug deposit was completely dissolved in the mixture.

2.2.3.6 Incubation

The candle jar holding the culture plates was incubated in an incubator (Hereus model number B5041) set to give an internal temperature of 37°C in the candle jar (Bellco Glass Inc., Vineland New Jersey) A damp paper towel was placed at the bottom of the jar below the separator mesh to ensure humidity during cultivation. Two medium-sized paraffin candles were lit and placed on each side of the stacked plates. The candle jar lid was replaced and good seal was maintained with Dow-Corning® high vacuum grease (Merck limited, England). The exhaust valve was left in an open position and closed only when the second candle was at the point of extinction. In order to test if there was no leakage, the vent was opened briefly and a hissing sound was indicative of no leakage. The jar and its contents were incubated for an average period of 30 hours.

2.2.3.7 Harvesting

After incubation, the supernatant in each test well was removed and discarded. A rubber tubing attached to a suction bulb was gently used to draw up and suspend the blood. In the absence of the latter a capillary tube was used. The red blood cell deposits were transferred to clean microscope slide for thick smear preparation. Harvesting was done starting with well A through to H. The prepared slides were allowed to air dry for at least 9 hours after which they were stained for 30 minutes in 2% Giemsa. The slides were viewed under oil immersion using a standard microscope with total magnification of X 1000.

2.2.3.8 Schizont maturation and morphologic criteria for data analysis

The tests were performed according to standard WHO procedures. Tests were considered successful if > 10% of the parasites in the control well developed into normal schizonts with ≥ 8 nuclei for the SP tests or with ≥ 3 nuclei for the CQ, MQ and QN tests. Counting was done starting from well A through to H. This was completed when trophozoite count reached 200.

The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration in which no schizonts were observed. Schizont growth at 8pmol CQ/well (1.6 μmol/L of blood), 64 pmol MQ/well (12.8 μmol/L of blood), and 256 pmol QN/well (51.2 μmol/L of blood) were taken to indicate resistance. For SP, the endpoint was 90% inhibition of normal schizonts with ≥ 8 nuclei, but a figure for resistance cut-off has not been reported, and comparative results only are given.

2.3 Results

2.3.1 *In vivo* antimalarial efficacy studies

2.3.1.1 Endemic sites

2.3.1.1.1 Mwea, 1997

Chloroquine (CQ)

In the 60 patients treated with CQ, parasitaemia range from 2,200 –69,700 parasites/μL with a geometric mean of 5,940 parasites/μL. Pre-treatment axillary body temperature varied between 37.6°C and 38.4°C (mean = 37.7°C). Fever was promptly resolved in the patients with treatment success. Note that all patients in this and other study sites, with fever ≥ 37.5°C received antipyretic paracetamol in accordance with the Kenyan National guideline. A mean age of 6.8 years was recorded for this group (see Tabel 2.3.1.1.1). Day 0 haemoglobin (Hb) level was 10.5 g/dL. There was a mean increase of 1.8 g/dL, over the trial period. This was higher in patients with initial lower Hb levels of (6-8g/dL), where an increment of 5.0 g/dL was observed, (anaemia Hb level of ≤ 5.0 g/dL were not included in the study).

Of the 60 children treated, 40 (67%) displayed adequate clinical response (ACR) and their infection was considered sensitive to CQ. Of the remaining 20 (33%), 1 case out of the 20 (5%) was febrile and had detectable parasitaemia by day 7 after initial parasite clearance. This is a case of LTF with RI type of parasitological response. The remaining 19 (95%) had detectable parasitaemia and fever by day 3, representing cases of ETF. Of these 11/ 19 (58%) cases had parasite density at 25% of pre-treatment density and 8 (42%) cases had parasite density >75% of the pre-treatment level respectively. These are ETF displaying RII and RIII parasitological response, respectively.

Sulfadoxine-pyrimethamine (SP)

In the 48 patients treated with SP, parasitaemia ranged from 3,960 parasites/μL to 60,300 parasites/μL with a geometric mean of 6,990 parasites/μL The body temperature before treatment ranged between 37.7°C and 38.3°C and a mean of 37.9°C, see Table 2.3.1.1.1. Patient age ranged between 11 months and 17

years (mean = 8.3 years). The mean Hb concentration was 9.95g/dL and had improved by day 7 with a mean increment of 1.7g/dL.

Of the 48 children, 34 (71%) displayed ACR and the infection was considered sensitive to SP. Of the remaining 14 (29%), 8/14 (57%) patients were febrile and had detectable parasitaemia by day 7 and at <25% of pre-treatment level indicating LTF with RI type of parasitological response. In the remaining 6 (43%) that were parasitaemic they had ≥ 25% of the pre-treatment level plus fever by day 3, suggesting ETF. Of these 6, 4 (67%) displayed RIII type of response with parasite density at ≥ 75% of the pre-treatment level and 2 (33%) displayed RII with parasite density at 25% of the pre-treatment level.

Patients who failed CQ or SP treatment were adequately re-treated with AQ.

Comparative analysis between CQ and SP

The incidence of clinical and parasitological failure was not significantly different between CQ and SP [20/60 (33%) with CQ versus 14/48 (29%) with SP] (OR 0.82 95% CI [0.33-2.02]; P = 0.798).

Reduction of fever in the 2 treatment regimens was similar, Table 2.3.1.1.1. The Hb level was measured on days 0, 3, 7 and 14 indicated a mean increase over the trial period in both the CQ and SP treatment modalities. No difference was observed for CQ, 1.8 g/dL and 1.7 g/dL in the SP group, (P > 0.05).

2.3.1.1.1 Mwea 1997: *In vivo* efficacy of chloroquine and SP in uncomplicated falciparum infection

Table 2.3.1.1.1: Clinical characteristics and response of uncomplicated falciparum malaria to chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) treated groups: Mwea, 1997

	CQ	SD	SP	SD
Admission parameters				
No. enrolled	76		89	
No. completing D_{14} test period	60		48	
[a] Mean age (years)	6.8	5.92^	8.3	6.71^
GMPD[a] D0	5,940 [2,200-69,700]	8,689	6,990 [3,960-60,300]	8,005
Temperature D0 (°C)[a]	37.7[37.6-38.4]		37.9[37.7-38.3]	
Mean Hb conc.(g/dL)[a]	10.5[8.9-11.6]	0.7[!]	9.95[8.7-12.1]	0.9[!]
Outcome				
Clinical response	**n [%]**		**n [%]*** **	
ETF	19[31]		6[12]	
LTF	1[2]		8[17]	
ACR	40[67]		34[71]	
Parasitological response				
S	40[67]		34[71]	
RI	1[2]		8[17]	
RII	11[18]		2[4]	
RIII	8[13]		4[8]	
*Mean fever clearance time (h)	48		48	
**Mean parasite clearance time (h)	48		48	
Mean Hb level (g/dL)	12.3	1.02[!!]	11.65	1.09[!!]
Major side effects	0		0	

Abbreviations: SP, sulfadoxine-pyrimethamine; GMPD, geometric mean parasite density
[a] Mean [range in parentheses].

*Mean fever clearance time [FCT, defined as the time from first drug administration until the axillary temperature fell to 37.2°C or below].
**Mean parasite clearance time [PCT, defined as the time from first drug administration until there was no patent parasitaemia].

***Values expressed as percentage of the total samples analysed [also done for other study sites and demonstrated in the respective tables].

- SD = standard deviation.
- For significance level [indicated as P value], 2-tailed *t*-test not assuming equal variances was used in all study sites.
- P value > 0.05 indicates no significant difference; a P value of <0.05 or < 0.001 indicates significant difference].

[!]P > 0.05; [!!]P >0.05; P < 0.001 between [!] and [!!] for CQ and SP pre- and post-treatment respectively.

^P > 0.05

As outlined above, the efficacy of antimalarial drugs, singly or in combination, was assessed in the following endemic sites: Tiwi, 1997; Busia, 1998; Tiwi, 1998, Oyugis, 1998 and Kendu Bay 1999. See Tables, (2.3.1.1.2 to 2.3.1.1.6) respectively.

2.3.1.1.2 Tiwi, 1997: *In vivo* efficacy of SP in uncomplicated falciparum malaria.

In the 38 children treated with SP, parasitaemia ranged between 5,600 and 103,200 parasites/μL with a geometric mean of 17,695 parasites/μL. The axillary body temperature before treatment was between 35.6 °C and 40°C (mean = 36.8°C). The age range was 0.5years to 21 years (mean = 5 years). The mean Hb concentration was 11.4 g/dL. By day 21, Hb concentration showed an increase of 1.4g/dL.

Of the 38 patients that completed the study, 13 (34%) displayed ACR and their infections were considered sensitive to SP. Of 25 resistant cases (66%), 23 (92%) were febrile and microscopy positive by day 7 with parasite density <25% of pre-treatment analysis indicating LTF and displaying an RI level of resistance. By day 3, 2/25 (8%) resistant infections, were febrile and parasitaemic at ≥ 25% of the pre-treatment parasite density suggesting cases of ETF. Of these 2, 1 (50%) displayed RIII type of response with parasite density at ≥ 75% of the pre-treatment level and 1/2 (50%) displayed RII with parasite density at 25% of the pre-treatment level. See Table 2.3.1.1.2.

2.3.1.1.3 Kendu Bay, 1999: *In vivo* efficacy of SP in uncomplicated falciparum malaria.

In the 120 patients treated with SP, parasitaemia ranged from 1,960 to 98,400 parasites/μL with a geometric mean of 14,600 parasites/μL. Pre-treatment axillary body temperature varied between 37.8°C and 41°C (mean = 38.2°C), see Table 2.3.1.1.3. A mean age of 7.8 years was recorded for this group. There was a mean increase in Hb of 1.6g/dL over the trial period from the initial mean Hb concentration of 12.1g/dL.

Of the 120 patients that completed the study, 97 (81%) displayed ACR and their infections were considered sensitive to SP. Of 23 resistant cases (19%), 14 (61%) were febrile and had detectable parasitaemia by day 7 with parasite density <25% of pre-treatment analysis and remaining so throughout the trial period indicating LTF and displaying an RI level of resistance. The remaining treated patients 9/23 (39%) had fever and detectable parasitaemia by day 3, $\geq$ 25% of the pre-treatment parasite density indicating ETF. Of these 9, 8 (89%) displayed RIII type of response with parasite density at $\geq$ 75% of the pre-treatment level and 1 (11%) displayed RII with parasite density at 25% of the pre-treatment level.

2.3.1.1.2 Tiwi, 1997: *In vivo* efficacy of SP in uncomplicated falciparum malaria.

Table 2.3.1.1.2: Clinical characteristics and response of uncomplicated falciparum malaria to sulfadoxine-pyrimethamine (SP): Tiwi, 1997.

Admission parameters	**SP**	**SD**
No. enrolled.	38	
No. completing D_{14} test period	38	
Mean age (yrs)[a]	5	4.75
GMPD D0[a-]	17,695[5,600-103,200]	25,573
Temperature D0 (°C)[a]	36.8 [35.6-40]	
Mean Hb level (g/dL)[a]	11.4[6.3-13.1]	1.49[I]
Outcome		
Clinical response	**n [%]*****	
ETF	2[6]	
LTF	23[60]	
ACR	13[34]	
Parasitological response		
S	13[34]	
RI	23[60]	
RII	1[3]	
RIII	1[3]	
*Mean fever clearance time (h)	48	
**Mean parasite clearance time (h)	48	
Mean Hb level (g/dL)	12.8	0.613[II]
Major side effects	0	

See Table 2.3.1.1.1 for key to abbreviations

$P < 0.05$ between [I] and [II] for SP pre- and post-treatment

2.3.1.1.3 Kendu Bay, 1999: *In vivo* efficacy of SP in uncomplicated falciparum malaria.

Table 2.3.1.1.3: Clinical characteristics and response of uncomplicated falciparum patients to sulfadoxine-pyrimethamine (SP): Kendu Bay, 1999.

Admission parameters	**SP**	**SD**
No. enrolled.	120	
No. completing D_{14} test period	120	
Mean age (yrs)	7.8	4.76
GMPD D0[a]	14,600[1,960-98,400]	23,200
Temperature D0 (°C)[a]	38.2[37.8-41]	
Mean Hb level (g/dL)[a]	12.1[6.9-13.6]	0.65[I]
Outcome		
Clinical response	**n [%]*****	
ETF	9[7]	
LTF	14[12]	
ACR	97[81]	
Parasitological response		
S	97[81]	
RI	14[12]	
RII	1[1]	
RIII	8[6]	
*Mean fever clearance time (h)	48	
**Mean parasite clearance time (h)	48	
Mean Hb level (g/dL)	13.7	1.10[II]
Major side effects	0	

See Table 2.3.1.1.1 for key to abbreviations

$P < 0.001$ between [I] and [II] for SP pre- and post-treatment

2.3.1.1.4: Busia, 1998: *In vivo* efficacy of CQSP and SP in uncomplicated falciparum infection.

In this site, *in vivo* efficacy studies involved the use of combination of SP and CQ given to one group while SP alone was given to another group. WHO *in vivo* efficacy procedure was employed, (WHO, 1996). Of the 121 patients recruited in Busia in 1998, 113 (93%) completed the study; and of these 48/113 (42%) received CQSP while 65/113 (58%) were given SP. A summary of admission parameters and therapeutic response to both regimens are shown in Table 2.3.1.1.4.

The response to treatment employed the 14 day response criteria using the modified WHO protocol (WHO, 1996).

Patients were randomized into 2 groups and were similar in age, weight, temperature, and sex at enrolment, (Table 2.3.1.1.4)

CQSP

In the 48 children treated with CQSP, parasitaemia ranged from 5,600 parasites/μL to 58,900 parasites/μL (with a geometric mean of 21,600 parasites/μL). The axillary body temperature before treatment ranged between 37.8°C and 38.2 °C (mean=38.0 °C). All patients in this group had their body temperature re-monitored at the end of the 12 hours after drug administration and were within the health centres' vicinity. The same applied to the SP treated group. Age ranged between 0.9yrs and 24 yrs with mean age of 7.2 yrs. The day 0 Hb concentration was 10.5g/dL and a mean increase was recorded at the end of the trial period to be 1.3 g/dL.

Of the 48 patients that completed the study, 34 (71%) displayed ACR and their infections were considered sensitive to CQSP. Of the 14 resistant cases (29%), 6 (43%) were febrile and parasite positive by day 7 with parasite density <25% of pre-treatment analysis indicating cases of LTF and were considered of RI level of resistance. The remaining 8/14 (57%) had fever and detectable parasitaemia by day 3 and at ≥ 25% of the pre-treatment value indicating ETF. Of these 8, 4 (50%) displayed RIII type of response with parasite density at ≥ 75% of the pre-treatment level and 4 (50%) displayed RII with parasite density at 25% of the pre-treatment level.

SP

Sixty-five patients completed the study. The Hb concentration at enrolment was 11.2g/dL but a mean increase of 1.2 g/dL was recorded over the trial period. Parasitaemia ranged from 4,960 parasites/μL to 65,800 parasites/μL with a geometric mean of 26,970 parasites/μL. The age range was 0.7yrs to 27 yrs with a mean age of 8.3 yrs. Pre-treatment axillary body temperature ranged between 37.7°C and 38.0°C (mean=37.9°C).

Of the 65 patients, 53 (81%) displayed ACR and their infections were considered sensitive to SP. Of the 12 resistant cases (19%), 5 (42%) were febrile and parasitaemic by day 7 with parasite density <25% of day 0. These were cases of LTF and with RI level of resistance. In the remaining treated patients 7/12 (58%) were febrile and had detectable parasitaemia by day 3 and at ≥ 25% of the pre-treatment value indicating ETF. Of these 7, 4 (57%) displayed RIII type of response with parasite density at ≥ 75% of the pre-treatment level and 3 (43%) displayed RII with parasite density at 25% of the pre-treatment level.

Comparative analysis of patients treated on CQSP and SP response

Considering parasitological parameters, the 2 treatment regimens used were not significantly different. The incidence of clinical and parasitological failure was not significantly different (14/48 (29%) with CQSP versus 12/65 (19%) with SP [RR 0.55; 95% CI [0.21-1.45]; P=0.266]).

The Hb level was measured on days 0, 3, 7, 14, 21 and 28 in 48 CQSP and 65 SP recruits. Initial (day 0) mean Hb concentration for CQSP was 10.5 g/dL, and SP 11.2g/dL. Mean increase over the trial period was 1.8 g/dL in the CQSP group vs 2.0 g/dL in the SP group.

Calculation of fever clearance times and conducted 12 hours after first drug administration showed that 19/34 (56%) of the CQSP-treated patients were reported as afebrile compared to 18/53 (34%) in SP treatment group (P=0.04). By 24 hours, 27/34 (79%) [CQSP] and 28/53 (53%) [SP] were afebrile (P=0.022). Mean fever clearance times are given in Table 2.3.1.1.4.

There was no patient whose condition deteriorated and thus requiring alternative treatment before day 7. There were no fatal cases nor severe malaria. The 2 regimens were well tolerated.

2.3.1.1.4 Busia, 1998: *In vivo* efficacy of CQSP and SP in uncomplicated falciparum infection.

Table 2.3.1.1.4: Clinical characteristics and response of uncomplicated falciparum patients to sulfadoxine-pyrimethamine (SP) and sulfadoxine-pyrimethamine and chloroquine (CQSP): Busia, 1998.

	CQSP	SD	SP	SD
Admission parameters				
No enrolled	52		69	
No. completing D_{28} test period	48		65	
Mean age (years)	7.2	6.79^	8.3	7.24^
Temperature (°C)[a]	38.0 (37.8-38.2)		37.9(37.7-38.0)	
No. of patients with>/ 38.0 (°C)	27 (56%)		33 (51%)	
Meean Hb level (g/dL)[a]	10.5 (8.9-11.7)	1.17[I]	11.2 (7.8-12.6)	1.19[I]
GMPD D0[a]	21,600 (5,600-58,900)	15,097	26,970 (4,960-65,800)	17,982
Outcome				
Clinical response	**n[%]*** **		**n[%]*** **	
ETF	8[16]		7[11]	
LTF	6[13]		5[8]	
ACR	34[71]		53[81]	
Parasitological response				
S	34[71]		53[81]	
RI	6[13]		5[8]	
RII	4[8]		3[5]	
RIII	4[8]		4[6]	
*Mean fever clearance time (h)	12		24	
**Mean parasite clearance time (h)	48		48	
Mean Hb level (g/dL)	11.8	0.57[II]	12.4	0.68[II]
Major side effects	0		0	

CQSP: CQ in a total dose of 25mg/kg for 3 days and sulfadoxine-pyrimethamine (sulfadoxine, 25 mg/kg) with (pyrimethamine, 1.25 mg/kg SP). See Table 2.3.1.1.1 for key to more abbreviations.

^ $P > 0.05$

[I] $P > 0.05$ [between CQSP and SP, pretreatment]; [II] $P > 0.05$ [between CQSP and SP, post treatment]

$P < 0.001$ between [I] and [II] for CQSP and SP pre- and post-treatment respectively

***In vivo* efficacy of co-trimoxazole and SP in uncomplicated falciparum infection in Tiwi and Oyugis.**

In 1998 comparative efficacy of sulfamethoxazole/trimethoprim (referred to as co-trimoxazole) and sulfadoxine/pyrimethamine (SP) was conducted in malaria endemic sites of Tiwi and Oyugis.

Patients in the 2 groups and at the 2 sites had similar parameters and (not significantly different) in age, weight, temperature, and sex ratio, at enrolment, however patients in Oyugis had a slightly higher parasite density, see Table 2.3.1.1.5-6.

2.3.1.1.5 Tiwi, 1998

In Tiwi, 246 patients were screened out of which only 142 (58%), satisfied the inclusion criteria; 56 (22.7%) had taken antimalarials, 39 (15.8%) had low parasitaemia (<1,000parasites/μL of blood), and 9 (3.7%) had very high parasitaemia (>200,000 parasites/μL). Seventy-six (53.5%) patients were eventually enrolled and treated with SP while 66 (46.5%) were treated with co-trimoxazole.

Co-trimoxazole

In the 66 children treated with co-trimoxazole, parasitaemia ranged from 3,290 to 46,780 parasites/μL (geometric mean value = 8,842 parasites/μL). The axillary body temperature ranged between 37.6°C and 38.9°C, (mean value of 38.2°C). The age range was between 2.6 yrs to 3.2,yrs, (mean age was 2.44 yrs). The day 0 Hb concentration was 9.8g/ dL (day 0 range was 7.9 to 9.9g/dL) and by day 14 there was haematological improvement with mean value of 1.8g/dL.

Of the 66 patients that completed the study, 58/66 (88%) displayed sensitive infections (were aparasitaemic and afebrile by end of study period); however 6/66 (9%) patients were aparasitaemic and febrile, therefore 64/66 (97%) patients in total displayed ACR to co-trimoxazole. Of the 8 parasitological resistant infections (12%), 4/8 (50%) were parasitaemic by day 7 with parasite density <25% of pre-treatment value RI level of resistance. Furthermore, all the other 4/8 (50%) cases were parasitaemic by day 3 at ≥ 75% of the day 0 parasite density

suggesting RIII type of response. One out of the 4 (25%) RI infection was febrile indicating a case of LTF. Similarly, 1 out of the 4 (25%) RIII infection was febrile and was considered a case of ETF.

SP

Seventy six patients completed the study. The mean Hb concentration at enrolment was 9.7g/dL [day 0 range 8.6-10.5 g/dL] but a mean increase of 2.0g/dL was recorded over the trial period. Parasitaemia ranged from 4,590 to 53,980 parasites/μL, with a geometric mean of 8,765 parasites/μL. The age range of patients in this population was between 2.6 years to 4.1 years (mean 3.0 years). Pre-treatment axillary body temperature varied between 37.6°C and 39.6°C (mean = 38.2°C). Fever was resolved within an average of 48 hours.

Of the 76 patients, 62 (82%) infections displayed sensitive infection to SP (aparasitaemic but afebrile by end of study period). However, 8 cases were tested febrile and aparasitaemic. This suggests that a total of 70/76 (92%) infections displayed ACR. Of the 14 parasitological resistant infections (18%), 6/14 (43%) were parasitaemic by day 7 with parasite density <25% of day 0. These were cases of RI level of resistance. In the remaining treated patients 8/14 (57%) had detectable parasitaemia by day 3 at ≥ 75% of the pre-treatment level displaying RIII type of response. 1 out of the 6 (17%) RI infections was febrile and was considered a case of LTF. Similarly 5/8 (63%) RIII infection were febrile and were cases of ETF.

2.3.1.1.6 Oyugis, 1998

189 patients were screened and 126 (67%) were enrolled into this study. Thirty six (19%) had taken antimalarials, 23 (12%) had low parasitaemia and 4 (2%) had high parasitaemia. Sixty-nine of the 126 (55%) patients were treated with SP while 57 (45%) received co-trimoxazole.

Co-trimoxazole

In the 57 children treated with co-trimoxazole, parasitaemia ranged from 4,560 to 61,690 parasites/μL, with a geometric mean of 17,552 parasites/μL. The axillary body temperature before treatment ranged between 37.9°C and 39.3°C,

(mean = 38.6°C). Age ranged between 2.9 yrs and 3.9 yrs (mean = 2.67 years). Day 0 Hb concentration varied between 7.9g/dL and 9.7g/dL (mean = 8.4g/dL). Haematological improvement was observed by day 14 with a mean increase of 2.4g/dL.

Of the 57 patients that completed the study, 51 (89%) displayed sensitive infections (aparasitaemic but also afebrile by end of study period) to co-trimoxazole. Four cases out of 57 (7%) were aparasitaemic and febrile indicating that a total of 55/57 cases (96%) as displaying ACR. Of the remaining, 6 parasitological resistant infections (11%), 5 (83%) were microscopy positive by day 7 with parasite density <25% of pre-treatment analysis indicating an RI level of resistance. One out of the 6 (17%) had detectable parasitaemia at ≥ 75% of the day 0 level indicating RIII. 2/5 (40%) RI infection were febrile and were considered as LTF.

SP

Sixty-nine patients completed the study. The mean Hb concentration at enrolment was 8.0g/dL [day 0 range 7.9-8.6g/dL] but a mean increase of 2.7g/dL was recorded over the trial period. Parasitaemia ranged from 6,700 to 56,800 parasites/μ/L with a mean value of 19,707 parasites/μL. The age ranged between 2.8 years and 3.4 years (mean = 2.16years. Pre-treatment axillary body temperature ranged between 37.6 and 39.6°C, (mean = 38.2°C).

Of the 69 patients, 60 (87%) displayed sensitive infection to SP. Seven out of the 69 (10%) cases were aparasitaemic and febrile therefore a total of 67/69 (97%) cases displayed ACR. Of the 9 parasitological resistant infections (13%), 4/9 (44%) were parasitaemic by day 7 with parasite density <25% of day 0 indicating RI level of resistance. By day 3 of follow-up, 1/9 (11%) had detectable parasitaemia at 25% of the pre-treatment value indicating RII infection. 4/9 (44%) displayed RIII type of response with parasite density at ≥ 75% of the pre-treatment level. 2/4 (50%) infections displaying RIII level of response were febrile amd were considered as cases of ETF.

Comparative analysis of patients treated on co-trimoxazole and SP response in Tiwi and Oyugis.

A comparative analysis of the 2 regimens in the two study sites was carried out evaluating the clinical and parasitological responses over the 14-day test period. The combined incidence of parasitological failure rate for the combined sites for co-trimoxazole was 14/123 (11%) and for SP 23/145 (16%) (RR 0.72, 95% confidence interval [CI] 0.31-1.46, P= 0.289). The 14 day clinical failure rate for the combined sites for co-trimoxazole was 4/123 (3%), and for SP 8/145 (6%), (RR = 1.69 at 95% Confidence interval [CI] (0.91-3.15). P=0.129). This comprehensive analysis would suggest that in both sites, the risk of parasitological and clinical failure to SP and co-trimoxazole are similar at P value=0.289 and P=0.129.

The haematological figures were similar in both groups, in Tiwi and Oyugis, as was the anaemia at presentation, (Table 2.3.1.1.5 and 2.3.1.1.6). Improvement on these features matched treatment success. In Tiwi, the mean increase over the test period 1.8 g/dL in the co-trimoxazole group and 2.0 g/dL in the SP group in Tiwi. Similarly, in Oyugis the mean increase was 2.4g/dL in the co-trimoxazole and 2.7g/dL in the SP group. The mean haemoglobin value after treatment was 11.6g/dL (co-trimoxazole) and 11.7g/dL (SP), in Tiwi and 10.8g/dL (co-trimoxazole) and 10.7g/dL (SP) in Oyugis.

There was no statistical difference in fever clearance time between the 2 treatment groups in Tiwi and Oyugis (Table 2.3.1.1.5 and 2.3.1.1.6). The mean fever clearance time in co-trimoxazole treated group in the 2 sites was 1.5 days while the corresponding value for SP treated group was 2 days in Tiwi; in Oyugis mean fever clearance time was 1.5 days for SP and co-trimoxazole. Overall, by 48 hours, all sensitive cases were afebrile and aparasitaemic.

2.3.1.1.5 Tiwi, 1998: *In vivo* efficacy of Co-trimoxazole and SP in uncomplicated falciparum infection

Table 2.3.1.1.5: Clinical characteristics and response of uncomplicated falciparum patients to co-trimoxazole and sulfadoxine-pyrimethamine (SP): Tiwi, 1998.

	Co-trimoxazole	SD	SP	SD
Admission parameters				
No. enrolled	66		76	
No. completing D_{14} test period	66		76	
Mean age (years)	2.44	1.69^	3.0	1.47^
GMPD D0*	8,842 [3,290-46,780]	8,413	8,765 [4,590-53,980]	8,025
Temperature D0 (°C)*	38.2 [37.6-38.9]		38.2 [37.6-39.6]	
Mean Hb level (g/dL)*	9.8[7.9-9.9]	1.8[I]	9.7[8.6-10.5]	1.56[I]
Outcome				
Clinical response	**n[%]*** **		**n[%]*** **	
ETF	1[1.5]		5[7]	
LTF	1[1.5]		1[1]	
ACR	64[97]		70[92]	
Parasitological response				
S	58[88]		62[82]	
RI	4[6]		6[8]	
RII	0		0	
RIII	4[6]		8[10]	
*Mean fever clearance time (h)	36		48	
**Mean parasite clearance time (h)	24		24	
Mean Hb level (g/dL)	11.6	1.32[II]	11.7	1.21[II]
Major side effects	0		0	

Key: See section 2.3.1.1.1 for key to abbreviations.

*P > 0.05

[I]P > 0.05 [between co-trimoxazole and SP, pretreatment]; [II] P > 0.05 [between co-trimoxazole and SP, post treatment]

P < 0.001 between [I] and [II] for co-trimoxazole and SP pre- and post-treatment respectively

2.3.1.1.6 Oyugis, 1998: *In vivo* efficacy of Co-trimoxazole and SP in uncomplicated falciparum infection

Table 2.3.1.1.6: Clinical characteristics and response of uncomplicated falciparum malaria to sulfadoxine-pyrimethamine (SP): Oyugis, 1998.

	Co-trimoxazole	SD	SP	SD
Admission parameters				
No. enrolled	57		69	
No. completing D_{14} test period	57		69	
Mean age (years)	2.67	1.46^	2.16	1.24^
GMPD D0[a]	17,552 [4,560-61,690]	15,4366	19,707 [6,700-56,800]	15,829
Temperature D0 (°C)[a]	38.6 [37.9-39.3]		38.2 [37.6-39.6]	
Mean Hb level (g/dL)[a]	8.4[7.9-9.7]	0.49[!]	8.0[7.9-8.6]	0.52[!]
Outcome				
Clinical response	**n[%]*** **		**n[%]*** **	
ETF	0[0]		2[3]	
LTF	2[4]		0[0]	
ACR	55[96]		67[97]	
Parasitological response				
S	51[89]		60[87]	
RI	5[9]		4[6]	
RII	0		1[1]	
RIII	1[2]		4[6]	
*Mean fever clearance time (h)	36		36	
**Mean parasite clearance (h)	24		24	
Mean Hb level (g/dL)	10.8	1.59[!!]	10.7	1.78[!!]
Major side effects	0		0	

Key: See section 2.3.1.1.1 for key to abbreviations.

^ $P > 0.05$

[!] $P > 0.05$ [between co-trimoxazole and SP, pretreatment]; [!!] $P > 0.05$ [between co-trimoxazole and SP, post treatment]

$P < 0.001$ between [!] and [!!] for co-trimoxazole and SP pre- and post-treatment respectively

2.3.2 *In vivo* efficacy of SP in uncomplicated falciparum malaria.

2.3.2.1 Epidemic sites

2.3.2.1.1 Mosoriot 1997:

Twenty one children completed the study. Parasitaemia ranged between 9,520 to 103,200 parasites/μL (geometric mean = 16,107 parasites /μL). The axillary body temperature before treatment ranged between 35.8°C and 40.0°C (mean = 38°C). Age ranged between 0.7 yrs to 71 years (mean = 14.5 years). The mean Hb level on enrolment was 12.3g/dL but a mean increase of 2.1g/dL was recorded over the test period. Eleven out of 21 (52%)) displayed ACR and their infections were considered sensitive to SP. Of the remaining, 10 resistant infections (48%), 8 (80%), were febrile but parasitaemic by day 7 with parasite density at < 25% of day 0 level. Infection in these patients was of LTF and was considered as cases of RI type of response. By day 3, 2/10 patients (20%) had fever and parasite density was detected at ≥ 25% of the day 0 level and were cases of ETF. Of these 2, 1 (50%) displayed RIII type of response with parasite density at ≥ 75% of the pre-treatment level and 1 (50%) displayed RII with parasite density at 25% of the pre-treatment level. See Table 2.3.2.1.1.

2.3.2.1.2 Mt. Elgon 1999:

Sixty one children were treated with SP. The day 0 mean Hb was 12.6g/dL, improving over the trial period with a mean increase of 1.6g/dL. Parasitaemia ranged from 3,400 to 32,600 parasites/μL, with a geometric mean of 16,980 parasites/μL. The axillary body temperature before treatment was initiated, was from 37.6°C to 38.4°C (mean = 38°C). Age ranged between 0.9 yrs to 54 yrs (mean age was 15.3 yrs). Infection in 53 (87%) of the 61 children displayed ACR and their infections were considered sensitive to SP. Of the remaining 8 resistant cases (13%), 3 out of the remaining 8 (37.5%) were febrile and microscopy positive by day 7 with parasite density <25% of pre-treatment analysis indicating LTF and considered as RI level of resistance. By day 3, 5/8 (62.5%) cases were febrile and had detectable

parasitaemia at ≥ 25% of the pre-treatment parasite density suggesting cases of ETF. Three of the 5 (60%) displayed RIII type of response with parasite density at ≥ 75% of the pre-treatment level and 2/5 (40%) displayed RII response with parasite density at 25% of the pre-treatment level. See Table 2.3.2.1.2.

2.3.2.1.3 Kisii 1999

In the 54 children treated with SP, parasitaemia ranged from 4,400 to 43,600 parasites/μL, with a geometric mean of 17,110 parasites/μL. The axillary body temperature before treatment was initiated was from 37.7°C to 38.4°C (mean = 38°C). Age ranged between 0.6 years and 51 years (mean age was 16.3 years). The Hb level showed mean increase over the trial at 1.8g/dL. The mean day 0 Hb concentration was 12.9g/dL.

Of the 54 patients that completed the study, 48 (89%) responded to the drug and displayed ACR and their infections were considered sensitive to SP. Of the remaining 6 resistant infections (11%), 1/6 patient (17%) had parasitaemia below 25% of pre-treatment density by day 7 and was febrile. This was therefore a case of LTF and was considered as an RI type of parasitological response. In 5 (83%) of the remaining patients, fever and detectable parasitaemia at ≥ 75% of initial density was observed by day 3 of follow up. These were noted as ETF cases and considered as resistant at RIII level of parasitological response. See Table 2.3.2.1.3.

2.3.2.1.1 Mosoriot, 1997: *In vivo* efficacy of SP in uncomplicated falciparum malaria.

Table 2.3.2.1.1: Clinical characteristics and response of uncomplicated falciparum patients to sulfadoxine-pyrimethamine (SP): Mosoriot, 1997

Admission parameters	**SP**	**SD**
No. enrolled	21	
No. completing D_{21} test period	21	
Mean age(years)	14.5	16.8
GMPD D0[a]	16,107[9,520-103,200]	26.978
Temperature D0 (°C)[a]	38[35.8-40.0]	
Mean Hb level.(g/dL)[a]	12.3[12.1-12.8]	0.35[I]
Outcome		
Clinical response	**n[%]*****	
ETF	2[10]	
LTF	8[38]	
ACR	11[52]	
Parasitological response		
S	11[52]	
RI	8[38]	
RII	1[5]	
RIII	1[5]	
*Mean fever clearance time (h)	48	
**Mean parasite clearance ttime (h)	48	
Mean Hb level (g/dL)	14.4	0.54[II]
Major side effects	0	

See Table 2.3.1.1.1 for key to abbreviations

$P < 0.001$ between [I] and [II] for SP pre- and post-treatment

2.3.2.1.2 Mt. Elgon, 1999: *In vivo* efficacy of SP in uncomplicated falciparum malaria.

Table 2.3.2.1.2: Clinical characteristics and response of uncomplicated falciparum malaria to sulfadoxine-pyrimethamine (SP): Mt. Elgon, 1999

Admission parameters	**SP**	**SD**
No. enrolled	61	
No. completing D_{14} test period	61	
Mean age(years)	15.3	10.07
GMPD D0[a]	16,980[3,400-32,600]	8,923
Temperature D0 (°C)[a]	38[37.6-38.4]	
Mean Hb level.(g/dL)[a]	12.6 [12.1-12.8]	1.4[I]
Outcome		
Clinical response	**n[%]*** **	
ETF	5[8]	
LTF	3[5]	
ACR	53[87]	
Parasitological response		
S	53[87]	
RI	3[5]	
RII	2[3]	
RIII	3[5]	
*Mean fever clearance time (h)	48	
**Mean parasite clearance time (h)	48	
Mean Hb level (g/dL)	14.2	1.02[II]
Major side effects	0	

See Table 2.3.1.1.1 for key to abbreviations.

$P < 0.001$ between [I] and [II] for SP pre- and post-treatment

2.3.2.1.3 Kisii, 1999: *In vivo* efficacy of SP in uncomplicated falciparum malaria.

Table 2.3.2.1.3: Clinical characteristics and response of uncomplicated falciparum malaria to sulfadoxine-pyrimethamine (SP): Kisii, 1999

Admission parameters	**SP**	**SD**
No enrolled	54	
No completing D_{14} test period	54	
Mean age (years)	16.3	12.75
GMPD D0[a]	17,110[4,400-43,600]	13,099
Temperature D0 (°C)[a]	38[37.7-38.4]	
Mean Hb level (g/dL)[a]	12.9 [12.4-13.6]	0.37[I]
Outcome		
Clinical response	**n[%]**	
ETF	5[9]	
LTF	1[2]	
ACR	48[89]	
Parasitological response		
S	48[89]	
RI	1[2]	
RII	0[0]	
RIII	5[9]	
*Mean fever clearance time (h)	48	
**Mean parasite clearance time (h)	48	
Mean Hb level (g/dL)	14.7	1.48[II]
Major side effects	0	

See Table 2.3.1.1.1 for key to abbreviations

$P < 0.001$ between [I] and [II] for SP pre- and post-treatment

2.3.2.1.4 Chogoria, 1997: Severe malaria in an outbreak

Twenty four patients with severe malaria were examined. Patient age ranged from 15 to 70 years (mean 26 years). These were cases presenting at Chogoria Mission Hospital. Medical records/ history show that these patients had had some form of therapy prior to presentation with severe malaria symptoms or on admission. Overall, 12 of the 24 patients were confirmed to have received CQ, SP or AQ and failed both first and second treatment regimens. All patients were however successfully treated with intravenous QN.

2.3.2.2: Haematological assessement in patients that responded or failed antimalarial drug treatment

Haemoglobin levels were measured before and after treatment (on days 0, 3, 7, 14, 21 or 28). Tables 2.3.2.2 a-c shows the range of the Hb levels. The mean Hb values as well as study sites, and drug used and number of patients failing or responding to treatment are indicated. The level of significance in both cases failing or responding to treatments is also shown.

Table 2.3.2.2 [a-c]: Range/ mean haemoglobin levels in patients responding or failing treatment in all study sites.

a.

Study site [year]	Drug used	Treatment outcome	
		Treatment success	**Treatment failures**
Tiwi [1997]	**SP** TxS: 13 TxR: 25	**Hb [g/dL]*** Pre-Tx Post-Tx [6.3-10.9] [11.9-12.9] Mean: 10.8 11.9 **P > 0.05**	**Hb[g/dL]** Pre-Tx Post-Tx [10-13.1] [12.2-13.7] Mean: 11.9 13.7 **P = 0.001**
Mwea [1997]	**SP** TxS: 30 TxR: 18	**Hb [g/dL]** Pre-Tx Post-Tx [8.7-11.6] [10.9-11.6] Mean: 9.6 11.4 **P = 0.491**	**Hb [g/dL]** Pre-Tx Post-Tx [8.9-12.1] [10.2-12.8] Mean: 9.7 11.9 **P = 0.612**
	CQ TxS: 40 TxR: 20	Pre-Tx Post-Tx [9-11.2] [11.7-13.9] Mean: 10.8 12 **P = 0.551**	Pre-Tx Post-Tx [8.9-11.5] [12.2-14.9] Mean: 10.2 12.6 **P = 0.699**
Tiwi [1998]	**SP** TxS: 62 TxR: 14	**Hb [g/dL]** Pre-Tx Post-Tx [8.6-10.5] [10.2-11.1] Mean: 10.4 11 **P = 0.994**	**Hb [g/dL]** Pre-Tx Post-Tx [8.9-9.7] [10.6-12.3] Mean: 9.2 12.2 **P < 0.001**
	Co-tri TxS: 58 TxR: 8	**Hb [g/dL]** Pre-Tx Post-Tx [7.9-9.9] [10.2-11.9] Mean: 9.9 10.3 **P = 0.681**	**Hb [g/dL]** Pre-Tx Post-Tx [8.5-9.7] [11.8-12.1] Mean: 9.5 12.6 **P = 0.012**

Key (Tables 2.3.2.2 a-c): TxS = Number of patients successfully treated; TxR = Number of patients who failed treatment; Pre-Tx = Pretreatment; Post-Tx = Post-treatment; SP = Sulfadoxine-Pyrimethamine; Co-tri = Co-trimoxazole;

* = Range of haemoglobin (Hb) concentration in g/dL; $P \leq 0.05$ level of significance.

b.

Study site [year]	Drug used	Treatment outcome	
		Treatment success	**Treatment failures**
Oyugis[1998]	**SP** TxS: 60 TxR: 9	**Hb [g/dL]** Pre-Tx Post-Tx [7.9-8.5] [10.8-11.6] Mean: 8.6 9.6 **P = 0.16**	**Hb [g/dL]** Pre-Tx Post-Tx [8.1-8.6] [10.2-12.9] Mean: 8.2 10.8 **P < 0.01**
	Co-tri TxS: 51 TxR: 6	**Hb [g/dL]** Pre-Tx Post-Tx [7.9- 9.7] [10.1-11.9] Mean: 7.8 10.2 **P = 0.291**	**Hb [g/dL]** Pre-Tx Post-Tx [8.1-9.2] [10.6-12.1] Mean: 8.2 11.2 **P < 0.01**
Busia [1998]	**SP** TxS: 53 TxR: 12	**Hb [g/dL]** Pre-Tx Post-Tx [10.8-12.6] [12.2-13.9] Mean: 12.4 12.8 **P > 0.05**	**Hb [g/dL]** Pre-Tx Post-Tx [7.8-10.2] [12.2-13.1] Mean: 10 12 **P = 0.031**
	CQSP TxS: 34 TxR: 14	**Hb [g/dL]** Pre-Tx Post-Tx [8.9-11.4] [11.2-12.9] Mean: 12 12.4 **P >0.05**	**Hb [g/dL]** Pre-Tx Post-Tx [9.7-11.7] [11.7-12] Mean: 9 11.2 **P < 0.01**

c.

Study site [year]	Drug used	Treatment outcome	
		Treatment success	**Treatment failures**
Kendu Bay [1999]	**SP** TxS: 97 TxR: 23	**Hb [g/dL]** Pre-Tx [6.9-12.6] Post-Tx [12.2-13.9] Mean: 12.2 13.7 **P = 0.19**	**Hb [g/dL]** Pre-Tx [7.8-12.2] Post-Tx [12.7-14.1] Mean: 12 13.6 **P = 0.21**
Mosoriot [1997]	**SP** TxS: 11 TxR: 10	**Hb [g/dL]** Pre-Tx [12.2-12.6] Post-Tx [13.4-14.1] Mean: 12 14 **P = 0.0031**	**Hb [g/dL]** Pre-Tx [12.1-12.8] Post-Tx [13.6-14.7] Mean: 12.5 14.7 **P = 0.0027**
Kisii [1999]	**SP** TxS: 48 TxR: 6	**Hb [g/dL]** Pre-Tx [12.4-13.1] Post-Tx [13.2-14.6] Mean: 12.3 14.5 **P < 0.01**	**Hb [g/dL]** Pre-Tx [12.5-13.6] Post-Tx [13.2-14.9] Mean: 13.5 14.9 **P = 0.210**
Mt.Elgon [1999]	**SP** TxS: 53 TxR: 8	**Hb [g/dL]** Pre-Tx [12.1-12.7] Post-Tx [12.9-14.1] Mean: 12.4 13.8 **P = 0.130**	**Hb [g/dL]** Pre-Tx [12.1-12.8] Post-Tx [13.1-14.7] Mean: 12.8 14.5 **P = 0.269**

In vitro antimalarial drug susceptibility studies in malaria regions of Kenya

2.3.3 *In vitro* antimalarial drug tests

Only 4 sites representing different endemicities (described earlier) were chosen at random for the *in vitro* study due to limited resources. The sites included Mwea, Busia, Oyugis and Tiwi 1997. From these sites a total of 220 patients were treated with SP *in vivo*. The number of isolates that were successfully tested in each site was Tiwi 1997 = 22; Mwea = 25; Oyugis = 24; Busia = 27, giving a total of 98. The remaining 122 (55%) of the isolates were not included in the analysis due to the following: 48 (39%) were contaminated; 41 (34%) did not have sufficient growth (<10% schizont growth) and finally 33 (27%) had no growth. The various effective concentrations of these drugs are in Appendix E.

2.3.3.1 Tiwi 1997

2.3.3.1.1 Chloroquine

Table 2.3.3.1 presents *in vitro* resistant or sensitive data. Isolates maturing into schizonts at 8pmol/well (1.6μmol/litre blood) or above are considered to be resistant to CQ. Results described here indicated that all the 22 isolates (100%) showed schizont maturation at CQ concentration of 64pmol/well (12.8μmol/litre blood), indicating a very high resistance *in vitro* to CQ. Of the 22 isolates, 8 displayed *in vitro* drug resistance also with SP.

2.3.3.1.2 Mefloquine

Successful schizont growth was exhibited by 11/22 (50%) isolates. The 11 were MQS with no schizont growth at 4pmol/well (0.80μmol/litre blood) concentration depicting MQ sensitivity in this locality. These 11 isolates were CQ resistant.

2.3.3.1.3 Quinine

Only 50% of the tests were successful and these tested sensitive to QN with no schizont growth observed at 32pmol/well, (6.4μmol/ litre blood) concentration. Eight of the 11 isolates that were QNS were also MQS isolates. Eight isolates that were sensitive to both QN and MQ were resistant *in vitro* to CQ.

2.3.3.1.4 Sulfadoxine-pyrimethamine SP

The antimalarial activity of SP on developing schizont in WHO *in vitro* micro-test (WHO, 1990), is less easy to determine than in the case of quinoline drugs. Schizonts with 3 nuclei or more develop even when the drug is effective; although these nuclei are often not well defined and the schizont may appear abnormally developed. The threshold for SP is 90% inhibition of schizonts with ≥ 8 nuclei, (WHO, 1990).

Twelve isolates failed to grow successfully in the control wells (<10% schizont growth in well A) and were therefore not included in the analysis. Of the 10 which grew, 2 were sensitive with no schizont growth at SDX 10pmol/PYR 0.125pmol per well (SDX 2μmol/ PYR 0.025μmol per litre blood). The remaining 8 isolates were apparently resistant having schizonts with 8 normal nuclei at SDX 3000pmol/ PYR 37.5pmol per well (SDX 600μmol/ PYR 7.5μmol per litre blood) SP concentration. Seven *in vitro*-resistant isolates displayed RI type of *in vivo* response, whereas 1/8 *in vitro* resistant isolate displayed an RII *in vivo* response. The 2 *in vitro* sensitive isolates were resistant *in vivo* and at RI level.

2.3.3.2 Mwea

2.3.3.2.1 Chloroquine

Table 2.3.3.2 presents the *in vitro* resistant or sensitive data. Isolates maturing into schizonts at 8pmol/well (1.6μmol/litre blood) are considered to be resistant to CQ. Successful schizont maturation was observed in 25/25 (100%) isolates cultured. Our results showed that all the 25 isolates (100%) showed schizont maturation at CQ concentration of 64pmol/well (12.8μmol/litre blood), indicating very high resistance *in vitro* to CQ.

2.3.3.2.2 Mefloquine

Of the 25 isolates, 9 were tested for sensitivity to MQ. The remaining 16/25 (64%) isolates did not achieve successful schizont growth. Isolates growing at 64pmol/well (12.8μmol/litre blood) are classed as resistant. No schizont growth was observed at this concentration indicating sensitivity to MQ *in vitro*. Interestingly, the 9 MQS isolates were CQR.

2.3.3.2.3 Quinine

Ten out of the 25 isolates (40%) tested showed satisfactory growth of normal schizonts and were used in the analysis. The remaining 15/25 (60%) did not achieve the 10% schizont growth in the control well and were therefore not included in this analysis. The 10 isolates were sensitive with no schizont growth at 32pmol/well (6.4μmol/litre blood) concentration.

2.3.3.2.4 Sulfadoxine-pyrimethamine SP

Sixteen isolates failed to grow successfully in the control wells (<10% schizont growth in well A and were therefore not included in the analysis. Of nine isolates, 5 were sensitive with no schizont growth at SDX 10pmol/PYR 0.125pmol per well drug level (SDX 2μmol/ PYR 0.025μmol per litre blood). The remaining 4 isolates were apparently resistant and having schizonts with 8 normal nuclei at SDX 3000pmol/ PYR 37.5pmol per well (SDX 600μmol/ PYR 7.5μmol per litre blood) SP concentration. Two of these were resistant at RI and 2 were sensitive *in vivo*. The 4 SP resistant isolates were also CQ resistant.

2.3.3.3 Busia 1998

2.3.3.3.1 Chloroquine

Successful schizont maturation was achieved in 27/27 (100%) isolates at 64pmol/well (12.8μmol/litre blood), indicating very high resistance to CQ, see table 2.3.3.3.

2.3.3.3.2 Mefloquine

Six isolates of the 27 (22%) tested for sensitivity achieved successful growth in the control well. The remaining 21 (78%) isolates did not achieve successful growth (schizont growth <10% in the control well) and were not included in the analysis. Isolates with schizonts growing at 64pmol/well (12.8μmole/litre blood) are considered MQR (WHO, 1973). All the 6 isolates did not achieve schizont growth at this concentration indicating sensitivity. Similarly, six isolates that were MQS, were resistant to CQ.

2.3.3.3.3 Quinine

Of the 27 isolates, 12 tested sensitive to QN and with no schizont growth observed at 32pmol/well (6.4μmol/litre blood). Fifteen isolates did not achieve successful growth (schizont growth < 10% in the control well) and were not included in the analysis. Of the 12 QNS isolates, 5 were MQS.

2.3.3.3.4 Sulfadoxine-pyrimethamine SP

Twelve isolates were found to be apparently resistant (3 isolates had schizonts with 8 nuclei at SDX 2000μmol/ PYR 25μmol per litre blood and 9 at SDX 600μmol/ PYR 7.5 μmol per litre blood). Only one isolate was found sensitive and no schizont growth was found in the SDX 10pmol/PYR 0.125pmol per well drug concentration. Of the 12 resistant isolates, 5 were resistant *in vivo* at RI level (*in vitro* resistant at at SDX 600μmol/ PYR 7.5μmol per litre blood), and 2 cases were of RII and resistant at SDX 2000μmol/ PYR 25μmol per litre blood. One case was RIII and having schizont growth at SDX 600μmol/ PYR 7.5μmol per litre blood. The remaining 4, were sensitive *in vivo* with 1/4 isolates having schizont growth *in vitro* at SDX 2000μmol/ PYR 25μmol per litre blood and 3/4 isolates having schizont growth at SDX 600μmol/ PYR 7.5μmol per litre blood.

2.3.3.4 Oyugis 1998: *In vitro* antimalarial drug susceptibility assay

2.3.3.4.1 Chloroquine

All the 24 isolates tested were resistant to CQ with schizont growth observed at 64pmol/well (12.8μmole/litre blood) concentration, see table 2.3.3.4. Similar observations were made in Mwea, Busia and Tiwi, areas of moderate to high malaria transmission.

2.3.3.4.2. Mefloquine

Schizont inhibition at 64 pmol/well (12.8μmol/litre blood) was found in 7 isolates out of the 24 isolates examined. The remaining 17 (71%) isolates did not achieve successful growth. Similar observations were seen in Mwea, Tiwi and Busia.

2.3.3.4.3 Quinine

Eight out of the 24 isolates (33%) grew satisfactorily. Inhibition of schizont growth at 32pmol/well (6.4μmol/ litre blood) was observed in 1/8 isolates examined. The remaining 7/8 isolates, no schizont growth was observed at 16pmol/well (3.2μmol/ litre blood), indicating sensitivity.

2.3.3.4.4 Sulfadoxine-pyrimethamine SP

Four out of the 24 isolates achieved successful growth with normal schizont growth in the control well. Of these, 3 were sensitive responding to drug concentration of SDX 10pmol/ PYR 0.125pmol/ well. The remaining one had 8 nuclei at SDX 3000pmol/ PYR 37.5pmol per well (SDX 600μmol/ PYR 7.5μmol per litre blood) [suggesting resistance]. The 3 SP sensitive isolates were CQR. The SP resistant isolate was also resistant *in vivo* and displayed an RI level of parasitological response.

2.3.3.1 Tiwi 1997: *In vitro* antimalarial drug susceptibility.

Study Number	*In vivo* Parasitological Response	Corresponding *in vitro* response to:			
		SP	CQ	MQ	QN
[redacted]	RI	**	R	**	**
KT.2	RI				
[redacted]	[redacted]	R	R	S	S
[redacted]	[redacted]	**	R	**	S
KT.5	RI				
[redacted]	[redacted]	R	R	S	S
[redacted]	[redacted]	**	R	S	S
[redacted]	[redacted]	R	R	S	S
KT.9	RI				
KT.10	RI				
[redacted]	RI	**	R	**	**
[redacted]	RI	R	R	S	**
[redacted]	RI	R	R	S	S
[redacted]	RI	R	R	S	**
[redacted]	RI	**	R	**	**
[redacted]	RI	R	R	S	**
[redacted]	RI	**	R	**	**
KT.18	RIII				
[redacted]	RI	R	R	S	S
[redacted]	RI	**	R	S	S
[redacted]	RI	**	R	S	S
KT.22	RI				
[redacted]	RI	**	R	**	S
[redacted]	RI	S	R	**	S
KT.25	S				
[redacted]			R		
KT.27	S				
[redacted]	S	**	R	**	**
[redacted]	S	**	R	**	**
KT.30	S				
[redacted]	[redacted]	S	R	**	**
KT.32	S				
KT.33	S				
KT.34	S				
KT.35	S				
KT.36	S				
KT.37	S				
KT.38	S				
Number s tested successfully		**10**	**22**	**11**	**11**

Key:

:** *In vitro* cultivation not successful

:Cleared portion = isolates not tested *in vitro*; shadowing indicate isolates tested

:MIC: minimum inhibitory concentration (MIC) was defined as the lowest drug concentration in which no schizonts were observed. Resistance thresholds for: CQ = 8pmol/well; MQ = 64pmol/well QN = 256pmol/well; >100pmol/well with normal schizonts having ≥ 8 nuclei.

:R = resistant isolates

:S = sensitive isolates [inhibition of schizont maturation at cut-off concentration]

2.3.3.2 Mwea 1997: *In vitro* antimalarial drug susceptibility.

Study Number	*In vivo* Parasitological Response	Corresponding *in vitro* response to SP	CQ	MQ	QN
KM001	S				
KM002	S				
[redacted]	S	**	R	S	S
[redacted]	S	**	R	S	S
[redacted]	RI	R	R	S	**
[redacted]	S	R	R		**
[redacted]	S	**	R	S	S
KM008	S				
KM009	S				
[redacted]	S	**	R	S	S
[redacted]	S	R	R	S	S
[redacted]	[redacted]	S	R	S	S
KM013	S				
KM014	S				
[redacted]	S	**	R	S	S
KM016	S				
[redacted]	S	**	R	S	S
KM018	S				
[redacted]	S	S	R	**	S
KM020	S				
KM021	S				
KM022	S				
[redacted]	[redacted]	**	R	**	**
[redacted]	RI	R	R	**	**
KM025	S				
[redacted]	RII	**	R	**	**
[redacted]	RIII	**	R	**	**
KM029	S				
[redacted]	[redacted]	**	R	**	**
[redacted]	S	**	**	**	**
[redacted]	RI	**	R	**	**
KM033	S				
[redacted]	RI	**	**	**	**
[redacted]	RIII	**	R	**	S
[redacted]	S	S	**	**	**
[redacted]	RIII	**	R	**	**
[redacted]	S	**	**	**	**
[redacted]	RI	**	R	**	**
KMO55	RII				
KM056	S				
[redacted]	[redacted]	**	R	**	**
[redacted]	S	S	R	**	**
[redacted]	RI	**	R	**	**
KM060	S				
KM061	S				
[redacted]	S	S	R	**	**
KM063	S				
[redacted]	RI	**	R	**	**
Numbers tested successfully		9	25	9	10

2.3.3.3 Busia 1998: *In vitro* antimalarial drug susceptibility.

Study Number	*In vivo* Parasitological Response	Corresponding *in vitro* response to SP	CQ	MQ	QN
KB562	S				
KB529	S				
KB520	S				
KB501	S				
KB578	S	**	R	**	S
KB589	S	**	R	**	S
KB594	S	**	R	S	**
KB627	S	**	R	S	S
KB629	S	**	R	**	**
KB632	S				
KB783	S				
KB748	S				
KB737	S				
KB729	RI	R	R	S	S
KB700	S	R	R	S	S
KB699	S				
KB650	RI	R	R	S	S
KB656	S				
KB661	S	R	R	**	**
KB668	S	R	R	S	S
KB672	S	R	R	**	S
KB675	S				
KB684	S				
KB686	S	**	R	**	**
KB784	S				
KB787	S				
KB795	RI	R	R	**	**
KB798	S				
KB835	S				
KB853	RI	R	R	**	**
KB936	S	S	R	**	S
KB922	S	**	R	**	**
KB905	S				
KB902	S				
KB901	S				
KB897	RI	R	R	**	S
KB963	S				
KB977	S				
KB981	RIII				
CKB1006	S				
KB1017	S				
CKB1138	RIII	R	R	**	**
CKB1069	RII	R	R	**	**
CKB1074	S				
CKB1106	RII	R	R		S
CKB1130	S				

[redacted]	S	**	R	**	**
[redacted]	S	**	R	**	**
KB024	S				
[redacted]	S	**	R	**	**
KB109	S				
KB142	S				
[redacted]	RII	**	R	**	S
[redacted]	S	**	R	**	**
KB403	S				
KB471	RIII				
AKB596	S				
[redacted]	S	**	R	**	**
AKB692	S				
AKB863	S				
AKB1347	S				
AKB857	S				
AKB1569	RIII				
[redacted]	S	**	R	**	**
AKB845	S				
Numbers tested successfully		**13**	**27**	**6**	**12**

2.3.3.4 Oyugis 1998: *In vitro* antimalarial drug susceptibility.

Study Number	*In vivo* Parasitological Response	Corresponding *in vitro* response to SP	CQ	MQ	QN
KY0061	S				
KY0120	S				
[redacted]	[redacted]	S	R	**	**
[redacted]	[redacted]	S	R	**	**
KY0134	S				
[redacted]	[redacted]	S	R	**	**
[redacted]		**	R	**	**
[redacted]	[redacted]	**	R	**	**
[redacted]		**	R	**	**
KY0151	S				
KY0247	S				
[redacted]	[redacted]	R	R	**	S
KY0167	S	**	R	S	**
KY0257	S				
KY0152	S	**	R	**	S
[redacted]	[redacted]	**	R	**	**
KY0205	S				
KY0229	S				
KY0246	RI				
[redacted]		**	R	**	**
[redacted]		**	R	S	**
KY0289	S				
KY0359	S				
[redacted]	[redacted]	**	R	S	**
[redacted]		**	R	**	**
KY1304	S				
KY0365	S				
KY0288	S				
KY0321	S				
[redacted]	[redacted]	**	R	S	S
[redacted]		**	R	**	**
KY0403	S				
[redacted]	[redacted]	**	R	**	S
KY0419	S				
KY0424	S				
[redacted]	[redacted]	**	R	S	**
KY0411	S				
KY1002	S				
KY1013	S				
[redacted]		**	R	**	S
KY1020	S				
[redacted]	[redacted]	**	R	S	S
[redacted]		**	R	**	**
KY0137	S				
KY1114	S				
[redacted]	[redacted]	**	R	**	**
KY1152	S				
KY1182	S				

KY0154	S				
KY1227	S				
KY1236	S				
KY1226	S				
KY1270	S				
KY1203	S				
KY1238	S				
[redacted]	[redacted]	**	R	S	S
KY1341	S				
KY1337	S				
KY1332	S				
KY1402	S				
[redacted]	[redacted]	**	R	**	S
KY1431	S				
KY1436	RIII				
KY1439	RIII				
KY1445	S				
KY1449	RIII				
KY1451	S				
KY1459	S				
KY1462	S				
Numbers tested successfully		**4**	**24**	**7**	**8**

When comparing *in vivo* efficacy to SP or CQ relative to *in vitro* system on samples from the 4 sites, an *in vitro* value ranged from 25% to 92% [SPR] to an *in vivo* value of 13% to 66% [SPR]. *In vitro* value of 100% [CQR] to an *in vivo* value of 33% [CQR] was observed. Taken together and on average, an *in vivo* value of 33% [CQR] and 27% [SPR] was observed whereas an *in vitro* value of 100% (CQR) and 69% [SPR], was found, see Table 2.3.3.5. Some of the factors that can account for *in vivo/in vitro* discordant results are host factors that play an important role in therapeutic failure such as pharmacokinetics and the level of acquired immunity. There may be other factors like host genetic factors unrelated to immunity and social behaviour of the host (for example, concomitant self-medication with other classical antimalarial drugs or traditional herbal medicine) that could account for the difference between *in vitro* and *in vivo* data.

Table 2.3.3.5 Sensitivities of *Plasmodium falciparum* isolates from malaria endemic areas of Kenya, to chloroquine, sulfadoxine-pyrimethamine.

Drug and site	No. of patients					
	In vivo Response by D_{14}			*In vitro* Test		
	Total	**S**	**R**	**N**	**S**	**R**
Chloroquine						
Mwea**	60	40	20	25	0	25
Tiwi	-			22	0	22
Busia	-			27	0	27
Oyugis	-			24	0	24
Total	**60**	**40**	**20(33%)**	**98**	**0**	**98 (100%)**
Sulfadoxine-Pyrimethamine						
Mwea**	48	34	14	9	5	4
Tiwi**	38	13	25	10	2	8
Busia**	65	53	12	13	1	12
Oyugis*	69	60	9	4	3	1
Total	**220**	**160**	**60(27%)**	**36**	**11**	**25(69%)**

Key: * = Under 5 years; ** = All age groups

2.4 Discussion

The introduction of macro/micro *in vitro* test in parallel with *in vivo* assessment of antimalarial drug efficacy (WHO, 1973) has tremendously increased our understanding of the determinants of treatment success. The *in vitro* method gives a result avoiding effects of immunity, or host-genetic factors that can affect drug metabolism or bioavailability or effects of mal-absorption of the antimalarial drug. In this chapter both tests were applied and the choice of sites complied with the sentinel sites identified by the Kenyan National Malaria Control Programme. These sites represent areas experiencing different levels of malaria transmission intensities including mesoendemic, hyperendemic and holoendemic areas. Sites were chosen in at random but with fair representation of endemic and epidemic regions of Kenya. Subjects were recruited from outpatients presenting uncomplicated febrile illness.

The chosen sites varied in size, population and other parameters. Sites are either urban or rural. Kendu Bay, Oyugis for instance are small in size and are rural. However, Busia is peri-urban while Mwea and Tiwi are urban. The epidemic sites, Mt.Elgon, Kisii and Chogoria are peri-urban while Mosoriot is urban.

In all the sites where SP was used, compliance presented no problem because of its single dose therapy given under supervision. Similarly, compliance for CQ single dosing over 3 days. Co-trimoxazole, on the other hand, was given twice daily over 5 days because of its short plasma half–life. Treatment requires supervision for 5 days. This limited the compliance problems that can be associated with multiple dosing over an extended period.

Antimalarial drugs either singly or in combination e.g., SP, co-trimoxazole, CQ, and in combination with SP, (CQSP) were assessed.

The first case of CQ resistant *P. falciparum* infection was described in a non-immune traveller in Kenya in 1978, (Kean, 1979). Since then resistance has spread to almost all countries in sub-Saharan Africa, particularly in East and Central Africa where parasitological failure rates for a standard dose of CQ (25 mg/kg) of 70% or more have been reported from Kenya (Brandling-Bennett *et al.*, 1988), Tanzania (Fowler *et al.*, 1993), Cameroon and Congo (Brasseur *et al.*, 1995) and Ethiopia (Tulu *et al.*, 1996). Similar observations have been reported

from the Kenyan Ministry of Health, (MOH report, 1997; Rapouda *et al.*, 1997) with *in vivo* CQ failure rates between 61% and 80% in western and coastal regions, respectively. Again in central Kenya, in an urban town of Mwea, failure rates of 30% to CQ are reported here. This reflects on heterogeneity in CQ resistance whereby parasitological resistance was found to vary between 61%, 33%, 80% in western, central and coastal Kenya, respectively. In another study carried out by Clarke and colleagues (1996) in Turkana District in northern Kenya, they found no clinical or parasitological failure in patients 1-40 years of age after 14 days of follow-up. Similarly, Guthman *et al.*, (1996) in Sudan found only 10% CQ resistance in children 6 months to 5 years although in other parts of the country high rates had already been reported in 1989 (Bayoumi *et al.*, 1989). Similar trends in geographical differences of therapeutic response is observed for SP.

Resistance to SP in the East African region is also on the increase. When SP was introduced as the second-line drug in Kenya in 1983, initial studies showed little or no resistance to SP. But later studies reported cure rates ranging from 54% to 92% (Nguyen-Dinh *et al.*, 1982; Spencer *et al.*, 1986; Hagos *et al.*, 1993; Anabwani *et al.*, 1996; Clarke *et al.*, 1996).

In the present study in Mwea, central Kenya, the efficacy of CQ was assessed in 1997 around the time a ban on the use of the drug as first line was imminent. There was 67% *in vivo* sensitivity to the drug and 33% treatment failure. Majority (31%), of the drug failure cases were of RII (18%) or RIII (13%) resistant type and were classified as early treatment failure. The 67% sensitivity recorded in Mwea would suggest that CQ could be used against primary infection only switching to a more efficacious drug in refractory cases (secondary infection). However, the 33% CQ drug failure in this area is a major concern to health providers. It is therefore, not surprising that a blanket ban on use of the drug was introduced recently (1997) and the drug, although showing a 67% efficacy, could not now be used in a place like Mwea.

Chloroquine replacement, SP, was also assessed in Mwea. Results show that 71% of cases examined responded adequately to the combination therapy. Interestingly both low level (RI) [17%] and high level RII [4%], and RIII [8%] resistance were detected among the remaining 29% who failed therapy. This is

very disturbing as this combination was recently introduced as first line therapy in place of CQ. As seen here in Mwea, the proportion of cases responding to each therapy is not significantly different, 67% CQ versus 71% SP. The encouraging observation is that failures to both therapies responded adequately to AQ treatment. The efficacy of AQ against CQ-resistant cases has been reported (Hawley *et al.*, 1996) and it is once more demonstrated here even against SP failures. This would suggest that the drug is a potential "back-up" or "stand-by" and its use alone should be limited to failed treatment, at least in Mwea or central Kenya.

It has been postulated that resistance to SP rises rapidly upon its introduction, with a useful therapeutic life estimated to be about five years (for example Nzila *et al.*, 2000). Results described here from different malaria endemicities (between 1997 and 1999) found different levels of SP failure. For example in the endemic sites of Mwea [1997] the number of cases that failed therapy was 29% to SP. In Tiwi-1997 the number of cases that failed SP therapy was 66% and by 1998, resistance to SP decreased to 18%. The source of SP was authentic Fansidar® in both studies. Perhaps the improvement can be linked to lower use of the drug in the area or maybe due to other unknown factors. However, similar observations have been made from other studies. For example, a study reported by (Ndyomugyenyi and Magnussen, 2000), showed improved efficacy to CQ in 1998 (CQ resistance in site one, 23%; site two, 37%; site three, 38%) compared to 1995 (CQ resistance in site one, 30%; site two, 58%; site three, 58%). Large differences in drug response are found geographically such as the variations in e.g., cotrimoxazole response in different parts of Uganda (Kilian *et al.*, 1998) and as observed in this study on SP sensitivity in different parts of Kenya. It is probable therefore that variations can occur in time too. It is also possible that the large reservoir of untreated (asymptomatic) infections available to infect the vector in sites of intense malaria transmission may make the survival of drug-resistant mutants difficult in the absence of regular drug pressure (Fischhoff, 1996). For example, presence of mutations in *dhfr* that result in parasite resistance to pyrimethamine might result in decreased catalytic efficiency and therefore a

competitive disadvantage (in the absence of pyrimethamine) compared with malaria parasites without the mutation, (Hastings, 1997).

In another site of western Kenya called Busia, where malaria transmission is holoendemic, resistance to SP was found to be 19%. Again in two sites in western Kenya and where malaria transmission is holoendemic, resistance to SP was found to be 13% in Oyugis and 19% in Kendu Bay. Other studies conducted in Kenya by the 1990s (van Dillen *et al* 1999), reported failure rates of 18.5% and 9.5% to AQ and SP, respectively, in a hospital in northern Kenya, and rates of 35.1% and 34.5% in another hospital in the west (van Dillen *et al.*, 1999).

Interestingly resistance to SP in the epidemic foci was similar to the situation described for western Kenya. For example resistance to SP was 11% (Kisii) and 13% (Mt.Elgon). However, resistance to SP was high in Mosoriot at 48%.

Clearly, there appears to be higher level of resistance to SP in urban than rural locations. In the endemic focus there was no statistical difference within the rural [P=0.541] and within the urban [P=0.147] locations. This allowed for pooling of SP therapeutic data in both the locations. Higher degree of *in vivo* failure to SP was reflected in urban towns of Mwea and Tiwi in contrast to the situation seen in rural areas namely Kendu Bay, Oyugis, and Busia [P= 0.05; OR = 2.35]. Similarly, evidence of high therapeutic failure to SP in the epidemic focus was noted in Mosoriot an urban town whilst high sensitivity to SP was reported in rural areas of Kisii and Mt. Elgon [P<0.001; OR = 6.62]. Again no significant difference [P= 0.966] was observed in the rural epidemic sites of Kisii and Mt. Elgon, allowing for results of sensitivity to be pooled together. Probable reasons for the higher level of SP resistance in urban areas compared to rural areas observed in these studies could be due to easy access and inadequate use of SP from informal sources in urban locations. Travel to and from urban locations is easier so transfer of resistant strains in travellers would be facilitated.

Where transmission is low, drug treatment will be widespread because of the high probability of an infection to evolve towards a clinical attack, i.e., most infections become symptomatic and this would select resistant strains that would spread rapidly because of the higher probability of self-fertilization. Together with

easy access to standard antimalarial drugs close to towns, this probably explains the situation observed in Mosoriot where SP resistance is almost 3 times higher than in the rural Mt. Elgon or Kisii areas.

An epidemic of malaria in the highland area of Chogoria (Omar *et al.*, 2001b) led to hospitalization of a group of patients. The outbreak followed heavy rainfall. During the outbreak, patients were treated with standard antimalarial drugs CQ, SP or AQ. However, upon failure of initial therapy, patients were re-treated with the next line drug of either SP [if treated with CQ] or AQ [if treated with SP and vice versa]. Twenty four patients who failed to respond to subsequent therapy and were developing signs of severe malaria were admitted and ultimately treated with intravenous QN. The Chogoria study is the first to report failure to first-line and second-line antimalarial drugs in a non-immune population in Kenya. Economic trade between the highland area of Chogoria and the mesoendemic area of Tharaka and other neighbouring endemic areas is very frequent. The possibility, therefore exists of the importation of resistant parasites in asymptomatic gametocyte carriers. The absence of acquired immunity in the local population and the failure of first-line drugs may have caused the severe form of malaria disease. Molecular characterisation of drug resistant determinants, also discussed in this thesis, revealed high degree of mutations in genes conferring resistance to SP and CQ from parasite isolates from the hospitalized patients.

Attention has recently switched to combination therapy as opposed to monotherapy for treatment of uncomplicated malaria. Combination of antimalarials that do not share the same resistance mechanisms have been postulated to reduce the chance of selection of the resistant parasite populations (White, 1999) and consequently of delaying spread of drug resistance. The rationale for combination treatment highlights important issues of clinical effectiveness, and of reducing individual drug pressure in order to prolong the useful lifespan of SP. In this chapter, different drug treatment regimens were employed and their therapeutic advantages assessed. Treatment with CQ and SP (CQSP) was compared with SP in the holo-endemic area of Busia. Similarly, a treatment regimen using co-trimoxazole was compared with SP in two sites of Oyugis and Tiwi.

In Busia, the effectiveness of SP alone versus the combination of CQSP in the treatment of uncomplicated malaria was assessed. The principle of using CQ together with SP is because, CQ, as well as its additional effect on the parasite, is known to have antipyretic activities and hence plays an important part against the pathogenesis of malaria fever. Its mode of action as an anti-inflammatory agent is not certain but it may interfere with production of tumour necrosis factor (TNF) by mononuclear cells stimulated with endotoxin (Kwiatkowski *et al.*, 1995), and modulation of cytokine production. Therefore, a treatment regimen, combining a 4-aminoquinoline with the parasiticidal activity of SP seems logical as it takes advantage of the antipyretic and anti-inflammatory effects of the 4-aminoquinoline.

A significant outcome to treatment with CQSP compared with SP was observed in fever clearance times in the initial 24 hours (P=0.022). Since all patients treated with SP were treated with paracetamol, we observed no added effect on the outcome of fever in this treatment group compared to the CQSP group as described above. And since paracetamol has been shown to have no anti-inflammatory effect (Adam and Stankov, 1994), unlike drugs such as aspirin (acetylsalicylic acid), which could therefore partly explain the above interpretation. The overall degree of anaemia (Hb) was 11.6 (CQSP) and 11.7 (SP) by end of study period. Patients with high parasitaemia had a large fall in haematocrit in the first 3 to 7 days following treatment in both groups. Similar observation has been been found in another study (ter Kuile *et al.*, 1995) and has been related to the destruction of parasitised and non-parasitised erythrocytes which is followed by haematological recovery on treatment successes.

Overall, the results of this study, in relation to previous investigation conducted in the Gambia, (Bojang *et al.*, 1998) confirm that CQSP versus SP alone, has an advantage through antipyresis. Perhaps resistance to CQ (MOH, 1997 Report) could explain the absence in the Kenyan context of the added advantage of CQ to the CQSP combination on parasitological response. However, the advantage on fever clearance times offered by CQ to CQSP combination supports the antipyretic role of CQ (Kwiatkowski *et al.*, 1995) and its importance in alleviating initial malaria disease. The evidence so far suggests that combination therapy with

CQ plus SP might achieve the aims of first-line treatment for uncomplicated malaria, in terms of faster relief of symptoms and sustained clearance of parasitaemia, better than either drug alone. However, the combination of CQ+SP may not be relevant in areas of multidrug resistance and perhaps in these areas efforts or research should consider trials based on agents that are effective locally. There is no evidence of serious side effects associated with the combination in treating uncomplicated malaria and in order to fully assess the risks for treatment, surveillance for evidence of treatment-associated toxicity must be maintained.

Efficacy of co-trimoxazole, as described here, was found to be comparable to that of SP. The studies were conducted in Tiwi (hyperendemic), situated in the coast and Oyugis (holoendemic), situated in western Kenya. The effectiveness of 6mg trimethoprim and 30mg sulphamethoxazole/kg/24hours (co-trimoxazole) over 5 days for the treatment of uncomplicated malaria showed no significant difference from that of SP with regard to treatment outcome. The combined incidence of parasitological failure rate for the two sites for co-trimoxazole was 14/123 (11%) and for SP 23/145 (16%) (RR 0.72, 95% confidence interval [CI] 0.31-1.46, P=0.289). The 14 day clinical failure rate for the combined sites for co-trimoxazole was 4/123 (3%), and for SP 8/145 (6%), [RR 1.69, 95% CI 0.91 to 3.15, P=0.129].

The results of this study represent the first report of a high level of clinical as well as parasitological effective response of co-trimoxazole to *P. falciparum* in Kenya, (Omar *et al.*, 2001c). Similarly earlier reports from *in vivo* studies carried out in Mozambique (Wolfensberger, 1970), Nigeria (Fasan, 1971) and South Africa (Hansford and Hoyland, 1982) had shown a high efficacy of various regimens of co-trimoxazle against uncomplicated malaria with parasitological cure rates above 95% after 7-14 days, although some authors observed recrudescence rates (after 28 days) of 10.7% (Wolfensberger, 1970). More recently Bloland and colleagues (1991) reported 100% parasitological cure (44 out of 44 children) after 14 day follow-up in Malawi using a 5 day regimen of co-trimoxazole and in The Gambia parasitological cure rate was found to be 96.7% and 92.3% after 6 and 21 days respectively (Daramola *et al.*, 1991). As Daramola and colleagues pointed out, the widespread use and availability of co-trimoxazole as an antibiotic may

lead to considerable drug pressure and the development of resistant parasites has to be anticipated.

The recent finding (from Tanzania) of 22% of 61 children being parasitaemic 14 days after a 5 day regimen of co-trimoxazole (Mutabingwa *et al.*, 1996), i.e., parasitological cure rate of 78%, may be interpreted as a first sign of resistance. However, clinical (Clyde *et al.*, 1971) as well as *in-vitro* studies (Petersen, 1987) suggest that such a cross-resistance may be limited.

Aggravation of clinical conditions requiring an alternative treatment is based on presence of parasitaemia (parasitological response) and or fever (clinical response). The revised WHO (1996) protocol that defines ETF as presence of parasitaemia on day 3 greater than on day 0, with fever sometimes leads to a wrong classification of patients. When left untreated, some patients with fever and positive thick film on day 3 ("treatment failure" according to the WHO classification) cleared the parasites and became afebrile on day 4 and remained so and aparasitaemic until day 14 (ACR). Such a scenario was observed in 2 cases in the epidemic area of Mt.Elgon. The 2 cases remained untreated and refused to complete the study but were followed up for assessment due to ethical considerations. These cases illustrate the limits of the *in vivo* test of resistance. However, this method is the valid measure of drug resistance that may be used to guide national antimalarial drug policy.

Analysis of Hb in patients responding or failing treatment to antimalarials examined in this study, show significant improvement in mean Hb levels of those who failed treatment in 3 endemic sites (Busia, Tiwi, Oyugis) and one epidemic site Mosoriot (Table 2.3.2.2 a to c). However, in the epidemic site of Mt. Elgon improvement was not significant in those failing or successfully treated. Similar observations were made in the meso-endemic Mwea and holo-endemic Kendu Bay, (Table 2.3.2.2 a and c). In another epidemic site, Kisii, improved Hb level was observed only in successfully treated patients, (Table 2.3.2.2.a). However, by the end of the study period there was an overall improvement in the Hb levels in all the sites irrespective of the treatment regimen.

Examination of the mean parasite density [MPD] in all patients per site shows a match between parasite clearance and haematological improvement e.g.,

in Busia, Tiwi [1997,1998], Oyugis and Mosoriot. Perhaps, the extensive reduction in MPD in the treatment failures, for example in Tiwi 1997, $MPD_{day\ 0}$ = 15,530 and $MPD_{day\ 14}$ = 2,925, could have prevented further destruction of infected red blood cells which would have resulted in low level Hb. Results presented here show that treatment of malaria does lead to haematological improvement, of malaria associated anaemia. This lends further support to observations made elsewhere that effective treatment of malaria infection could improve haemoglobin status, (Bloland *et al.*, 1993). It appears from these observations that analysis of the Hb levels was important clinically and indicates a marked improvement on treatment, but the results do not necessarily correlate with its success or failure.

In vitro assays on 4 antimalarial drugs (CQ, SP, MQ, QN) and in 4 sites (Tiwi, Mwea, Busia, Oyugis) were carried out. The present study confirmed high level of CQ resistance (MOH, 1997; Shretta *et al.*, 2000). Isolates maturing into schizonts at 8pmol/well (1.6μmol/litre blood) are considered to be resistant to CQ. All isolates showed maturation at the relevant cut-off of 8pmol/well (1.6μmol/litre blood) indicative of resistance.

Resistance to CQ in Kenya is "stabilised" with *in vivo* resistance of between 61-80% (MOH, 1997). An inherent biological advantage of CQ-resistant parasites has been suggested as one explanation for the rapid spread of CQ resistance (Payne, 1987). However, the continued use of CQ selects for resistant parasites, encouraging their survival and propagation, and this is likely to contribute to a rapid expansion of the reservoir of resistant strains.

Sensitivity to MQ was evidenced from the 4 studied sites. Mefloquine is not used as a standard regimen in Kenya. The absence of MQ drug pressure could perhaps account for the observed parasite susceptibility to MQ *in vitro*. Interestingly all CQR isolates examined were sensitive to MQ. It is unclear why CQR isolates are MQS (Cowman, *et al.*, 1994). However molecular investigations have shown the role of a mutant *pfmdr1* at codon 86 (asparagine to tyrosine) in *P. falciparum* isolates, associated with CQR and MQS in Gambian isolates (Duraisingh *et al.*, 2000). The obvious advantage to this observation would be the use of MQ against CQR infection. This offers an attractive control option for use

of MQ or a related drug, perhaps in combination, in areas where CQ resistance has stabilised.

Similarly all isolates tested were sensitive to QN. These findings are in agreement with those of Haruki, *et al.*, (1998) also conducted in Kenya. In another study, Pasvol, *et al.*, (1991) found no isolate that was resistant to QN. They reported QN sensitivity at IC_{99} and IC_{50} of 2.72 and 0.364uM, respectively. All the isolates tested were also CQR. In situations of CQ and SP failure, therefore, QN will be a suitable alternative antimalarial to amodiaquine for treating multi-drug resistant cases, at least in the Kenyan situation. Perhaps quinine's restricted use in hospitals for the management of severe and complicated falciparum malaria and treatment failure, and its short half-life, could explain absence of QN resistance over the years. Full sensitivity *in vivo* and *in vitro* to QN has previously been reported from Nigeria both in CQS and CQR isolates (Salako *et al.*, 1988). However, there are reports of QN resistance in Africa (Jelinek, 1995; Brasseur *et al.*, 1988; Brandicourt *et al.*, 1986) but it is still uncommon and the drug still remains a reliable treatment. With no manifest resistance to MQ and QN, and in the absence of widespread MQ and QN drug pressure, sensitivity to the 2 drugs *in vitro* is expected.

Some isolates tested were highly resistant to SP in the *in vitro* system, and perhaps this could act as a "warning signal" of reduced efficacy to SP. Out of a total of 36 isolates, 25 (69%) were SP resistant and 11/36 (31%) were SP sensitive. Overall SP failure rate observed clinically in this study was a mean of 27% in endemic and epidemc sites suggesting the importance of close monitoring and surveillance of SP use and hence of its efficacy in different malaria areas of Kenya. Such a study is part of an ongoing and joint effort with the National Malaria Control Programme, (NMCP). In summary the *in vitro* assessments indicate the presence of resistance to CQ (100%), of 69% resistance to SP and of very high sensitivity to MQ and QN in the 4 malaria endemic sites in Kenya between 1997 and 1998.

Both *in vitro* and *in vivo* tests of resistance have their limitations and the results described here show that the *in vitro* test of resistance is a complementary tool and may act as a "signal" of emerging resistance. The use of *in vitro* tests is

limited to research purposes to provide baseline data on drug response and to monitor cross-resistance patterns and is unlikely to replace the *in vivo* test for therapeutic efficacy.

As a consequence of this work the importance of designating priority areas for surveillance and for continual monitoring of parasite resistance to antimalarial drugs in different malaria ecologies, is emphasized. The resulting information must be utilized at peripheral levels in order to optimize case detection and improve case management to achieve appropriate and effective preventive and therapeutic measures.

CHAPTER 3

Effectiveness of "home treatment" in reducing parasite burden

3.1 Introduction

The use of formal health facilities as the first step for malaria treatment in Africa has been shown to be very low, (Dabis *et al.*, 1989; Deming *et al.*, 1989; Ruebush *et al.*, 1995), while a high proportion of malaria episodes are treated at home unmonitored. Home diagnosis and appropriate treatment using antimalarials is potentially beneficial. This is due to the fact that malaria treatment initiated promptly at home may minimize the incidence of severe episodes, (Pagnoni, *et al.*, 1997).

In Kenya, a major obstacle to the effective implementation of malaria control strategy is the fact that antimalarials are usually given inappropriately either due to non-compliance or in inadequate doses. Ultimately, this leads to the parent or guardian seeking medical assistance from the hospital or health centres as a result of persistence of sickness at a time when, perhaps, the infection has progressed to a threatening level.

One of the major symptoms that prompt carers to initiate treatment at home is the sign of fever. In a malaria endemic area, fever or locally termed "homa" is usually the initial feature used to indicate a possible malaria infection although the underlying cause of the fever may be different. The use of fever alone to diagnose malaria can result in unnecessary use of antimalarials creating drug pressure for resistance selection (Olivar *et al.*, 1991). In one such study in Western Kenya, where malaria is holoendemic, Ruebush *et al.*, (1995), self-treatment was extremely common among the residents of the rural community. They reported that, of 138 episodes of febrile illness, 60% were treated at home with herbal remedies or medicines purchased at local shops, and only 18% received treatment at a health centre of hospital. No treatment was sought by the remainder (22%). In another study in coastal Kenya, Mwenesi *et al.*, (1995), found that of 118 mothers who had diagnosed their children as having malaria, 26% had given antimalarials, 27% had given antipyretics or other medications, 23% said they had taken the child to a health centre, 6% had given a home remedy, and 18% had not given any treatment or done anything about the illness.

In highly endemic areas, two factors: self-medication and inaccurate drug histories, due to various social and cultural reasons (Nwanyanwu *et al.*, 1996),

represent serious constraints in performing and interpreting the *in vivo* tests. This could be true when some antimalarial drugs like CQ and SP have long elimination half-life; a mean of 4-8 weeks for CQ; [5 weeks (Frisk-Holmberg *et al.*, 1984)] but up to 2 months have been reported (Ette *et al.*, 1989)]. Similarly, half-life reported for SP is between 4-10 days (Dollery 1991). The available urine tests only detect antimalarials up to 14 days post drug intake, and the use of high performance liquid chromatography (HPLC) in the field is impractical. It is assumed that a negative urine test means no therapeutically effective level of antimalarial remains. The assumption is probably mistaken.

To give some indication of the home treatment on parasite burden and the reliability of using fever as an index of malaria infection, analysis of urine samples of patients reporting febrile illness or malaise was carried out. Parasite density of microscopically positive cases was determined in home pre-treated patients and those that had not received treatment prior to presentation. Parasite density was taken as an estimate of parasite burden.

3.2 Materials and Methods

3.2.1 Patients

Patient weight, age and home address were recorded on presentation at the outpatient departments of Mwea, Tiwi and Busia district hospitals. History of drug intake was taken and type or amount of drug ingested was recorded. Fever (locally known as homa) was taken to be body temperature of $\geq 37.5^{o}C$. The axilla or buccal temperature was taken and recorded for each child. Thermometers were swabbed clean with 70% ethanol between each use. History of other signs and symptoms (headache, vomiting, coughing, headache, malaise, diarrhoea) was taken. A score of three pluses (+++) was assigned to highest recorded symptom, that is > 50% of patients with such symptom. Two pluses (++) for the next common symptom and one + (plus) for the least recorded symptom.

3.2.2 Urine test

Urine (approximately 5ml), was collected into sterile universal bottles. The Saker-Solomons' urine test (Mount *et al.*, 1989), for the determination of chloroquine was adopted, while a modification of the Bratton-Marshall sulfonamide test (De Almeida and De Souza, 1983) was performed on each sample (see Appendix F, G).

3.2.2.1 Saker-Solomon test

This was performed to detect presence of CQ and desethylchloroquine (Appendix F). One ml of phosphate buffer solution, pH 8.0, was transferred to a 15ml screw cap sterile centrifuge tube and 0.2 ml of 0.05% Tetrabromophenolphthalein ethyl ester, (TBPEE)-in-chloroform reagent solution was added [see Appendix F on preparation]. This was followed by 2 ml of patient urine sample. The tube and its contents was shaken vigorously for about 15 seconds and allowed to stand for 15 minutes. After phase separation, a yellow green colouration of the chloroform layer is indicative of a negative test while a red-purple colouration is indicative of a positive test.

3.2.2.2 Bratton-Marshall BM test (Lignin test)

A modified version of this test was performed to detect previous intake of sulfonamide or sulfonamide containing compounds (Appendix G). One ml of urine sample was placed in a sterile screw cap tube. Then a drop of the prepared sodium nitrate solution was added followed by 2 drops of concentrated HCl. The tube and its contents was shaken and allowed to stand for 1 min. Then 3 drops of Bratton-Marshall solution was added, tube shaken to mix the contents and then allowed to stand for 1 min. Persistent purple colouration of the solution is indicative of a positive test while absence of purple colouration or purple to brownish colour change is indicative of a negative test.

3.2.3 Standard controls

For standards and positive controls, authentic drugs [Fansidar, (Roche®) 1.25mg/kg pyrimethamine and 25 mg/kg sulfadoxine and chloroquine base, 25mg/kg of CQ (Avloclor®, ZENECA)] were used. Varying levels of drug concentration were tested in order to ensure that broad spectrum of drug levels in the urine samples is detected.

3.2.4 Statistical analysis

Epi-Info version 6 was used in data analysis. The statistical significance and odds ratio [OR] at 95% confidence interval CI, was calculated using STATCALC from the 2 X 2 tables for cases with or without fever or parasitaemia and which tested positive or negative for antimalarials.

3.3 Results

Table 3.3.1 presents a summary of baseline information at the time samples were collected. The superscript denotes the year study was conducted. However, only 3 sites: Tiwi, Mwea, and Busia were chosen for analysis. The chosen sites differ in malaria transmission intensities and endemicity. Tiwi is hyperendemic, Mwea is mesoendemic while Busia is holoendemic. Overall, 171 samples from these sites were examined with 37, 42 and 92 from each site respectively.

The relationship between previous antimalarial drug intake and its influence or impact on parasite density or burden was assessed. Fever as an index of malaria infection was evaluated.

Table 3.3.1 Urine analysis in 5 endemic sites of Kenya.

	Mwea[97]	Tiwi[97]	Busia[98]	Tiwi[98]	Oyugis[98]	Kendu B[99]
CQ^{P} SP^{P}	8	9	6	13	12	9
CQ^{P} SP^{N}	27	19	17	34	8	18
CQ^{N} SP^{P}	9	6	69	9	16	5
$*CQ^{N}$ SP^{N}	15	14	12	11	18	21
$**CQ^{N*P/F}$ $SP^{N*P/F}$	*108*	*38*	*113*	*142*	*126*	*120*

CQ^{P} SP^{P} : CQ positive and SP positive

CQ^{P} SP^{N} : CQ positive and SP negative

CQ^{N} SP^{P} : CQ negative and SP positive

$*CQ^{N}$ SP^{N} : Control group [positive or negative for parasitaemia or fever]

$**(CQ^{N*P/F}$ $SP^{N*P/F})$: CQ negative and SP negative [positive fever and parasitaemia]

Superscript: Year of study

3.3.2 Patients

Table 3.3.2 presents the overall number (per site) testing positive or negative for either or both CQ and SP. In Busia, only 13%, of the samples examined tested negative to both drugs. The corresponding values for Tiwi and Mwea are 37.8% and 35.7% respectively. Values for Tiwi and Mwea are similar and much higher than those of holoendemic Busia. Values testing positive for CQ in both Tiwi (40%) and Mwea (43%) are higher than that for Busia (12%), but interestingly the holoendemic Busia has higher values for samples testing positive for SP, (69%) than in the mesoendemic Mwea (2%) and hyper-endemic Tiwi (11%). Samples testing positive for both drugs were found in all 3 sites, but the number is not significantly different from site to site.

In Busia samples were collected from patients aged 9/12-13 years, mean age of 8 years. In Tiwi and Mwea, samples were collected from patients aged 1 to 13 years and 6/12 to 9 years, respectively with mean ages of 5.6 years and 5.2 years respectively.

Table 3.3.2 Number of samples testing positive or negative

CQ/SP	Busia (%) n=92	Tiwi (%) n=37	Mwea (%) n=42
$CQ^N SP^{N*}$	12(13)	14(38)	15(36)
$CQ^P SP^N$	11(12)	15(40)	18(43)
$CQ^N SP^P$	63(68)	4(11)	1(2)
$CQ^P SP^P$	6(6)	4(11)	8(19)

n = number of patients testing positive or negative for CQ and or SP

3.3.3 Parasite burden

Table 3.3.3 presents mean parasite density of patients from whom urine samples were collected. Parasite burden [indicated as the mean parasite density MPD] in all three sites for CQ^NSP^N group was high with MPD ranging from 4,380 to 7,330 parasite/μL of blood. In Busia, MPD per treatment group falls within the

same range with no significant difference between the values, although the CQSP treated group had the lowest burden, (4,005 parasite/μL). In comparing the holoendemic Busia to mesoendemic Mwea, the corresponding values show a pattern with $CQ^{P}SP^{P}$ having the lowest parasite burden. Interestingly, in Mwea the CQ-negative and SP-positive group bears the highest parasite burden with a value about twice that of CQ-positive and SP-negative group and indeed higher than the untreated group. The parasite density or burden of the $CQ^{P}SP^{N}$ and $CQ^{N}SP^{P}$ treated groups in hyperendemic Tiwi is not significantly different from the untreated group. However, the SP-positive group had the highest parasite burden. Interestingly, the $CQ^{P}SP^{P}$ group had no detectable parasitaemia suggesting that this group carried no parasite burden.

The P-values [significance level] for combined sites were calculated relative to the control group. Results from the 3 sites and for the group that tested positive to CQ and SP, showed on presentation a lower proportion with parasite burden (parasitaemia) relative to the control group ($P<0.0016$ odds ratio [OR] = 0.15 [0.04 to 0.16]). No significant difference was observed in the proportion with parasite burden and testing $CQ^{N}SP^{P}$ or $CQ^{P}SP^{N}$ relative to the control group, ($P=0.9596$ OR 1.03 [0.34 to 3.02; $P=0.2839$ OR 0.58 [0.19 to 1.76]), respectively.

Table 3.3.3 Mean parasite density: indicator of parasite burden per group in study sites

Drugs	$CQ^{N}SP^{N}$	$CQ^{P}SP^{N}$	$CQ^{N}SP^{P}$	$CQ^{P}SP^{P}$
Busia [N=92]	12	11	63	6
Parasite density (parasite/μL)				
• Range	3,400-29,600	2,010-5,200	1,900-5,400	2,100-4,300
• Mean	7,330	4,964	5,057	4,005
Tiwi [N=37]	14	15	4	4
Parasite density (parasite/μL)				
• Range	3,400-13,000	1,890-5,200	2,300-6,200	0
• Mean	4,742	4,107	5,460	0
Mwea [N=42]	15	18	1	8
Parasite density (parasite/μL)				
• Range	2,040-7,600	1,600-2,980	4,880	1,300-2,100
• Mean	4,383	2,628		1,749

3.3.4 Fever

Axillary temperature was recorded in most patients supplying urine sample, even in the control group (the control group was negative for antimalarial intake as urine test revealed). In Busia the proportion of those with fever ranged from 41.7% in the untreated group to 83.3% in the CQ^PSP^P group (see Table 3.3.4). Similarly, it ranged from 0% in the CQ^NSP^P group to 50% in CQ^PSP^P in Mwea and Tiwi, respectively.

The P-values for combined sites were calculated relative to the control group. Results from the 3 sites and for the group CQ^NSP^P and CQ^PSP^P, showed lower temperature (P=0.0002 at 95% CI, OR 5.4 [2.06 to 14.48]; P=0.0086 at 95%CI 5.59 [1.45 to 22.49])), relative to the control group [CQ^N SP^N], respectively. No difference in temperature was observed for CQ^PSP^N relative to the control group, (P=0.15 at 95% CI, OR 2.24 [0.78 to 6.52]). The chi square (χ^2) trend for fever in the holoendemic site of Busia, followed by Mwea then Tiwi, is shown in Table 4.5.4, (P=0.015, 0.029, 0.539), respectively.

Table 3.3.4 Proportion of patients with fever per group

	Busia		**Mwea**		**Tiwi**	
	Num	**n(%)**	**Num**	**n(%)**	**Num**	**n(%)**
CQ^N SP^N	12	5(41)	15	1(7)	14	3(21)
CQ^P SP^N	11	3(27)	18	5(28)	15	9(60)
CQ^N SP^P	63	41(65)	1	0	4	0
CQ^P SP^P	6	5(83)	8	4(50)	4	2(50)
Total	92	54(59)	42	10(24)	37	14(38)
χ^2 Trend	p=0.015		P=0.029		P=0.539	

Fever = Axillary temperature $\geq$ 37.5°C

3.3.5 Malaria indices most frequently reported by carers

See Table 3.3.5 for symptoms considered as indicative of malaria infection by carers. Fever ranked highest at 52% followed by cough, with diarrhoea ranking lowest.

Table 3.3.5 Malaria symptoms

Symptom	Proportion N=171[%]	Grade
Fever	89[52]	+++
Coughing	46[27]	++
Headache	19[11]	+
Malaise	9[5]	+
Vomiting	5[3]	+
Diarrhoea	3[2]	+

3.4 Discussion

This study investigated the recognition of malaria by carers in the home setting. Assessment of anti-malarial drug use, in 5 study sites endemic for malaria in Kenya was determined. In addition the proportion of urine positive for drugs amongst all age groups reporting at the health centres was assessed.

From the 3 sites of Busia, Mwea and Tiwi, 44/171 (26%) of the patients were found to be positive for 4-aminoquinolines, 68/171 (40%) for sulfadoxine, and 18/171 (11%) for both of these drugs, Table 3.5.2.

The relationship between fever and the level of parasitaemia is at the core of recent attempts to find suitable case definitions of malaria attacks for use in epidemiologic studies (Smith *et al.*, 1994; Greenwood *et al.*, 1997). Both antimalarials and antipyretics may influence the relationship between fever and parasitaemia since carers treat fever by using antipyretics and / or antimalarials that may be taken before a visit to the clinic to reduce the fever, e.g. as observed in Ghana, (Ahorlu, *et al.*, 1997).

Three areas of different malaria transmission intensities (mesoendemic, hyperendemic and holoendemic), were chosen in order to study the presence of drug in urine (positive for SP and or CQ) and its association with fever and parasitaemia prior to seeking formal treatment.

Overall, evidence of use of both CQ and SP (CQ^PSP^P) was associated with lower parasitaemia on presentation, (P value of <0.0016; OR 0.15 (95%CI 0.04-0.61) compared to CQ or SP administered singly. Similarly, when SP was taken alone (CQ^NSP^P), there was a significantly lower temperature compared to the control group (this constituted the group without detectable drug in urine CQ^NSP^N), (P=0.0002; OR 5.4 [2.06-14.48]). This was followed by a group that showed both CQ and SP (CQ^PSP^P), (P=0.0086; OR 5.59 [1.45-22.49]) There was no significant temperature difference when CQ (CQ^PSP^N) was detected alone (P=0.15; OR2.24 [0.78-6.52]). This supports the idea that CQ is no longer effective in Kenya due to high *in vivo* failure rate, >70% (MOH, 1997). Significant proportion that had lower temperature and SP or CQSP in the urine, as explained above, were from Busia, (χ^2 trend for Busia, Mwea and Tiwi was P=0.0116, 0,202 and 0.551, respectively).

Mean parasite density when sulphonamide or 4-aminoquinoline was detected in urine ranged between 1,749 and 7,330 parasites/μL of blood. Certainly treatment initiated from home appears to be crucial in preventing the attainment of dangerous parasitaemic levels especially in the case of young children, in whom serious complications of malaria can arise quickly. The holoendemic Busia had the highest parasite burden. This is consistent with the endemicity as with Mwea and Tiwi. Again in Busia, no difference in parasite burden between groups whose urine showed $CQ^{P}SP^{P}$ or $CQ^{N}SP^{P}$ could be found ($P>0.05$). See also chapter 2, the *in vivo* studies for the effects of CQ and SP in observed treatment trials.

Detection of parasitaemia, when drug can be confirmed in urine may mean under-dosage or the presence of resistance. Likely problems with malaria diagnosis and administration of antimalarials at home include (1) under-dosage which may contribute to drug resistance, (2) danger of over-dosage especially in children, although this has been found to be uncommon in areas with a high rate of self medication, (Deming *et al.*, 1989).

As seen in this study, the decision to treat with antimalarials by carers was mostly based on fever [52% (89/171), compared to other symptoms], (Table 3.5.7). Results from this study indicate that highest proportion of subjects testing positive to CQ and or SP was observed in young children (<10 yrs), with a mean age of 7.26 yrs (Busia); 5.6 yrs (Tiwi); 4.76 yrs (Mwea). This is not surprising since young and non-immune population are most susceptible to malarial disease in the endemic sites.

Coughing (presumed to indicate malaria by carers) was ranked second in the list of clinical symptoms. In malaria endemic countries clinical features of pneumonia are often confused with malaria due to overlapping symptoms (O'Dempsey *et al.*, 1993). In Kenya, lower respiratory tract infections have been reported to be the second-most prevalent infection affecting mostly children, (MOH 1994). Perhaps this could explain the high rating (27%) by carers diagnosing "coughing" as indicative of malaria infection. Diarrhoea was considered the least characteristic (3%) symptom caused by malaria.

The urine tests used here are simple, sensitive and useful for field investigations on drug-use. Basically, if an individual has taken 25mg/kg CQ in

response to a febrile episode, the urine assay would test positive for approximately 10 days, with 1μg/ml detection limit (Mount *et al.*, 1989). For patients who have been on a CQ 300mg weekly prophylactic regimen, the test has been found to remain positive for 5 days after the most recent dose (Steketee *et al.*, 1988). This tool is therefore useful to monitor treatment compliance and evidence of recent drug use. It is however not clear, whether these tests could pick up local remedies used as a preventive measure. The modified Saker-Solomon test adopted here has been reported to cross-react with QN and proguanil (Mount *et al.*, 1989). However, in *in vivo* and *in vitro* CQ sensitivity trials, the objective of the assay to exclude recent antimalarial drug use would still be fulfilled. On the other hand, the modified Bratton Marshall urine test for sulfonamides, has been reported to produce false positive results with nitrazepam, clonazepam, flunitrazepam and other drugs which carry an aromatic primary amino group or a drug which is metabolized to such compounds (D'Almeida and De Souza, 1983). So these drug tests are not highly specific, but experience here suggests they are relevant.

The findings of the study have important clinical implications for a community-based care policy. Early and appropriate treatment of malaria detected in children by carers will prevent complications that arise as a result of persistence of symptoms and attainment of high parasitaemic levels leading to severe malaria or anaemia. The institution of home diagnosis and treatment as a tool for malaria control has long been advocated (Deming *et al.*, 1989; Foster, 1995; Ruebush *et al.*, 1995; Hamel *et al.*, 2001). An important difference between home and health centre-diagnosed malaria, is that, in the case of the former, treatment is initiated using home- prepared herbs, analgesics and usually inadequate doses of antimalarials purchased at local drug shops. The final recourse, if the illness does not respond to home treatment, is the formal health sector (Dabis *et al.*, 1989; Deming *et al.*, 1989; Agyepong and Anderson, 1994; Ruebush *et al.*, 1995; Ahorlu *et al.*, 1997). Such cases may finally appear in the health centre/hospital with severe malaria. Therefore if home diagnosis and treatment strategy is to have a positive impact on malaria control and child survival, a strong and effective educational campaign will have to be instituted to address the problem of inadequate and inappropriate treatment especially for children. Increasing the

carers' awareness of the symptoms and signs of simple and potentially life-threatening malaria and their appropriate management would have a much needed impact in controlling malaria morbidity and mortality.

CHAPTER 4

Molecular Characterisation of Drug-resistance determinants in Kenyan Epidemic and Endemic Malaria: I

Fansidar® resistance

4.1 Introduction

In Kenya, more than 30% of parasite isolates from malaria cases are resistant *in vivo* to CQ (MOH, 1997, Rapouda *et al.*, 1997; Shretta, *et al.*, 2000) and up to 40% of the outpatient attendance in some areas is due to suspected cases of malaria (MOH, 1997). Guidelines on the treatment of malaria cases are still being worked out. Drug pressure (total drug use) is probably directly related to the development of drug resistance, as is the size of the parasite population exposed. Early detection of treatment failures and associated genetic changes will warn of the emergence of drug resistance, and allow revision of treatment recommendations for better control of the disease. This chapter examined the genetic determinants of drug resistance to SP. Varied genotypes of *dhfr* and *dhps* have been found in different geographical areas where malaria is endemic. And in this chapter molecular characterization of SP drug resistant determinants in *P. falciparum* in endemic and epidemic regions of Kenya was investigated with the aim of assessing predictive value for PCR screening in treatment outcome.

4.2 Materials and Methods

4.2.1 Dihydrofolate reductase [*dhfr*] and dihydropteroate synthetase [*dhps*] genes

4.2.1.1 Sample collection

Pre- and post-treatment parasite samples were collected from the patients as dry blood spots on glass fibre membrane [GFM] (Titertek) supported by 55mm diameter Whatman filter paper disc (BDH) (Warhurst *et al.*, 1991). Samples were labelled and kept dry in resealable bags containing a desiccant. Bags were labeled to match samples. Bags were stored temporarily at room temperature, in the field before transportation to Malaria Unit, Centre for Biotechnology Research Institute, Kenya Medical Research Institute for genetic characterization where cold storage was maintained. Samples were later transported at ambient temperature to London School of Hygiene and Tropical Medicine (LSHTM) for further analysis and maintaining cold storage until needed.

Parasite DNA extraction procedure was as described by Warhurst *et al.*, (1991). Blood spots were initially outlined with pencil to clearly mark the spot. Each GFM was supported on a fresh 2.5cm Whatman filter paper disc and placed on a sintered glass vacuum filter (Millipore). Blood was lysed by washing with 2-3 mls sterile distilled water. Blood proteins and haemoglobin were removed by washing with 2.3mls of sterile normal saline. The prepared GFM was dried at room temperature for 1 to 2 hours and then stored in fresh labelled self-seal bags at 4°C with desiccant to maintain dryness.

4.2.1.2 Polymerase Chain Reaction

Parasites were examined for antifolate-resistance associated point mutations in the *dhfr* (chromosome 4) and *dhps* (chromosome 8) genes using a nested PCR/RFLP method described by Duraisingh *et al*, (1998), (see appendix H for primer sequence). The nested PCR approach was used to amplify regions of *dhfr* and *dhps* genes containing antifolate associated point mutations. The nest I reaction, 50μl reaction volume, contained a sector of the prepared GFM, 0.25μM of each nest 1 primer, standard PCR buffer [1.5mM $MgCl_2$, 50mM KCl, 10mM Tris HCl (pH 8.3), 0.5% DMSO], 200μM of each of the dNTPs, 1U of *Taq* polymerase (BioLine).

The reaction was allowed to proceed for 1 cycle at 94°C for 3mins, then 40 cycles at 94°C for 1min, 50°C for 2mins, 72°C for 2mins and finally at 72°C for 10mins.

The nest II reaction components, 0.25μM of each nest II primer, standard PCR buffer [1.5mM $MgCl_2$, 50mM KCl, 10mM Tris (pH 8.3), 0.1% Triton X-100 [Bioline]), 200μM of each of the dNTPs, 1U of *Taq* polymerase, and 3 μl of nest I PCR products as template. Mineral oil overlay was added to avoid evaporation and aerosol formation. Tubes were briefly microfuged and placed in thermal cycler, (Teddington, Mdx, UK (Hybaid Cycler, UK). The reaction was cycled 35 times at 94°C for 1min, 45°C for 1min, 72°C for 2 mins. An initial denaturation step of 94°C for 2 mins and a final extension step of 72°C for 10 mins was included.

4.2.1.3 Restriction Fragment Length Polymorphisms

4.2.1.2.1 Dihydrofolate reductase, *dhfr*

The method of Duraisingh was adopted (Duraisingh *et al.*, 1998), see appendix I for restriction sites and pattern of restriction digests. Restriction digestion of the 522bp M3-F/ fragment with the enzyme *NlaIII* discriminates between the "wild type" allele Ala (GCA) and the "mutated" allele Val (GTA) at codon 16. The M3-F/ product contains an additional *NlaIII* site that produced a 376bp and a 146bp fragment serving as an internal control site for digestion. Presence of Ala at *dhfr* codon 16 permits digestion of the 146bp fragment by *NlaIII* yielding two fragments of 93bp and 53bp. Standard laboratory lines of *P. falciparum* were used as controls for "wild-type" (negative control) and "mutant-type" (positive control) for each polymorphic sites on *dhfr* gene. The Thai strain K1 and the Gambian FCR3 strains were used as "wild-type" and "mutant-type" controls, respectively for *dhfr* codon 16.

Amplification containing codon 51 was digested with *Tsp5091*. The enzyme discriminates between the "wild type" allele Asn (AAT) and the "mutated" allele Ile (ATT) at codon 51. If the enzyme cuts the M3-F/ fragment, a 154bp and 64bp pattern, indicative of wild type sequence is obtained. W2 and K1 strains of *P. falciparum* were used as the positive control [mutant sequence] and negative control [wild type], respectively.

XmnI restriction enzyme was used to distinguish between mutant and wild type codon 59. The presence of a restriction site, allows digestion giving 163bp, 137bp and 26bp fragments indicative of mutant (Arg-59; CGT-59). A 189b and 137b pattern, was indicative of wild type (Cys-59; TGT-59). *P. falciparum* strains K1 and T9/96 served as mutant and wild type controls.

Similarly, for codon 108, the PCR products from M3-F and M4-F amplification primers, were used to detect polymorphisms. AluI restriction enzyme was used to distinguish between wild type (Ser-108; AGC-108); *BsrI* can distinguish mutant (Asn-108; AAC-108) and *BstNI* distinguished mutant (Thr-108; ACC-108). AluI was used on fragment M4-F while M3-F was for *BsrI* and *BstNI*. *P. falciparum* strains K1 and T9/96served as mutant and wild type controls for Ser108 and Asn108, respectively. Whereas strain FCR3 was used for Thr108 positive control.

A *DraI* mismatch engineered in the F/ primer permitted the distinction of the wild type (Ile-164; ATA-164) from the mutant (Leu-164; TTA) at codon 164 and using an M3-F/ amplified fragment. Two additional *DraI* sites are contained in the amplified fragment (control sites). V1/S and K1 strains of *P. falciparum* were used as mutant and wild type controls, respectively.

4.2.1.3.2 Dihydropteroate synthetase, *dhps*

Codon 436 is contained in the K-K/ PCR product. Presence of wild type Ser-436 (TCT-436) was identified by *MnlI* digestion of the product giving 278bp, 121bp and 39bp fragments. The mutant (Ala-436; GCT-436) was identified by a pattern of 317bp and 121bp. For mutant and wild-type controls, T9/96 and K1 digest pattern were used, respectively. Determination for the presence of the amino acid sequence of Phe residue at codon 436, was not conducted as all the isolates examined harboured Ser or Ala residue or both.

Polymorphism at codon 437 was detected using *MwoI* restriction enzyme which detected presence of wild type (Ala-437; GCT-437) while *AvaII* identified the mutant (Gly-437; GGT-437) contained in the K-K/ PCR fragment. Mutant and wild type controls were KI and FCR3, respectively.

Codon 540 wild (Lys-540; AAA-540) to mutant (Glu-540; GAA-540) change was detected by *FokI* digestion of K-K/ PCR fragment. A digest comprising 320bp, 85bp and 33bp is indicative of Glu-540 while 405bp and 33bp is indicative of Lys-540. For mutant and wild type control, TN-1 and K1 were used respectively.

For codon 581, a change from wild type (Ala-581) to mutant type (Gly-581) was identified by the digestion of the L-L/ PCR product using the *BstUI* and *BslI*, respectively. T9/96 and K1 were used as wild type and mutant controls, respectively.

Codon 613 wild type (Ala-613; GCC-613), wild type sequence contained in L-L/ PCR fragment was identified by digesting the fragment with *MwoI*. The mutant sequence Ser or Thr-613, was identified by restricting the product with *BsaWI* (Ser-613) or *AgeI* (Thr-613) restriction enzymes, respectively. K1 and W2 were used as positive and negative controls for Ser-613, respectively; V1/S and W2 were used as negative and positive controls for Thr-613, respectively.

4.2.1.4 Electrophoresis

PCR products or restriction digests were electrophoresed in 1.5%-2% gel (3:1 Ultrapure agarose (Gibco): Nusieve GTG (FlowGen, UK). A 100bp ladder marker was used for size estimation. Undigested PCR product and a negative control (without DNA material) were electrophoresed along with the digests. Electrophoresis was at 100V for 1.5-2 hours. Gels were stained in 10μg/ml ethidium bromide and visualized and photographed on an ultra-violet transilluminator (312nm) or documented using "GRABIT" gel documentation system.

4.2.1.5 Statistical Analysis

Statistical analysis of association between allele prevalence and drug failure was carried out using the STATCALC programme of Epi Info version 6, calculating chi square on 2x2 tables using Fisher's exact test (or Yate's correction, where indicated) in a two-tailed comparison. Significant association is assumed if $P \leq 0.05$ or lower.

Sensitivity SN: Percentage of drug failures correctly predicted by the positive test result: (TP/TP+FN) x 100.

Specificity SF: Percentage of successful treatments correctly predicted by the negative test (TN/TN+FP) x 100).

Positive predictive value PPV: Percentage of positive tests correctly predicting an unsuccessful treatment (TP/TP+FP) x 100).

Negative predictive value NPV: Percentage of negative tests correctly predicting a successful treatment (TN/TN+FN) x 100).

Key: TP = True Positives: FN = False Negatives: TN = True Negatives: FP = False Positives.

4.2 Results

4.2.1 *dhfr* and *dhps*

All the samples examined for gene polymorphisms from the endemic and epidemic sites possessed "wild type" *dhfr* at codons 16 (Ala-16) and 164 (Ile-164). Similarly, they carried the "wild type" codons 581 (Ala-581) and 613 (Ala-613) of the *dhps* gene. Mutations described in this thesis mean codon changes altering the amino acid coded for.

4.3.2 Prevalence of *dhps* and *dhfr* point mutations

4.3.2.1 Endemic

4.3.2.1.1 Tiwi (1997)

An overall prevalence of 33/38 (87%) mutant *dhfr* sequences with 24/38 (63%) mutant *dhps* sequences was observed. There was a higher prevalence of mutant *dhfr* sequences. Mixed alleles were detected in 9/38 (24%) for *dhfr* and 11/38 (29%) for *dhps*. Twenty three out of 38 cases showed RI resistance to SP. There was one RII and one RIII case respectively. Table 4.3.2.1.1 displays the diverse genotypes of pre-treatment samples from this endemic region. The RII (T.8) was an infection in a 4-year old child. The isolate had a quintuple mutant genotype, 3 in *dhfr* at codons 51, 59 and 108 and 2 in *dhps* at codons 437 and 540, ($dhfr^{51,59,108}dhps^{437,540}$). Similarly, the RIII case was a 6.5-year old child and the isolate displayed Ser108Asn and Cys59Arg mutations of the *dhfr* gene. The mean age of treatment failures in Tiwi was significantly different from those successfully treated (3.6 ± 3.1 yrs and 8.08 ± 6.2 yrs): P=0.036. Thus, older patients in Tiwi were more likely to clear their infections.

4.3.2.2 Epidemic

4.3.2.2.1 Mosoriot (1997)

SP failures in Mosoriot were 10/21 (48%). Eight of these cases were RI type and one each of RII and RIII. Of the 21 isolates, 2 (10%) had mutant *dhfr* genotype and 8 (38%) had mutant *dhps* genotype. Mixed genotypes were not detected in this focus. Mutant genotypes for 8-RI failures were very diverse with mutant and or wild type sequence in both the *dhfr* and *dhps* gene, see Table

4.3.2.2.1. The genotypes for RII case was *dhfr*: Ser108Asn and *dhps*: Ser436Ala, Lys540Glu. For the RIII case the *dhfr* was Ser108Asn and *dhps* Ser436Ala. The mean age of failures did not differ significantly from those that were successfully treated (13.0 ±10.3 and 15.9 ± 21.6 yrs): P=0.696. Therefore age had no detectable influence on outcome of treatment in the epidemic site.

Prevalence of mutant *dhfr* and *dhps* genotypes in endemic Tiwi and epidemic Mosoriot is shown in Table 4.3.2.

Table 4.3.2 Prevalence of mutant/ wild type genotypes of *dhfr/ dhps* genes in malaria endemic (Tiwi) and epidemic (Mosoriot) region of Kenya.

		Tiwi 1997 N = 38			Mosoriot 1997 N = 21		
		w	**m**	**mx**	**w**	**m**	**mx**
Gene	**Codons**						
d*hfr*	*51*	30	6	2	20	1	0
	59	18	20	0	18	3	0
	108	6	23	9	9	12	0
dhps							
	436	32	4	2	16	3	2
	437	25	10	3	17	3	1
	540	23	6	9	18	3	0

Key:
w: wild type
m: mutant type
mx: mixed

Table 4.3.2.1.1. Prevalence of antifolate associated point mutations in Kenyan *Plasmodium falciparum*, Tiwi 1997

Study No.	Age (yrs.)	*Dhfr*			*dhps*			
		51	59	108	436	437	540	R/S
T.1	2	N	R	N	S	G	K/E	RI
T.2	2.7	N	R	S/N	S	G	K/E	RI
T.3	4	N	R	N	S	A	E	RI
T.4	1.5	N	R	N	S	A	K	RI
T.5	0.5	I	R	N	S	G	E	RI
T.6	3	I	C	N	S	A	K/E	RI
T.7	1.5	N	C	N	S	A	E	RI
T.8	4	I	R	S/N	S	G	E	RII
T.9	3	N	C	S	S	A	K	RI
T.10	5	I	R	N	S	A/G	K/E	RI
T.11	2.8	N	R	N	S	G	E	RI
T.12	13	N	R	S/N	S	A	K/E	RI
T.13	2.5	N	R	N	S	A	K	RI
T.14	5	N	R	N	A	A	K	RI
T.15	2	N	R	N	S	A	K	RI
T.16	12	I	R	N	S	A	K	RI
T.17	5	N	R	N	A	A	K	RI
T.18	6.5	N	R	N	S	A	K	RIII
T.19	4.9	N	R	N	S	A	K	RI
T.20	0.9	N	R	N	S/A	A	K	RI
T.21	2	N	R	S/N	S	G	E	RI
T.22	1	N	R	N	S	A/G	K	RI
T.23	0.92	I	C	N	S/A	A	K	RI
T.24	0.8	N/I	C	S/N	S	A	K	RI
T.25	7	N	C	N	S	A	K	S
T.26	4	N	C	N	A	A	K	S
T.27	8	N	C	N	S	A	K	S
T.28	6	N	C	S	S	A	K	S
T.29	15	N	C	S/N	S	G	K/E	S
T.30	2.25	N	C	S	S	A	K	S
T.31	4	N/I	R	N	S	A/G	K/E	RI
T.32	2.25	N	C	S/N	S	A	K/E	S
T.33	2.67	N	C	S/N	S	A	K	S
T.34	21	N	C	S	S	G	K/E	S
T.35	2.5	N	C	N	S	G	K	S
T.36	16	N	C	S/N	S	G	K	S
T.37	3.7	N	C	S	A	A	K	S
T.38	11	N	C	S	S	A	K	S

Key: white: wild type; grey: mutant type; R [RI, RII, RIII] = resistant; S = sensitive; N108=Asn108 [mutant type]; S108=Ser108 [wild type]; C59=Cys59 [wildtype]; R59=Arg59 [mutant type];K540=Lys540 [wild type]; E540 = Glu540 [mutant type];S436=Ser436 [wildtype]; A436=Alanine[mutant type]; A437=Alanine437[wildtype]; G437=Gly437 [mutant type].

Table 4.3.2.2.1 Prevalence of antifolate associated point mutations in Kenyan *Plasmodium falciparum*, Mosoriot 1997

Study No.	Age (yrs).	*dhfr*			*Dhps*			
		51	59	108	436	437	540	R/S
M.1	34	N	C	N	A	A	E	RI
M.2	19	N	C	N	S	A	K	RI
M.3	26	N	C	N	S	G	E	RI
M.4	11	N	C	N	A	A	K	RIII
M.5	12	N	C	N	S/A	A/G	K	RI
M.6	9	I	R	N	S	A	K	RII
M.7	2.9	N	R	N	S	G	K	RI
M.8	8	N	R	N	S	G	K	RI
M.9	4	N	C	N	S/A	A	K	RI
M.10	13	N	C	N	S	A	K	S
M.11	6	N	C	N	S	A	K	S
M.12	71	N	C	N	A	A	E	S
M.13	5	N	C	S	S	A	K	S
M.14	12	N	C	S	S	A	K	S
M.15	6	N	C	S	S	A	K	S
M.16	4	N	C	S	S	A	K	S
M.17	0.7	N	C	S	S	A	K	S
M.18	3.9	N	C	S	S	A	K	R1
M.19	9	N	C	S	S	A	K	S
M.20	5	N	C	S	S	A	K	S
M.21	43	N	C	S	S	A	K	S

Key:
white: wild type; grey: mutant type; R [RI, RII, RIII] = resistant; S = sensitive; N108=Asn108 [mutant type]; S108=Ser108 [wild type]; C59=Cys59 [wildtype]; R59=Arg59 [mutant type]; K540=Lys540 [wild type]; E540 = Glu540 [mutant type];S436=Ser436 [wildtype]; A436=Alanine[mutant type]; A437=Alanine437[wildtype]; G437=Gly437 [mutant type].

4.3.2.3 Predictive value of *dhfr* and *dhps* point mutations in Tiwi and Mosoriot (1997)

Wild type *dhfr* Ser-108 was seen in only one of the treatment failures from each location. Pre-treatment samples from all successful drug treatments from Tiwi and Mosoriot carried the wild type codons Asn-51 and Cys-59 while mutant Asn-108 was seen in some cases responding to SP in both areas (in Tiwi: T.25, T.26, T.27, T.31, T.35; in Mosoriot: M10, M11, M12).

Failure was associated with presence of mutated *dhfr* in Tiwi (P=0.003) and Mosoriot (P=0.008), (Tables 4.3.2.2.2a and b) and the same relationship was seen when Asn-108 was analysed alone. In Tiwi the presence of 2 or more mutations in *dhfr* was very highly associated with SP failure (P<0.001). Here, the combination of 2 or more *dhfr* mutations and any mutation in *dhps* was also highly associated with (P=0.0006). Only 3 doubly mutated examples of *dhfr* were seen in the Mosoriot group, but those were among the 10 drug failures. Drug failure here was associated with presence of mutated *dhps* (P=0.008) but the association was not significant in Tiwi. Failure was associated with the combined presence of a mutation in *dhps* and in *dhfr* only in Mosoriot (P=0.008) and there was an association of failure here with the presence of *dhps* Gly-437 (P=0.035).

For Mosoriot, the positive predictive value of *dhps* Gly-437 for drug failure was 100% (a measure of the utility of the test: Table 4.3.3.3a), and this was also the case when *dhps* Gly-437 occurred together with *dhfr* Asn-108. Positive predictive value was retained at 88% when any *dhfr* mutations were considered with any *dhps* mutations.

In Tiwi, (Table 4.3.2.2.2.b) the lowest positive predictive value is 69% (*dhps*: Gly-437) and the highest is 100% for 2 or more *dhfr* mutations. The combination of 2 or more *dhfr* mutations and mutated *dhps* also has a 100% positive predictive value.

Table 4.3.2.2.2a: Association between alleles, alone and in combination, with SP failure in Tiwi

Association	2 x 2 Table		P value	SN %	SF %	PPV %	NPV %
	Mt	Mt -					
Dhfr mutation vs Res	25	0	0.003	100	39	76	100
Resistance Sen	8	5					
Dhfr 108N vs resistance	24	1	0.003	100	39	76	100
	8	5					
2 or more *dhfr* mutations	23	2	0.001	92	100	100	87
	0	13					
2 or more *dhfr* mutations +	16	9	0.001	64	100	100	59
mutated *dhps*	0	13					
Dhps mutation vs resistance	17	8	NS	68	46	71	43
	7	6					
Dhps 437G vs resistance	9	16	NS	36	69	69	36
	4	9					
Dhfr 108N + *dhps* 437G vs	9	16	NS	36	77	75	39
resistance	3	10					
Mutated *dhfr* and *dhps* vs	17	8	NS	68	62	77	50
resistance	5	8					
Dhfr vs. *dhps* mutation	6	2	NS				
	9	10					
Combined TIWI/MOSORIOT	12	2	0.01				
Dhfr vs. *dhps* mutation	13	19					

Key: *Sensitivity* SN: Percentage of drug failures correctly predicted by the positive test result: (TP/TP+FN) x100; *Specificity* SF: Percentage of successful treatments correctly predicted by the negative test (TN/TN+FP) x100); *Positive predictive value* PPV: Percentage of positive tests correctly predicting an unsuccessful treatment (TP/TP+FP) x100); *Negative predictive value* NPV: Percentage of negative tests correctly predicting a successful treatment (TN/TN+FN) x100); TP = True Positives: FN = False Negatives: TN = True Negatives: FP = False Positives. Mt = mutation present; Mt - = mutation not present; Res = resistant; Sen = sensitive.

2 X 2 Table showing an example of presence or absence of *dhfr* mutation in resistant and sensitive field samples .

Statistical analysis of association between allele prevalence and drug failure was carried out using the STATCALC programme of Epi Info version 6, calculating chi square on 2x2 tables using Fisher's exact test (or Yate's correction, where indicated) in a two-tailed comparison. Significant association is assumed if $P \leq 0.05$ or lower.

	Genotype mutant [+]	mutant [-]
Outcome resistant	25	0
sensitive	8	5

When applied on an EpiInfo Version 6:

```
          + Disease -
  +------------------+
+ |     25   |       0 |    25
  +----------+-------|
- |      8   |       5 |    13
  +------------------+
Exp    33             5       38
```

Analysis of Single Table ; Odds ratio = Undefined; Exact limits preferred.Relative risk = 1.63 (1.06 <RR< 2.50); Taylor Series 95% limits for RR. Fishers' exact test recommended since one table value is zero.

Chi-Squares		P-values
Uncorrected:	11.07	0.0008763
Mantel-Haenszel:	10.78	0.0010255
Yates corrected:	7.96	0.0047767
Fisher exact:	1-tailed P-value: 0.0025640	
	2-tailed P-value: 0.0025640	

Table 4.3.2.2.2.b: Association between alleles, alone and in combination, with SP failure in Mosoriot

Association	2 x 2 Table		P value	SN %	SF %	PPV %	NPV %
	Mt	Mt -					
dhfr mutation vs resistance Res	9	1	0.008	90	73	75	89
Sen	3	8					
dhfr Asn-108 vs resistance	9	1	0.008	90	73	75	89
	3	8					
2 or more *dhfr* mutations vs resistance	3	7	N.S.	30%	10 0	100	61
	0	11					
dhps mutation vs resistance	7	3	0.008	70	91	88	77
	1	10					
dhps 437G vs resistance	4	6	0.035	40	10 0	100	65
	0	11					
dhfr 108N + *dhps* 437G vs resistance	4	6	0.035	40	10 0	100	65
	0	11					
Mutated *dhfr* and *dhps* vs resistance	7	3	0.008	70	91	88	77
	1	10					
dhfr vs. *dhps* mutation	6	0	0.01				
	4	9					

Key: *Sensitivity* SN: Percentage of drug failures correctly predicted by the positive test result: (TP/TP+FN) x100; *Specificity* SF: Percentage of successful treatments correctly predicted by the negative test (TN/TN+FP) x100); *Positive predictive value* PPV: Percentage of positive tests correctly predicting an unsuccessful treatment (TP/TP+FP) x100); *Negative predictive value* NPV: Percentage of negative tests correctly predicting a successful treatment (TN/TN+FN) x100); TP = True Positives: FN = False Negatives: TN = True Negatives: FP = False Positives. Mt = mutation present; Mt - = mutation not present; Res = resistant; Sen = sensitive.

4.3.3 Pre-treatment prevalence of *dhps* and *dhfr* point mutations

4.3.3.1 Endemic

4.3.3.1.1 Busia (1998)

Out of the 12 cases that failed SP treatment, 5 were RI, 4 were RII and 3 were the RIII type of parasitological response. Of the 5 RI cases, 2 had triple *dhfr* mutations (*dhfr*^{51,59,108}), and 3 possessed quadruple mutations (*dhfr*^{51,59,108}*dhps*^{437}). Pre-treatment analysis of the 4 RII cases, 2 were triply mutated (*dhfr*^{51,59,108}), 1 had 4 mutations (*dhfr*^{51,59,108}*dhps*^{437}), and 1 possessed 5 mutations (quintuple *dhfr*^{51,59,108}*dhps*^{437,540}). All the 3 RIII infections had quintuple mutations (*dhfr*^{51,59,108}*dhps*^{437,540}).

Pre-treatment assessment on the 53 sensitive cases revealed 19 isolates with mutations; of these 6 had quintuple mutations (*dhfr*^{51,59,108}*dhps*^{437,540}), and 4 with quadruple mutations (*dhfr*^{51,59,108}*dhps*^{437}), and 9 were triple mutated, (*dhfr*^{51,59,108}). All the remaining 39 sensitive cases harboured the wild type sequence of the residues examined.

4.3.3.1.2 Tiwi (1998)

Similarly, in Tiwi, there were 14 treatment failures to SP, 6 were of RI, and 8 were of RIII type parasitological response. Of the 6 RI cases, 5 had triple mutations (*dhfr*^{51,59,108}), and 1 had quadruple mutations (*dhfr*^{51,59,108}*dhps*^{437}). Of the 8 RIII cases, 2 possessed triple mutations, (*dhfr*^{51,59,108}), 2 had quadruple mutations, (1 with *dhfr*^{51,59,108}*dhps*^{436}; 1 with *dhfr*^{51,59,108}*dhps*^{437}) and 4 had quintuple mutations (3 with ^{51,59,108}*dhps*^{437,540}; 1 with *dhfr*^{51,59,108}*dhps*^{436,540}).

Of the 62 cases responding to treatment, 6 had quintuple mutations (^{51,59,108}*dhps*^{437,540}), 8 possessed quadruple mutations (1 with *dhfr*^{51,59,108}*dhps*^{436}; 7 with *dhfr*^{51,59,108}*dhps*^{437}) and 7 had triple mutations (*dhfr*^{51,59,108}). Two isolates were mixed clones of wild and mutant types at codon 108 and 51. The remaining 39 samples were of wild type.

4.3.3.1.3 Oyugis (1998)

Of the 9 SP failures, 4 were RI, 1 was RII and 4 were RIII. Pre-treatment analysis of these samples revealed that of the RI response samples 1 was with triple mutations (*dhfr*^{51,59,108}), and 3 were quadruple mutants (*dhfr*^{51,59,108}*dhps*^{437}). The only RII case had quadruple mutations (*dhfr*^{51,59,108}*dhps*^{437}). Out of the 4 RIII type, all had quintuple mutations (*dhfr*^{51,59,108}*dhps*^{437,540}).

Pre-treatment analysis of the 60 sensitive infections showed the following mutant genotypes; 5 quintuple (*dhfr*^{51,59,108}*dhps*^{437,540}), 7 quadruple, 2 with (*dhfr*^{51,59,108}*dhps*^{436}), 5 with (*dhfr*^{51,59,108}*dhps*^{436}), 12 triple (*dhfr*^{51,59,108}). Three *P. falciparum* infections were mixed (wild and mutant types) at codons 108, 59 and 51. All the remaining 33 sensitive infections were wild type in both *dhfr* and *dhps* genes.

4.3.3.1.4 Kendu Bay (1999)

There was a total of 23 failures in Kendu Bay with 14 cases RI, 8 were RII and 1 was RIII. Pre-treatment analysis on the 14 RI type revealed, 3 had triple mutations (*dhfr*^{51,59,108}); 7 had quadruple mutations, 4 with (*dhfr*^{51,59,108}*dhps*^{436}), 3 with (*dhfr*^{51,59,108}*dhps*^{437}) and 4 had quintuple mutation (^{51,59,108}*dhps*^{437,540}). Of the 8 RII cases, 6 had quadruple mutations: 1 with (*dhfr*^{51,59,108}*dhps*^{436}); 5 with (*dhfr*^{51,59,108}*dhps*^{437}), 2 had quintuple mutants (^{51,59,108}*dhps*^{437,540}). The only RIII case had quintuple mutations.

Out of the 97 sensitive cases, the following had polymorphisms on *dhfr* and *dhps*, 9 quintuple (*dhfr*^{51,59,108}*dhps*^{437,540}), 4 quadruple (*dhfr*^{51,59,108}*dhps*^{437}) and 19 triple (*dhfr*^{51,59,108}). Four isolates were mixed infections at codon-108 with wild (Ser-108) and (Asn-108). The remaining 61 sensitive isolates were wild type.

4.3.3.2 Epidemic

4.3.3.2.1 Kisii (1999)

Of the 54 SP-treated patients, 6 failed to respond. Out of these 6 SP-resistant cases, 1 was RI and 5 were RIII. Pre-treatment analysis revealed that the 1 RI case had triple mutation *dhfr*^{51,59,108}. Of the 5 RIII cases, 1 had double mutations: *dhfr*^{59,108} and 4 had triple mutations: *dhfr*^{51,59,108}.

Pre-treatment analysis of the 48 sensitive infections showed that 8 had mutations on the *dhfr* and *dhps* genes, 2- triple $dhfr^{51,59,108}$, 1-double $dhfr^{51,108}$ and 5 single $dhfr^{108}$. Forty isolates were wild type.

4.3.3.2.2. Mt.Elgon (1999)

Eight cases out of 61 failed treatment to SP, and of these, 3 were of RI, 2 were of RII type and 3 were of RIII type of response. Pre-treatment analysis revealed that of the 3 RI type, 2 had triple mutations ($dhfr^{51,59,108}$) and 1 had double mutations ($dhfr^{59,108}$). Of the 2 RII, 1 had triple mutations ($dhfr^{51,59,108}$) and 1 had double mutations ($dhfr^{51,108}$). Of the 3 RIII cases, all had triple mutations ($dhfr^{51,59,108}$).

Pre-treatment analysis of the 53 sensitive infections showed 2 with double mutations ($dhfr^{59,108}$) and 2 with single 108 *dhfr* mutations. Two isolates were mixed (wild and mutant) infections at codons 108 and 51. The remaining 47 were wild type in both *dhfr* and *dhps* genes.

4.3.3.3 Association between *dhfr* and *dhps* genotypes and outcome

Taking the results from the endemic sites together, (Busia, Tiwi, Oyugis and Kendu Bay) there was a correlation between treatment failures and presence of four point mutations- 3 *dhfr* ($dhfr^{51,59,108}$) and 1 *dhps* ($dhps^{437}$). The association between the quadruple mutations and treatment failure is significant ($P < 0.001$ and $\chi^2 = 55.01$). There was also an association with quintuple mutations *dhfr* ($dhfr^{51,59,108}$ and $dhps^{437,540}$) and treatment failure. The association of the quintuple mutations and SP treatment failure was also significant ($P < 0.001$; $\chi^2 = 14.76$) (see Table 4.3.3.3e).

Similarly, analysis for association between treatment failure and *dhfr* and *dhps* gene polymorphisms in the epidemic foci of Mt.Elgon and Kisii was carried out and the association was significant when there were triple or double mutations in *dhfr* ($dhfr^{59,108}$ or $dhfr^{51,108}$), ($P < 0.001$; χ^2 50.85 [triple]; $P = 0.0075$;χ^2 8.02 [double]) (see Table 4.3.3.3e).

Sensitivity, specificity and predictive values are also shown in table 4.3.3.3e.

Table 4.3.3.3a: Sulfadoxine/ pyrimethamine sensitivity profile of epidemic and endemic malaria in Kenya.

Endemicity	Site	N	Sensitive	Resistant			
				RI	RII	RIII	Total
Endemic							
	Busia	65	53	5	4	3	12
	Tiwi	76	62	6	0	8	14
	Oyugis	69	60	4	1	4	9
	Kendu Bay	120	97	14	8	1	23
		330	272 (82%)	29	13	16	**58** (18%)
Epidemic							
	Kisii	54	48	1	0	5	6
	Mt. Elgon	61	53	3	2	3	8
		115	101 (88%)	4	2	8	**14** (12%)

Key: N = number of patients; R = Parasitological failure (grade RI,RII, RIII).

Table 4.3.3.3b: Prevalence of pre-treatment *dhfr* and *dhps* polymorphisms in SP-resistant cases of epidemic and endemic malaria.

Site	N	Quintuple *dhfr* 51, 59, 108 *dhps* 436(or 437), 540	Quadruple *dhfr* 51, 59, 108 *dhps* 436(or 437)	Triple* *dhfr* 51, 59, 108	R_N
Endemic					
Busia	12	4	4	4	12
Tiwi	14	4	3	7	14
Oyugis	9	4	4	1	9
Kendu Bay	23	7	13	3	23
Total	**58**	**19**	**24**	**15**	**58**

Epidemic		Triple *dhfr* 51, 59, 108	Double *dhfr* 59 (or 51),108	Single *dhfr* 108	
Kisii	6	5	1	0	6
Mt. Elgon	8	6	2	0	8
Total	**14**	**11**	**3**	**0**	**14**

Key: * = Infections with Triple *dhfr* genotypes were the same infections with double ($dhfr^{59,108}$) genotypes
N = Total resistant cases per site. R_N Total resistant cases with multiple mutations

Table 4.3.3.3c: **Prevalence of pre-treatment *dhfr* and *dhps* polymorphisms in SP-sensitive cases of epidemic and endemic malaria**

Site	S	Quintuple *dhfr* 51, 59, 108 *dhps* 436(or 437), 540	Quadruple *dhfr* 51, 59, 108 *dhps* 436 (or 437)	Triple* *dhfr* 51, 59, 108	N_S
Endemic					
Busia	53	6	4	9	19
Tiwi	62	6	8	7	21
Oyugis	60	5	7	12	24
Kendu Bay	97	9	4	19	32
Total (35%)	**272**	**26**	**23**	**47**	**96**
Epidemic					
		Triple *dhfr* 51, 59, 108	Double *dhfr* 59(or 51),108	Single *dhfr* 108	
Kisii	48	2	1	5	8
Mt. Elgon	53	3	2	1	6
Total (14%)	**101**	**5**	**3**	**6**	**14**

Key: S = Total sensitive cases per site. N_S Total sensitive cases with mutations in *dhfr* and d*hps* genes.

Table 4.3.3.3d: Proportion of pre-treatment *dhfr* and *dhps* gene mutations in SP-resistant cases of epidemic and endemic malaria

Endemic

	Quintuple	Quadruple	Triple
RI (29)	4	14	11
RII (13)	3	8	2
RIII (16)	12	2	2
	19	**24**	**15**

Epidemic

	Triple	Double	Single
RI (4)	3	1	0
RII (2)	1	1	0
RIII (8)	7	1	0
	11	**3**	**0**

Key:

Single = *dhfr* ^{108}

Double = *dhfr* $^{59(\text{or } 51),108}$

Triple = *dhfr* $^{51, 59, 108}$

Quadruple = *dhfr* $^{51, 59, 108}$ *dhps* $^{436 \text{ (or } 437)}$

Quintuple = *dhfr* $^{51, 59, 108}$ *dhps* $^{436(\text{or } 437), 540}$

Table 4.3.3.3e: Indices of pre-treatment association of *dhfr* and *dhps* gene polymorphism with resistance in endemic and epidemic malaria in Kenya

	Genotype	P	χ^2 (Yates)	SN	SF	PPV	NPV
Endemic							
	Quintuple	<0.001	19.92	33	90	42	86
	Quadruple	<0.001	39.77	41	92	51	88
	Triple	0.182	1.78	26	83	24	84
Epidemic							
	Triple	<0.001	49.66	79	95	69	97
	Double	0.023	5.15	21	97	50	89

Key:

SN = sensitivity

SF = specificity

PPV = positive predictive value

NPV = negative predictive value

4.3.4 Prevalence of *dhfr* and *dhps* mutations in severe malaria cases, Chogoria-Kenya

As explained earlier, association between genotypes conferring resistance to SP and treatment outcome could not be examined here since all the hospitalised patients progressed to severe malarial disease and were ultimately treated with QN. This section therefore, examined only the prevalence of genotypes of *dhfr* and *dhps* on admission in severe malaria patients. All isolates carried the wild type codons 16, 164 (*dhfr*) and 436, 581, 613 (*dhps*). Only one isolate (C19) [see Table 4.3.4] had the wild-type codons 51, 59, and 108 (*dhfr*) and 437 and 540 (*dhps*); the remaining 23 isolates had the mutant codons 51 and 108 of *dhfr* gene and codons 437 and 540 of *dhps* gene; one isolate (C24) had mixed genotype (mutant and wild type) of codon 108. Isolate C10 carried the wild type codon 59 of *dhfr* gene and wild type codons 437 and 540 of the *dhps* gene.

Table 4.3.4 Prevalence of *dhfr* and *dhps* polymorphism in severe malaria patients, Chogoria (1997).

S/N	*dhfr* gene			*Dhps* gene		Therapy
	51	59	108	437	540	
C1	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C2	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C3	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C4	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C5	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C6	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	CQ>PSD
C7	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	AQ>PSD
C8	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	PSD>AQ
C9	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C10	\\\\\\\\		\\\\\\\\			CQ>PSD
C11	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	PSD>AQ
C12	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C13	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C14	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	CQ>PSD
C15	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C16	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	AQ>PSD
C17	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	CQ>AQ
C18	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	PSD>AQ
C19						PSD>AQ
C20	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	PSD>AQ
C21	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	CQ>PSD
C22	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\	\\\\\\\\	?
C23	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	\\\\\\\\	?
C24	\\\\\\\\	\\\\\\\\	▬▬▬	\\\\\\\\	\\\\\\\\	?

Key: ND = NOT DETERMINED; CQ = CHLOROQUINE; AQ = AMODIAQUINE; PSD = FANSIDAR®; ? = NO RECORD OF DRUG TREATMENT;

\\\\ = MUTANT; BLANK = WILD TYPE; ▬ = MIXED WILD TYPE AND MUTANT GENOTYPES

> = a change from first to second treatment regimen.

Figure 4a-b *dhfr* gene polymorphisms

Figure 4a *dhfr* ($^{Ser}108^{Asn}$)

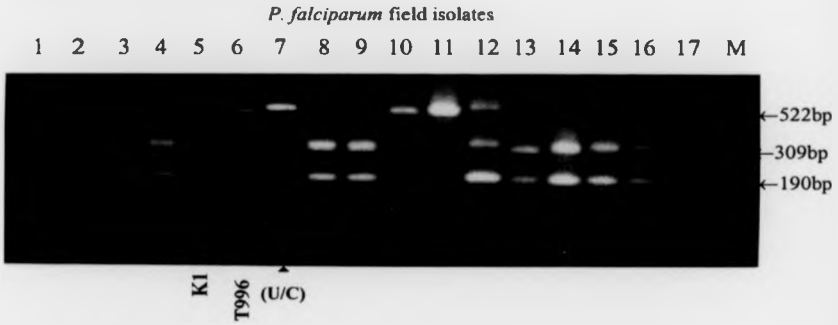
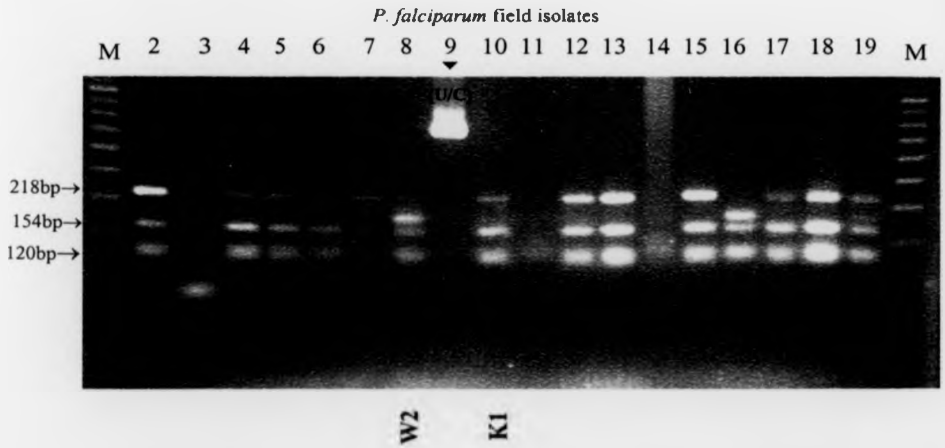


Figure 4b *dhfr* ($^{Asn}51^{Ile}$)



A representative number of *P. falciparum* field isolates is shown in Figures 4a-b (is shown). The labels signified as M = 100 bp ladder marker; ▲, ▼, = indicates uncut (UC) PCR product; → approximates the band size (in base pair). Laboratory clones used as controls are shown for each figure. A band profile similar to the control K1 isolate indicates presence of mutant Asn–108 whereas a band profile similar to T996 control isolate indicates wild type Ser-108, see Figure 4a. Also as shown in Figure 4b, a band profile similar to W2 control isolate indicates presence of mutant Ile-51 whereas a band profile similar to K1 isolate is indicative of wild type Asn-51.

Figure 4c-d *dhps* gene polymorhpisms

Figure 4c *dhps* ($^{Lys}540^{Glu}$)

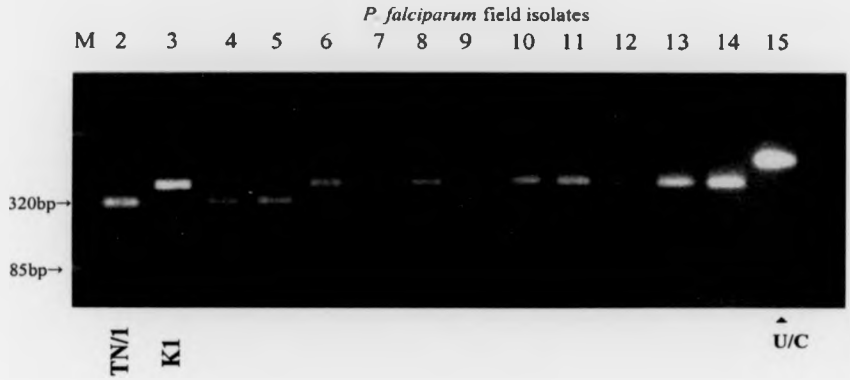
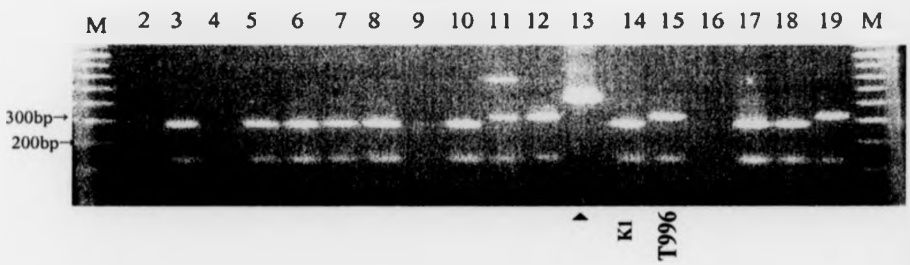


Figure 4d *dhps* ($^{Ser}436^{Ala}$)



A representative number of *P. falciparum* field isolates is shown in Figures 4c-d. The labels signified as M = 100 bp ladder marker; ▲, ▼, = indicates uncut (UC) PCR product; → approximates the band size (in base pair). Laboratory clones used as controls are shown for each figure. As shown in Figure 4c, a band profile similar to the control TN/1 isolate indicates presence of mutant Glu–540 whereas a band profile similar to K1 control isolate indicates wild type Lys-540. Similarly in Figure 4d, a band profile similar to K1 control isolate indicates presence of wild type Ser-436 whereas a band profile similar to T996 control isolate is indicative of mutant type Ala-436.

4.4 Discussion

In analysing pre-treatment samples for *dhfr* and *dhps* genotypes, it was found that samples from all successful treatment cases from Tiwi and Mosoriot (1997) carried the wild type codons 51 and 59 of the *dhfr* gene while mutant codon 108 was seen in some cases responding to SP in both epidemic and endemic areas. This indicates, as expected, that Asn-108 was often not sufficient alone to determine resistance to the SP combination.

Resistance was associated with presence of mutated *dhfr* in Tiwi (P=0.003) and Mosoriot (P=0.008), (Tables 4.3.3.3a and b) and the same relationship was seen when Asn-108 was analyzed alone. In Tiwi the presence of 2 or more mutations in *dhfr* was very highly predictive of resistance (P<0.001). Here, the combination of 2 or more *dhfr* mutations and any mutation in *dhps* was also highly predictive of resistance (P<0.001). Only 3 doubly mutated examples of *dhfr* were seen in the Mosoriot group, but those were among the 10 drug failures. Resistance here was associated with presence of mutated *dhps* (P=0.008) but this association was not significant in Tiwi. Resistance was associated with the combined presence of a mutation in *dhps* and in *dhfr* only in Mosoriot (P=0.008) and there was an association here with the presence of *dhps* Gly-437 (P=0.035). Perhaps *dhps* mutation may be more crucial when fewer *dhfr* mutations are present.

For Mosoriot, the positive predictive value (a measure of the utility of the test) was 100% for mutations in *dhps* and this was the case also when *dhps* Gly-437 occurred together with *dhfr* Asn-108. This was retained at 88% when any *dhfr* mutations were considered with any *dhps* mutations.

In the Tiwi sample the lowest positive predictive value is 69% (*dhps*: Gly-437) and the highest is 100% for 2 or more *dhfr* mutations. The combination of 2 or more *dhfr* mutations and mutated *dhps* also has a 100% positive predictive value. Together with the value of 77% for any mutation in *dhfr* and *dhps*, this also supports the results from Mosoriot suggesting that changes in *dhps* substantially influence the therapeutic response to SP, and this agrees with laboratory *in vitro* data (Triglia *et al.*, 1997). The association seen between *dhps* and *dhfr* (effectively linkage disequilibrium) support the suggestion that drug selection is acting on the enzyme products of both genes.

Acquired immunity in endemic areas may override the effect of mutations, which should determine drug failure. Since the younger age group in Tiwi was more likely to show drug failure, and the association of drug failure to "resistant" mutations, at least in *dhps*, was less strong in this location. It is possible that a proportion of infections in this location that responded to SP treatment did so in spite of parasites which were drug-refractory.

However, studies on presence of mutations in recrudescent infections revealed correlation between mutations in *dhfr* and *dhps* (Brooks *et al.*, 1994) and SP treatment failure (Jelinek *et al.*, 1999).

Results from the endemic (Busia, Tiwi (1998), Oyugis and Kendu Bay) and epidemic sites (Mt. Elgon and Kisii) showed that mutations in pre-treatment cases in both *dhfr* and *dhps* genes are associated with SP treatment failures, indicating the predictive value of these mutations [see Table 4.3.3.3d]. The sensitivity and specificity of the quintuple mutations in predicting treatment outcome, for the combined endemic sites, was calculated to be 33% and 90% respectively. However, the positive predictive value (that is the proportion of subjects who failed to respond to SP and had quintuple mutations in pre-treatment), for the combined endemic sites, was 42%. Similarly, the negative predictive value NPV was 86% in the combined endemic sites. The low (42%) PPV and low sensitivity (33%) could indicate patients' acquired immunity contributing to parasite clearance in spite of those parasites carrying mutated genes. It appears therefore, that use of these molecular markers in predicting treatment failure in endemic areas suffers this limitation. In the epidemic foci, where the immune effect is almost zero, the sensitivity was very high at 79% with a matching high specificity of 95%. The PPV was equally high at 69%. Interestingly, the NPV (the proportion of subjects that responded to SP and did not have the quintuple mutations) stands at 97%. Therefore, these molecular markers, to a very large extent, could be used as predictors of treatment failure in epidemic sites as evident from this work. Negative predictive value results indicate that prediction of treatment success would be reliable even in endemic areas.

Overall, the association of resistance phenotype and *dhfr/dhps* before treatment mutations seen in this study is significant both in the endemic [$P<0.001$,

χ^2 = 77.84] and epidemic foci [P<0.001, χ^2 = 50.85], suggesting usefulness of these mutant genotypes in predicting treatment outcome to SP. This was higher for quintuple and quadruple mutations (P<0.001, χ^2 = 13.57) than triple mutations (P=0.07, χ^2 = 3.24) [Table 4.3.3.3e] in endemic sites. The findings reported here, would suggest that multiple mutation in *dhfr* and *dhps* genes determines high level resistance to Fansidar® even though some sensitive isolates had mutations on *dhfr/dhps* genes [see Table 4.3.3.3c]. See Figures 4a, 4b, 4c, and 4d for a representation of field isolates showing mutant and wild type *dhfr* and *dhps* gene polymorphism.

In the epidemic sites, association was observed between isolates with double (P=0.023) or triple mutations (P=0.05) in *dhfr* gene and SP failure. This suggests that the presence of infections with either double or triple mutations on *dhfr* is sufficient to predict SP failure.

The patient samples in Chogoria were different compared to other epidemic sites. Patients from this site had failed treatment to CQ, SP and AQ and progressed to severe malaria and were admitted. Examination of polymorphisms on *dhfr* and *dhps* on admission revealed that 22/24 (92%) Kenyan parasites from Chogoria epidemic, carried triple point mutations in codons 51 (Asn to Ile), 59 (Cys to Arg) and 108 (Ser to Asn) of the *dhfr* gene; and also carried double point mutations in codons 437 (Ala to Gly) and 540 (Lys to Glu) of the *dhps* gene. Overall the high prevalence of mutant genotypes and the probable absence of acquired immunity to malaria could have resulted in failure of standard antimalarial therapy in this locality.

Mutations in the *dhps* enzyme of *P. falciparum* are clearly of major importance in the mechanism of resistance to sulfa drugs (Triglia *et al.*, 1997; Wang *et al.*, 1997b), but a second component has been identified that appears to allow the parasite to more efficiently use folate to assist in bypassing the antimalarial effect of these drugs (Wang *et al.*, 1997b). It is not clear what the factor is, but it may be a more efficient folate uptake system or alterations in enzymes that enable increased use of folate. It is not clear how important the folate factor is in resistance to sulfa antimalarial drugs in the field. Current data suggest

that in the presence of pyrimethamine, the folate effect that compromises susceptibility to sulfadoxine is much reduced, compared to its level when parasites are challenged with sulfadoxine alone (Wang *et al.*, 1997b). The *dhfr/dhps* point mutations observed here most likely underly SP resistance in the study area. SP was only recently recommended as a replacement for chloroquine as first line drug in Kenya (MOH Report 1999). This would suggest that sentinel areas of the country should be closely monitored for SP resistance-associated point mutations in *P. falciparum*.

Overall, these findings call for a critical review of the policy adopting SP as first line drug in Kenya. There is obviously an urgent need to search for alternatives. Perhaps in trying SP/CQ, SP/ artesunate, amodiaquine/ artesunate and other new combinations it is hoped that success may follow these approaches. Outlets and use of such drugs should be closely monitored by the government if full scale resistance to any of the drugs is to be delayed or avoided.

CHAPTER 4 II

Molecular Characterisation of Drug-Resistance Determinants in Kenyan Epidemic and Endemic Malaria: II Chloroquine resistance

4.7 Introduction

Efforts made in the understanding of the genetic basis of CQ resistance led to the identification of *pfmdr1* (chromosome 5) and *cg2* (chromosome 7) genes. Codon changes in these genes were linked to the CQ resistance phenotype. However, the association of these changes with resistance was found not to be absolute, either those of *pfmdr1* or *cg2*, was not absolute, suggesting another essential genetic difference. The mechanism of CQ resistance was earlier suggested to be multigenic (Foote *et al.*, 1990b). In 1990, an American group had reported association of CQ resistance with changes in chromosome 7 in a laboratory cross (Wellems *et al.*, 1990). In 1997 (Su *et al.*, 1997), the group identified a candidate gene (*cg2*) on chromosome 7. While changes in *cg2*, as confirmed by other workers (Basco and Ringwald, 1999; Durand *et al.*, 1999; Adagu and Warhurst 1999b; McCutcheon *et al.*, 2000), were linked to CQ resistance *in vitro*, the association was not complete. Transfection studies (Fidock *et al.*, 2000a) ruled out *cg2* as the determinant of CQR. Recently, Fidock *et al.*, (2000b) identified another gene (*pfcrt*) on chromosome 7 coding for the *Plasmodium falciparum* chloroquine resistance transporter as the main genetic determinant of *in vitro* resistance. *Pfcrt* encodes a 10 transmembrane-domain protein in the lysosomal membrane, but its precise function remains unclear. A change in codon 76 of the *pfcrt* gene, Lys-76-Thr (AAA to ACA) appears to be strongly linked to the CQ resistance phenotype with other changes within the gene probably playing a complementary role.

In this section *pfcrt* changes were examined in Kenyan isolates obtained from malaria patients in Mwea (1997: endemic), central Kenya. A parallel assessment of the *pfmdr1* codon 86 and 1246 status of the isolates was carried out and a comparison was drawn as to the degree of involvement of each gene in CQ resistance. Analysis was carried out revealing the extent of the match between *pfmdr1* and *pfcrt* with CQ resistance. Samples from recrudescence were also analysed for evidence of selection.

In addition, samples from severe malaria patients in Chogoria in 1997 (epidemic) were examined for drug-resistant determinants in *cg2*, *pfmdr1*. *Pfcrt* polymorphisms were not examined in the Chogoria samples as Chogoria study was

already completed by the time the *pfcrt* gene was identified. The molecular analysis of isolates from Mwea was carried out in collaboration with the University of Maryland (see Appendix J for personnel involved) while the *in vivo* work was conducted in Kenya.

4.8 Materials and methods

4.8.1 Endemic

4.8.1.1 Mwea

4.8.1.1.1 Analysis of *pfcrt* and *pfmdr1*

Reagents for PCR were purchased from Gibco-BRL (Gaithersburg, MD) and restriction endonucleases from New England Biolabs (Beverly, MA), see appendix J. All genotypic analysis of the isolates was performed by PCR amplification of parasite DNA, allele-specific restriction analysis (ASRA) at codons of interest. PCR primers and reaction conditions for the ASRA of polymorphic codons of *pfcrt* (76, 220, 271, 325, 356 and 371) were as described (Djimde *et al.*, 2001). *Pfmdr1* was also analyzed at codons 86, 1034, 1042 and 1246 as described (Djimde *et al.*, 2001), but with some modifications and additions. For analysis of codon 1246, a nested PCR was performed, utilizing a primary PCR with primers mdr1246-1 (5'-GGGGATGACAAATTTTCAAGATTA-3') and mdr1246-2 (5'-GGGGACTAACACGTTTAACATCTT-3'), and a secondary PCR with primers mdr1246-D1 (5'-AATGTAAATGAATTTTCAAACC-3') and mdr1246-D2 (5'-CATCTTCTCTTCCAAATTTGATA-3'). The secondary product was analyzed by digestion with *Bgl II* as described (Djimde *et al.*, 2001). For size discrimination the secondary product was determined in 2% NuSieve agarose (FMC, Rockland, MD). Programs for the above PCR were as follows. Primary PCR: initial denaturation at 95°C for 5 mins, followed by 45 cycles of denaturing at 92°C for 30 seconds [sec], annealing at 45°C for 30 sec, and extension at 65°C for 45 sec; with a final extension at 72°C for 15 mins. Secondary PCR programs were as above but for 15-20 cycles, and with the extension time decreased to 30 sec.

4.8.2 Epidemic

4.8.2.1 Parasite samples

Chogoria Mission Hospital is situated on the eastern slope of Mt. Kenya at altitude of 1,585 meters. The hospital has a large catchment area ranging from Tharaka division to southern and central Meru districts (see chapter 2 for detailed description of study sites).

Diagnostic parasite samples were obtained from febrile patients (aged 15 to 70 years) admitted to the Hospital in May 1997, during a malaria epidemic. Many of the cases had failed first and second line drug treatment (CQ, followed by AQ or SP). A blood sample obtained from each of the patients was blotted on glass fibre membrane and allowed to dry and then stored dry at 4°C until needed for analysis. The dry blood samples were prepared as described by Warhurst *et al.*, (1991), see 4.2.1.1.

4.8.2.2 *Pfmdr1* gene

4.8.2.2.1 Semi-nested PCR for codon 184 and 1034

The novel allele specific, one tube, semi-nested PCR was used to amplify regions of the *pfmdr1* gene containing CQ resistance associated point mutations (see Appendix H) (Grobusch *et al.*, 1998). Each reaction contained 3 oligonucleotide primers (outer forward, inner forward [mutant, or wild type specific] and reverse).

Although allele specific PCR method of Grobusch *et al.*, (1998) was used in analysing codon 86, 184, 1034, 1042 and 1246 there was a limitation in getting amplified products from some of the samples. Therefore, a PCR-RFLP was also employed utilizing primer P3 and the outer primer 184R used by Adagu and Warhurst (1999a). These primers flanked *pfmdr1* codons 86 and 184. Primers 1034F and P2 were used to amplify a fragment containing codons 1034, 1042 and 1246.

P3/184R PCR product was digested with *AflIII* to detect changes in codon 86. The 1034F/P2 product was digested with either *Vsp1* or *EcoRV* to detect changes in codon 1042 and 1246.

4.8.2.2.2. Restriction Fragment Length Polymorphism for codons 86 and 1246

The enzyme reactions were performed following the conditions described by manufacturers. For codon 86, 1042, and 1246 the following restriction enzymes were used, *AflIII, VspI*, and *EcoRV*. The enzyme digestions were calculated for a final volume of 20µl using 5µl of the nested PCR product, 1U of the enzyme, with buffer and temperature conditions as described by the manufacturers (New England Biolabs, Hitchen, UK). The samples were left to digest overnight (approximately 12 hours) after which digestion was stopped by the addition of 2µl loading buffer (50mM Tris [pH 8.0], EDTA [pH 8.0], 0.5% SDS, 40% sucrose, 0.25% orange G (Sigma)). See Appendix G for details on the restriction pattern for each enzyme.

4.8.2.3 *Cg2* gene polymorphisms

The method described by Adagu and Warhurst, (1999b) was used to screen the 24 samples for *cg2* polymorphisms (chromosome 7) associated with chloroquine resistance. Primers Hub1and Hub2 were selected to flank κ repeat region and the three point polymorphisms upstream of the 5' end of the repeat. Similarly, Luq1 and Luq2 primers span the γ repeat and a point polymorphism at the 3' end of the repeat.

Primer name	Primer sequence
	κ repeat region
Hub1	5'..TAT GAA TTT GGG TAA ATC G..3'
Hub2	5'..ATA TAT CAC TAA GTT GTA GCT..3'
	γ repeat region
Luq1	5'..GTG GAA AGG TTC CTT TAC T..3'
Luq2	5'..ATA ATC TGG AGA CAC AAC T..3'

Each of these two primers was used at a final concentration of 0.8 µM in a reaction mixture of 50µl, containing 150µM of each of the four dNTP, 1.25 U of *Taq* polymerase (Bioline, Springfield, NJ) and standard PCR buffer (1.5mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl [pH 8.3], 0.01% (w/v) gelatin). Forty rounds of amplification were performed.

4.8.2.3.1 Restriction Fragment Length Polymorphisms

Digestion of the Hub1/Hub2 PCR products with *Tse*1 (New England Biolabs, Beverly, MA) (0.75U at 65°C (see Adagu and Warhurst, 1999*b*) produced restriction patterns indicative of either the Dd2 or HB3 type of the *cg2* gene. A 10μl digest mixture (containing 4μl Luq1/Luq2 PCR product, 1μl reaction buffer) incubated with *Rca*1 (Boehringer Mannheim, Mannheim, Germany) (1U at 37°C) produced patterns indicative of the 7G8 or HB3 types of this gene. Dd2 (South–east Asian) or 7G8 (South American) served as the CQ-resistant/mutant controls, and HB3 as the CQ-sensitive/wild-type control. The Hub1/Hub2 PCR products contain *Tse*1 restriction site - 2 sites in the Dd2 type *cg2* gene and 1 site in the HB3 type gene. Enzymatic digestion of the PCR products produced restriction patterns indicative of either Dd2 or HB3 type *cg2* gene (see Adagu and Warhurst, 1999b).

Luq1/Luq2 PCR products, 7G8 type, contain one *Rca*1 site while the HB3 type PCR products contain two *Rca*1 sites. Enzymatic digestion of the products produced patterns indicative of 7G8 or HB3 type *cg2* gene. Digestion was carried out at 37°C utilizing 1U of *Rca*1. Dd2 (South East Asian) or 7G8 (South American) were used as CQ resistant or mutant positive controls while HB3 served as CQ sensitive or wild type positive control. Primers were obtained from Life Technologies. A *Rca*1 digest pattern of 194bp and 97bp is indicative of 7G8 type while a 119bp, 106bp and 45bp is indicative of HB3 type. Note that the 119bp and 106bp products co-migrate as one band.

Undigested PCR products' size polymorphisms of 291bp and 270bp are respectively indicative of 7G8 and HB3 γ repeat, whereas the size of the *cg2* gene κ repeat, using Hub1 and Hub2 primers, is 716 bp (Dd2) and 700 bp (HB3). The size of the κ repetetive unit is 16 bases.

4.9 Results

4.9.1 Endemic

4.9.1.1 Mwea (1997)

The *pfcrt*-Lys76Thr polymorphism was carried by all the 60 pretreatment isolates. Similarly, Ala220Ser (57/57), Gln271Glu (57/57), and Arg371Ile (60/60) (secondarily associated with Lys76Thr) were harboured by these isolates. Changes in codons 326 (Asn326Ser) and 356 (Ile356Thr), which were imperfectly linked to the CQ resistance phenotype (Fidock *et al* 2000b, Djimde *et al.*, 2001), were detected respectively in 56 of 59 (95%) and in 1 of 57 (2%) isolates tested. Two samples presented mixed (wild and mutant) alleles at 326. Heterogeneity at the 326 and 356, contrasting the tight linkage of 76, 220, 271 and 371, was also observed in Mali samples (Djimde *et al.*, 2001), as well as in isolates from Malawi (Kublin *et al.*, unpublished). As *pfcrt* mutations were present in all the pre-treatment samples, the post-treatment samples were not examined. However, the post-treatment samples were examined for *pfmdr1* mutation even though 93% initially showed the 86Tyr mutation (see Table 4.9.1.1).

The Asn86Tyr *pfmdr1* mutation previously implicated in CQ resistance was detected in 92% of CQS cases and 91% of the resistant cases at D0. Two of these exhibited a mixture of Asn86 and 86Tyr. Interestingly, samples taken at D14 recurrent cases, all (100%) carried the mutant sequence of codon 86. Only the wild-type alleles were observed at codons 1034 (Ser1034, 52/52) and 1042 (Asn1042, 52/52) in the D14 recurrent infections. In contrast, there was marked heterogeneity at codon 1246 in the pre-treatment [D0] isolates, 41 of 58 (71%) samples had the Asp1246Tyr mutation, four of which exhibited a mixture of the two alleles and the remaining isolates exhibited wild type sequence. All (100%) the D14 recurrent infection had the Asp1246Tyr mutation.

To analyze the *pfmdr1* polymorphisms of the pre-treatment [D0] isolates with respect to subsequent parasitological outcome, we first segregated the isolates based on the WHO parasitological grading criteria. Those in the sensitive grade (S), comprised 40 of the 60 (67%) isolates, and 20 (33%) were resistant at RI or RIII level.

As Table 4.9.1.1 details, when examined individually, the D0 and D14 parasite isolates had mutations in the *pfmdr1* codons 86 and 1246; [Tyr86: D0 93%, D14 100%; Tyr1246 D0 71%, D14 100%]. However, isolates that harboured both the Asn-86-Tyr and Asp-1246-Tyr comprised 47% (17/36) of the sensitive infections and 75% (15/20) of the resistant infections (P=0.083). Of the 20 D7 recurrent isolates, 90% harboured the Asn86Tyr and 70% harboured the Asp1246Tyr in mono-allelic or mixed fashion, similar to the prevalence in the pre-treatment infections. There was no clear selection of combinations of the *pfmdr1* polymorphisms relative to the D0 sensitive isolates; only a modest increase in the frequency of the paired 86Tyr/1246Tyr was observed (D0 sensitive 47%; D7 post-treatment 65%). However, all D14 recurrent cases, (8/8; 100%), had Asn86Tyr and Asp1246Tyr mutant genotypes on the *pfmdr1* suggesting 100% selection for both genotypes together (P <0.001 [for 86Tyr] and P=0.76 [for 1246Tyr], (see Table 4.9.1.1). P value (also 2-tailed) for combined 86Tyr and 1246Tyr selection was significant [P=0.020].

Table 4.9.1.1: Prevalence of *pfmdr1* and *pfcrt* genotypes in CQ treatment failures from Mwea 1997.

Isolates	N	*pfcrt* 76T(%)	*Pfmdr1* 86Y(%)	*pfmdr1* 1246Y(%)	86Y+1246Y(%)
Day 0	60	100	93	71	63
Day 0 S	40	100	92	65	47
Day 0 R	20	100	91	76	71
RI	1	NA	100	83	83
RII	11	NA	100	100	100
RIII	8	NA	83	59	50
RI-RIII	20	NA	91	70	68
Day 7	20	NA	90	70	65
Day 14	8	NA	100	100	100

Key: NA = not done; S = Sensitive; R (I, II, III) = Resistant;

All the parasites harboured *pfcrt*76Thr. In contrast to *pfcrt*, presence of wild-type Asn86 and Asp1246 in treatment failures was 9% and 30%, respectively. However, none of the isolates carrying *pfmdr1*86Tyr or 1246Tyr had the wild-type *pfcrt* Lys76 as all tested isolates harboured 76Thr (mutant) genotype. It was therefore not possible to examine the two genes for linkage.

4.9.2 Epidemic

4.9.2.1 Chogoria (1997)

Although records of drug treatment for half of the samples were not available, Table 4.9.2.1 shows that 11/12 patients received, prior to parenteral QN, Fansidar® and chloroquine or AQ to which the infection was not sensitive. All the infections responded to parenteral QN therapy. Where available, records of drug treatment show that patients had received at least 2 antimalarial treatment regimens prior to parenteral quinine therapy.

4.9.2.2 Drug resistance associated polymorphisms

As presented in Table 4.9.2.1, the allele specific PCR (AS-PCR) results show that 22 of the 24 samples had the mutant codon 86 while the remaining 2 (C1 and C7) had the wild type sequence. All the 24 samples had mixed wild type and mutant codon 184 (not included in the Table). Twenty-two of the parasites carried the wild type sequence of codon 1034 and the remaining 2 (C15 and C20) had mixed sequences of mutant and wild type. Interestingly, while all the 24 parasites had the wild type sequence of codon 1042, 20 carried the mutant sequence of codon 1246 and the remaining 4 which carried mutant codon 86, had the 1246 wild type sequence though one, C20 (see above), had the mixed genotype of codon 1034. The PCR/RFLP results confirmed the AS-PCR results for codons 86, 1042 and 1246.

The *cg2* κ repeat analysis, based on size variation and restriction fragment length polymorphism shows, in all, that 18 of the 21 samples examined (86%) carried the Dd2 type while 3 (14%) had the HB3 type. For technical reasons, 3/24 samples were not determined. Similarly, 4/24 samples were not examined for *cg2* γ repeat. Size and RFLP analysis of the 20 samples examined showed that 18 (90%) of these carried the 7G8 type *cg2*. Presence of Dd2 or 7G8 *cg2* genotype is reported to be associated with chloroquine-resistance while an HB3 *cg2* genotype is associated with sensitivity.

PCR products of 291bp and 270bp respectively indicate of 7G8 and HB3 γ repeat.

Table 4.9.2.1: Genetic profile and treatment regimens of 24 severe malaria cases from Chogoria, 1997.

S/N	*Pfmdr1* gene			*cg2* gene				Therapy
	86	1034	1246	κ-size	κ-RFLP	γ-size	γ-RFLP	
C1			\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	?
C2	\\\\\\\		\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	?
C3	\\\\\\\		\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	?
C4	\\\\\\\		\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	?
C5	\\\\\\\		\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	?
C6	\\\\\\\			\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	CQ>PSD
C7			\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	AQ>PSD
C8	\\\\\\\		\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	PSD>AQ
C9	\\\\\\\			\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	?
C10	\\\\\\\		\\\\\\\				\\\\\\\	CQ>PSD
C11	\\\\\\\		\\\\\\\	ND	ND	ND	ND	PSD>AQ
C12	\\\\\\\		\\\\\\\	\\\\\\\	\\\\\\\		\\\\\\\	?
C13	\\\\\\\			\\\\\\\	\\\\\\\	ND	\\\\\\\	?
C14	\\\\\\\		\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	ND	CQ>PSD
C15	\\\\\\\	≡≡≡≡	\\\\\\\	\\\\\\\	\\\\\\\		\\\\\\\	?
C16	\\\\\\\		\\\\\\\	ND	\\\\\\\	ND	\\\\\\\	AQ>PSD
C17	\\\\\\\		\\\\\\\	ND	\\\\\\\	ND	ND	CQ>AQ
C18	\\\\\\\		\\\\\\\	\\\\\\\	ND	\\\\\\\	\\\\\\\	PSD>AQ
C19	\\\\\\\		\\\\\\\	ND				PSD>AQ
C20	\\\\\\\	≡≡≡≡						PSD>AQ
C21	\\\\\\\		\\\\\\\	\\\\\\\	\\\\\\\	ND	\\\\\\\	CQ>PSD
C22	\\\\\\\		\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	\\\\\\\	?
C23	\\\\\\\		\\\\\\\	ND	ND	ND	\\\\\\\	?
C24	\\\\\\\		\\\\\\\		\\\\\\\	ND	ND	?

KEY:

S/N = SERIAL NUMBER; ND = NOT DETERMINED; CQ = CHLOROQUINE; AQ = AMODIAQUINE; PSD = FANSIDAR®; ? = NO RECORD OF DRUG TREATMENT; \\\\ = MUTANT; BLANK = WILD TYPE; ≡≡ = MIXED (WILD TYPE AND MUTANT GENOTYPES).

> = a change from first to second treatment regimen.

Figure 4e-f *pfmdr1* gene polymorphisms

Figure 4e *pfmdr1* ($^{Asp}1246^{Tyr}$)

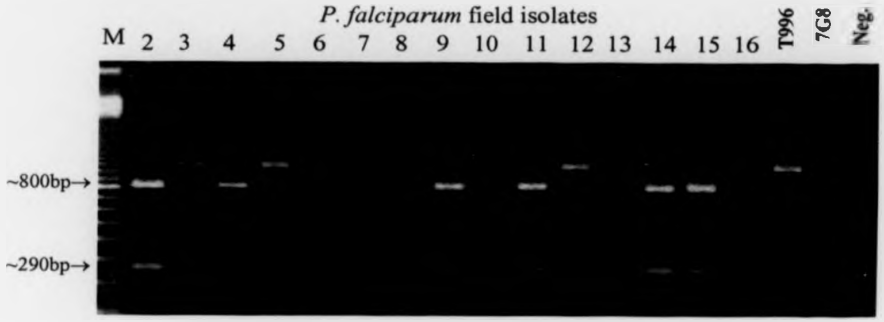
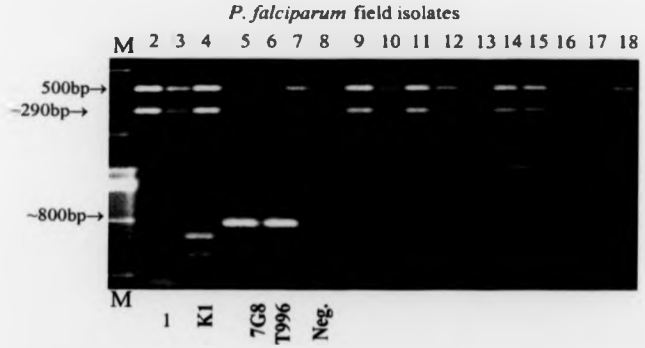


Figure 4f *pfmdr1* ($^{Asn}86^{Tyr}$)



Polymorphism on *pfmdr1* showing a representative number of *P. falciparum* field isolates. The labels signified as M = 100 bp ladder marker; ~ = approximates the band size (in base pair). Laboratory clones used as controls are shown for each figure.

4.10 Discussion

The surveillance of chloroquine-resistant malaria would be greatly facilitated by identification of reliable molecular markers of drug resistance that would be applicable in the field. Ideally presence of these markers or determinants could correlate with *in vivo* treatment outcome. The studies described here examined the roles played by putative drug-resistant determinants in CQ resistance.

In the Mwea study, the *pfcrt* mutation Lys76Thr was present in 100% of the CQR isolates but could not be definitively implicated in resistance here since 76Thr was also present in all *in vivo* susceptible isolates. Evidence of the ancillary role of *pfmdr1* to *pfcrt* (Warhurst, 2001b; Babiker *et al.*, 2001) and linkage disequilibrium has been reported (Adagu and Warhurst 2001). However, lack of wild type *pfcrt* in this site did not permit an evaluation of this suggestion.

As observed here while there were 40/60 CQ sensitive cases and in some other studies in East Africa (e.g., 18/23 CQ sensitive cases from Mozambique Mayor *et al.*, 2001), the *pfcrt* codon 76 mutation indicative of *in vitro* resistance is present in all isolates examined. Report of Dorsey *et al.*, (2001) in Uganda indicated presence of this mutation in all the 114 isolates tested, half of which were responsive to CQ *in vivo*. It is possible that, in these areas, where CQ-resistance level is fairly high, the Thr-76 change may not be discriminative enough to allow its use as predictor of drug failure. These observations are consistent with those of Pillai *et al.*, (2001) in Laos and Durand *et al.*, (2001) from samples collected from malaria endemic regions of sub-Saharan Africa, South East Asia and South America. However, in other areas like Mali, (Djimde *et al.*, 2001), Sudan (Babiker *et al.*, 2001) and Nigeria (Adagu and Warhurst, 2001), absence of *pfcrt*76Thr was an excellent predictor of successful CQ treatment. In Mali for instance, for *pfcrt*76Thr, the PPV (percentage of mutation-positive isolates correctly predicting a treatment failure) was 30% and the NPV (percentage of negative tests correctly predicting a successful treatment) was 98%. A test result showing apparent absence of *pfcrt*76Thr was an excellent predictor of successful CQ treatment. When results of patient population under 10 years of age in Mali was examined the PPV improved appreciably. Where the prevalence of *pfcrt*76Thr before treatment

was 41%, it rose to 100% with no mixtures in samples taken after treatment failure (Djimde *et al.*, 2001). It appears therefore that the mutant *pfcrt*76Thr is completely selected by CQ treatment, indicating crucial role in resistance, while incomplete selection of *Pfmdr1*86Ty occurs. Absence of *pfcrt*76Thr, predicts treatment success (Warhurst, 2001b).

As seen in Mwea and Chogoria, *pfmdr1* codon 1246 mutation, which is rare in Africa, but highly associated with South America isolates, (Foote *et al.*, 1990b; Povoa *et al.*, 1998; Zalis *et al.*, 1998) is emerging in this part of Africa. Its arrival would suggest increasing level of CQ resistance in Kenya. The mutation has also recently been detected in a few samples (13/50) from South Africa (McCutcheon *et al.*, 1999). In Chogoria, isolates contain mixed wild and mutant genotypes of codon 184. Changes in this codon appear to have no correlation with CQ resistance (Foote *et al.*, 1990b). Only 2 samples carried mixed mutant (Cys1034) and wild type (Ser1034) genotypes. Interestingly, these samples had mutant sequence of codons 86 and 1246 of *pfmdr1* gene shown in Table 4.8.3.2.1 [see Figure 4e, and 4f]. The low prevalence of 1034 mutation in Africa is expected, since this and the 1042 mutation are found mainly in S. America (Foote *et al.*, 1990b, Povoa *et al.*, 1998; Zalis *et al.*, 1998). The 4 Chogoria samples carrying wild type codon 1246 had mutant codon 86 and 3 of these had *cg2* point polymorphisms. At least 18 (75%) of the samples had *cg2* (see below) and *pfmdr1* CQ resistance related mutations. This study on hospitalized cases is the first time codon 1246 mutation has predominated in samples from East Africa. In Zaria, Nigeria (1993), codon 86 (Asn to Tyr) mutation was the only *pfmdr1* sequence variation detected in the area (Adagu *et al.*, 1997) and it was associated with low level CQ resistance. Reed *et al.*, (2000) found in transfection studies that *pfmdr1* Tyr-1246 significantly increased CQ-resistance of an already slightly resistant clone i.e., a clone with mutant *pfcrt*. The combined presence of these 2 mutations in *pfmdr1* could suggest that a higher degree of CQR is developing in Kenya.

The Chogoria samples were also examined for changes in *cg2* gene (Su *et al.* 1997). The κ and γ repeat regions of the gene were examined in this study. Eighteen (86%) of the 21 samples examined had the Dd2 type sequence of κ repeat while 90% of the 20 samples examined for γ repeat had the 7G8 type *cg2*

sequence. A match between *pfmdr1* 1246 mutation and *cg2* γ repeat polymorphisms was obtained for sixteen (89%) of the 18 samples that were examined for sequences changes in both genes. This high level match between 2 South American CQ resistance associated markers, on different chromosomes, would suggest that the mutations that were once rare in Africa are emerging in the continent particularly in East and perhaps South Africa. Overall, 18 (75%) of the 24 samples tested had the *cg2* and *pfmdr1* polymorphisms and this would strongly suggest CQ resistant status (Adagu and Warhurst, 1999b). It is not surprising therefore that many of the patients had failed initial treatment.

The allelic exchange experiments and drug profile study of transfectants (Fidock *et al.*, 2000a) suggest that the *cg2* polymorphisms do not primarily determine CQ resistance but the results are due to physical linkage of *cg2* and *pfcrt* on the same chromosome (Adagu and Warhurst, 2001). Other findings (Adagu and Warhurst, 1999b, Basco and Ringwald, 1999, Durand *et al.*, 1999 and McCutcheon *et al.*, 2000) found incomplete association of these polymorphisms with CQ resistance.

Presence of the *pfcrt*76Thr may be necessary for *in vitro* resistance but it is not sufficient to predict *in vivo* treatment outcome in areas where resistance prevalence is relatively high, >60% in Kenya. Mutations on *pfmdr1* may be important here in increasing resistance to CQ and perhaps additional polymorphisms on *pfcrt* may be involved in modulating CQ response as previously suggested (Fidock *et al.*, 2000b). The need for complementary mutations in *pfcrt* and probably *pfmdr1* may explain the slow development and spread of CQ resistance. Factors including drug absorption, pharmacokinetics of individual patients, parasite dynamics and patient immunity may indeed be additional factors that can influence treatment outcome.

There are obvious public health implications and it will be important to carry out more extensive surveys across Kenya as CQ therapy is reduced following the national ban on its use. Recent findings in Malawi suggest that mutant *pfcrt* is deleterious to the parasite and diminishes in prevalence when CQ pressure is reduced, with a consequent rise in CQ efficacy (Kublin *et al.*, unpublished observations). Similar observations were made in Thailand where gradual increase in *in vitro* CQ sensitivity on samples collected between 1978 and 1986, coincided

with the withdrawal of CQ for treatment of falciparum malaria, (Thaithong *et al.*, 1988). Perhaps, after proper and absolute withdrawal of CQ, it may be possible to re-introduce CQ in Kenya, maybe this time in combination with another "appropriate" antimalarial.

CHAPTER 5

Genetic Diversity of *Plasmodium falciparum* in Endemic and Epidemic areas of Kenya

5.1 Introduction

Study of the population structure of *P. falciparum* using genetic and molecular biology approaches has provided some of the most accurate information about parasite evolution and population genetics (Escalante *et al.*, 1998). In this chapter variations in 3 well characterized merozoite surface proteins, 1 and 2 (coded by *msp1* and *msp2* genes) and glutamate rich protein (*glurp* gene) were used as molecular markers to determine the diversity among *P. falciparum* in endemic and epidemic areas of Kenya. The genes code for highly immunogenic antigens (Anders and Smythe, 1989) are very polymorphic in nature and are regarded as suitable for parasite typing. Variations in degree of genetic diversity in different areas (Creasey *et al.*, 1990; Babiker and Walliker, 1997) have been suggested to be due to differences in transmission intensity (Creasey *et al.*, 1990; Paul *et al.*, 1995; Babiker and Walliker, 1997). A high level of diversity in *P. falciparum* has also been reported in hypo-endemic regions (Babiker *et al.*, 1991; Paul *et al.*, 1998). Studies on *P. falciparum* diversity reported in Kilifi and Sokoke areas of Kenya which experience seasonal malaria with peaks of transmission during the rainy period, (Kyes *et al.*, 1997), showed equal (50%) prevalence of IC and FC alleles of the *msp2* gene in clinical and asymptomatic malarial infections. Another study conducted in Kenya was in the western part of the country (Qari *et al.*, 1998). The group examined *msp1* alleles and identifiede amino acid substitution due to crossover recombinational events.

Malaria outbreaks in Kenya are becoming more frequent leading to high morbidity and mortality, (MOH, 1994-2001). It was proposed that epidemic infections, as seen in the highlands, are likely caused by one clone and polyclonality and extensive diversity as seen in endemic infections are not expected in epidemic outbreaks.

Genetic diversity studies based on 3 unlinked variants of the glutamate rich protein gene (*glurp*) and merozoite surface protein gene (*msp1* and *msp2*) were carried out using samples from severe malaria patients admitted in Chogoria Mission Hospital (an epidemic site). Studies on uncomplicated malaria cases from additional endemic and epidemic sites in Kenya used the *msp2* marker. Studies in Kenya examining genetic diversity among isolates causing epidemic malaria have

not previously been done. Each of the 3 genes contains a highly polymorphic region composed of repeated units: block 2 of *msp1* gene (Tanabe *et al.*, 1987; Kimura *et al*, 1990), block 3 of *msp2* gene (Smythe *et al.*, 1990) and region 2 of *glurp* protein gene (Borre *et al.*, 1991; Triglia *et al.*, 1992). In addition to size differences, the allelic variants of *msp1* and *msp2* were classified into 3 (K1, MAD20 and RO33) families of *msp1* and 2 *msp2* (FC27 and 3D7/IC) as defined by the sequence of the repeat units.

5.2 Materials and methods

5.2.1 Study site and population

5.2.1.1 Endemic areas

Tiwi, Mwea and Kendu Bay areas were the chosen endemic sites (see Chapter 2 for details on sites and patients description). These sites differ in malaria transmission intensities and endemicity. Tiwi is hyperendemic, Mwea is mesoendemic and Kendu Bay is holoendemic.

5.2.1.2 Epidemic sites

The epidemic sites include Mosoriot, Mt. Elgon, Kisii and Chogoria where malaria outbreaks were experienced.

5.2.2 Sample collection and DNA preparation

Blood sample obtained from each patient was blotted onto 25mm diameter glass fibre membrane, GFM (Titertek), (Warhurst *et al.*, 1991), see 5.2.1.1 for detailed protocol on sample/DNA preparation.

5.2.3 Polymerase chain reaction

The PCR was nested as described by Snounou *et al.*, (1999). See Table 5.2.3 for primer sequences.

Nest I reaction

A single-tube reaction using each of the three sets of nest I primers, (M1-OF/M1-OR; M2-OF/M2-OR; G-OF/G-OR) and containing a sector of the prepared GFM was used in a 50µl reaction volume. The reaction mixture consisted of standard PCR buffer [1.5mM $MgCl_2$, 50mM KCl, 10mM Tris HCl (pH 8.3), 0.1% Triton X-100 [Bioline]), 125nM of each primer, 125µM of each dNTP (Pharmacia Biotech), and 1U of *Taq* polymerase (Bioline). The reaction mixture was overlaid with mineral oil. The PCR was allowed to run as follows: an initial denaturation of 5 minutes (min) at 95°C, followed by 25 cycles of annealing [58°C for 2 min for *glurp*; 61°C for *msp1, msp2*]; extension 72°C for 2 min, and denaturation 94°C for 1 min. A final extension cycle of 72°C for 5 min was included.

Nest II reaction

To analyse for each individual polymorphic gene, 6 separate nest II reactions were performed. One µl of the nest I product was used as template in nest II reaction. The final volume of the reaction was 20µl. Reaction mixture contained 2µl standard PCR buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl [pH8.3], 0.1% Triton X-100), 250nM of each primer, dNTPs 125µM each dNTP, and 1.0U AmpliTaq Polymerase (Perkin Elmer Cetus, USA). The PCR assays were carried out in thermal cycler (Perkin Elmer Cetus, USA, [in Kenya] and Hybaid, Teddington, Mdx, UK [whilst in London School], with the following conditions: PCR was allowed to run as follows: an initial denaturation of 5 minutes (min) at 95°C, followed by 30 cycles of annealing [58°C for 2 min for *glurp*; 61°C for *msp1*, *msp2*]; extension 72°C for 2 min, and denaturation 94°C for 1 min. A final extension cycle of 72°C for 5 min.

Controls

Standard laboratory lines of *P. falciparum* were used as positive controls in all the reactions. For the KI allelic family of *msp1*, the laboratory strains K1, T9/96 from Thailand and 3D7 (unknown origin) were used as positive controls. MAD20 (New Guinean laboratory clone) and RO33 originally isolated in Ghana, strains [courtesy Ali Alluoeche] were used as positive controls for MAD20 and RO33 *msp1*-allelic family. Positive controls for *msp2*-FC27 and IC allelic family employed use of K1 and T9/96 or 3D7 strains, respectively. For *glurp* reactions the K1 was used as the positive control although any laboratory strain could be used. Negative control involved PCR reactions with no template DNA.

5.2.4 Electrophoresis and analysis

Msp1 and *glurp* band sizes were resolved in gels containing 1.75% Ultra pure agarose (Gibco) and 0.75% NuSieve® GTG agarose (FlowGen, UK) in TBE buffer (0.1M Tris base, 0.1M Boric acid, 50mM EDTA [pH 8.0]) or TAE (40mM Tris acetate, 1mM EDTA). A 1.5% ultra pure agarose and 0.5% NuSieve® GTG agarose were used for separating products of *msp2* and *glurp* genes. Gels with *msp1* and *msp2* products were run for 1.5 hours while *glurp* products were run for 2 hours, at a

constant voltage of 100V. A 100bp-ladder (Pharmacia Biotech) was used as the molecular size marker. Gels were stained with ethidium bromide in TAE buffer and results visualized and photographed on an ultra-violet UV transilluminator (312nm) using a gel documentation system, "GRABIT".

Table 5.2.3 Primer sequences for *msp1*, *msp2* and *glurp* genes

	Gene	Primer sequence	Sequence (5'→3')
Nest I	*glurp*	G-OF(fwd)	TGAATTTGAAGATGTTCACACTGAAC
		G-0R(rev)	GTGGAATTGCTTTTTCTTCAACACTAA
	msp1	M1-OF(fwd)	CTAGAAGCTTTAGAAGATGCAGTATTG
		M1-OR(rev)	CTTAAATAGTATTCTAATTCAAGTGGATCA
	msp2	M2-OF(fwd)	ATGAAGGTAATTAAAACATTGTCTATTATA
		M2-OR(rev)	CTTTGTTACCATCGGTACATTCTT
	Allele		
Nest II	*glurpII*	G-NF (fwd)	TGTTCACACTGAACAATTAGATTTAGATCA
		G-OR(rev)	GTGGAATTGCTTTTTCTTCAACACTAA
	msp1 (K1)		
		M1-KF(fw)	AAATGAAGAAGAAATTACTACAAAAGGTGC
		M1-KR(rev)	GCTTGCATCAGCTGGAGGGCTTGCACCAGA
	msp1 (MAD20)		
		M1-MF(fwd)	AAATGAAGGAACAAGTGGAACAGCTGTTAC
		M1-MR(rev)	ATCTGAAGGATTTGTACGTCTTGAATTACC
	msp1 (RO33)		
		M1-RF(fwd)	TAAAGGATGGAGCAAATACTCAAGTTGTTG
		M1-RR(rev)	CATCTGAAGGATTTGCAGCACCTGGAGATC
	msp2 (IC/3D7)		
		M2-ICF(fwd	AGAAGTATGGCAGAAAGTAAKCCTYCTACT
		M1-ICR(rev)	GATTGTAATTCGGGGGATTAGTTTGTTG
	msp2 (FC27)		
		M2-FCF(fwd)	AATACTAAGAGTGTAAGGTGCARATGCTCCA
		M2-FCR(rev)	TTTTATTTGGTGATTGAGAATTGAA

5.2.5 Statistical analysis

This was calculated for each typing reaction, *msp-1*, *msp-2* and *glurp* (Chogoria) and *msp2* (in other sites). Relative sizes of the amplified DNA from the test samples were determined from a regression equation relating logarithm of size of the markers to distance moved in millimetres. Overall frequencies were calculated as a ratio of samples found to harbour a particular variant over the total number of samples collected in any one survey. The multiplicity of infection (MOI) [probable minimum number of clones represented] was calculated as the number of distinct fragments per PCR positive sample (Beck *et al*, 1997).

5.3 Results

The nest II primer sequences were designed to amplify and produce a single fragment per allele. The presence of more than one band was therefore indicative of multiple alleles. The 3 polymorphic markers were used on samples from Chogoria. However, only *msp2* marker was used on samples from Tiwi, Mwea and Kendu Bay, Mosoriot, Mt. Elgon and Kisii sites. The success rate for samples genotyped from each site is shown in Table 5.3.

5.3.1 Endemic

Tiwi (1997)

In the 37 Tiwi samples examined, there were 7 and 6 types of IC and FC alleles of *msp2* respectively. Of 37 sampled infections 6 isolates were clonal infections with either IC or FC *msp2* allele (MOI=1). For mixed infections (defined as infections containing more than one clone) the range of MOI was between 2 and 4 (mean=2.35; standard deviation SD=0.797), observed in 31 isolates.

Kendu Bay (1999)

Eleven types of IC and 6 types of FC were seen in 31 samples from Kendu Bay. Seven isolates out of these 31 samples were clonal infections (MOI=1) of IC or FC *msp2* allele. The MOI in the remaining 24 mixed infections was between 3 and 4 (mean=3.51; SD=0.510).

Mwea (1997)

Genotyping of 28 isolates from Mwea revealed 7 types of IC and 6 types of FC. Seven out of 28 infections were clonal (MOI=1). The range of multiplicity in 21 mixed infections was between 3 and 4 clones (mean=3.37; SD=0.497).

5.3.2 Epidemic

Mosoriot

In the 20 Mosoriot samples examined, there were 3 and 5 types of IC and FC alleles of *msp2* respectively. Similarly, 5 out of these 20 were assessed as clonal infections (MOI=1) and for the remaining 15 mixed isolates, the range of multiplicity was found to be between 2 and 3 clones (mean=2.53; SD=0.516).

Mt. Elgon

In the Mt. Elgon region, 30 samples were genotyped and of these, 4 and 3 types were of IC and FC *msp2* allele respectively. Seven out of the 30 samples were clonal infections (MOI=1). MOI in the remaining 23 mixed infections was between 1 and 2 clones (mean=1.53; SD=0.510).

Kisii

A total of 36 isolates were examined for parasite diversity in the *msp2* locus. The results showed 8 and 5 types to be IC and FC *msp2* allele respectively. Nine out of 36 infections were clonal infections MOI=1. The MOI was calculated to be between 1 and 2 (mean=1.38; SD=0.492) in the remaining 27 mixed infections.

Overall, the frequencies of the different allelic families of *msp2* were not seen to vary appreciably between 2 epidemic sites of Mosoriot [2-4 genotypes per infection] and Chogoria [3-4 genotypes per infection] (see section 5.3.3) and 3 endemic sites of Kendu Bay, Mwea and Tiwi [2-4 genotypes per infection] ($P=0.482$, students *t*-test). However, there was significant difference between the endemic sites [2-4 genotypes per infection] and the 2 epidemic sites of Kisii and Mt. Elgon [1-2 genotypes per infection] ($P<0.001$, *t*-test).

In summary, overall analysis of DNA from isolates tested from malaria endemic and epidemic sites indicates the presence of more than one clone in the majority of samples.

5.3.3 *Msp1, msp2* and *glurp II* markers in Chogoria

The allelic families of the *msp-1* (KI, MAD20, RO33), *msp2* (IC, FC) and *glurp* fragments were substantially diverse, see Figure 5.3.3 (a to f). The prevalence of each allelic family was estimated by calculating the percentage of fragments assigned to one family by PCR with family-specific primers within the overall number of fragments detected for that locus in the group considered.

All the distinguishable allelic variants for each marker were marked numerically starting with the largest in size; 7 for *glurp* (PCR product size range 700bp-1300 bp), 12 for *msp2* (comprising 4 FC27-type variants (PCR product size range 300 bp-400 bp) and 8 3D7-IC-type variants (PCR product size range 480 bp-690 bp), and finally 14 variants for *msp1* with 10 Kl-type variants (PCR product size range 200 bp-330 bp) and 3 MAD20-type variants (PCR product size range 230 bp-210 bp) and only 1 RO33 allelic variants of 160 bp, see Figure 5.3.3.

A minimum number of intra-allele size-variant antigens was detected. This was 14 in *msp-1* block 2, 12 in *msp-2* block 3 and 7 in the *glurp* II fragment. For the multiplicity of infection per individual sample, the minimum and maximum number of clones detected was 3 and 4 respectively (mean=3.12). Furthermore, since most isolates contained multiple genotypes, it was impossible to determine in which specific combination these alleles occurred in individual parasites. See Figure 5.3.4a-b for a representation of field isolates.

Frequency calculations (explained in materials and methods) for each variant of a particular marker family was done, see Figure 5.3.3. Of the *msp1* K1-allele, the most frequent fragment was 250bp (25%); MAD20 220bp (21%); RO33 160bp (50%). For *msp2* FC allele, 320bp (17%) was most frequent and of the *msp2*–IC type a 500bp fragment (33%). In the case of *glurpII* fragments, the 900bp was most prevalent, (33%).

Table 5.3. Comparisons of observed variation of *msp2* among individuals living in areas with different levels of malaria endemicity.

Malaria	Area	n	n![%]	Mean (MOI)	Multiple Infection (%)
Holoendemic	Tiwi	38	38[100]	2.35	84*
Holoendemic	Kendu Bay	30	30[100]	3.51	76*
Mesoendemic	Mwea	40	28[70]	3.37	69*
			Mean	**3.08**	
Epidemic	Mosoriot	20	20[95]	2.53	55*
"	Mt. Elgon	30	30[100]	1.53	58*
"	Kisii	36	32[89]	1.38	56*
"	Chogoria	24	24[100]	3.12	64**
			Mean	**2.91**	

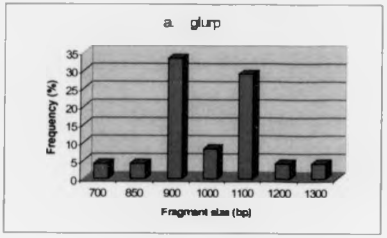
Key:
* = % of multiple infection from uncomplicated malaria (harbouring 2 clones [in epidemic areas] and [3 clones in endemic areas])
** = severe [complicated] malaria [harbouring 3 clones in epidemic Chogoria].
! = Number of samples successfully genotyped
MOI = Multiplicity of infection

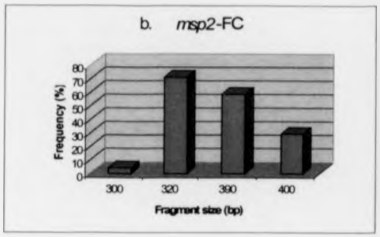
Table 5.3.1.2 Variable sequence of *msp-2* in *Plasmodium falciparum* field Isolates from malaria endemic and epidemic areas.

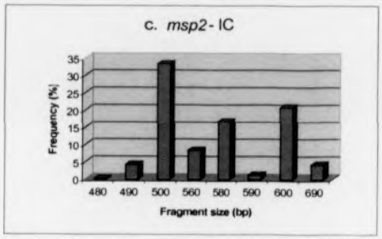
Site	Number of isolates	Variable sequence	Allele	Intra-allele size variant*	Minimum number of variants*
Endemic					
• Tiwi	38	490-680 300-390	IC FC	7 6	13
• Mwea	28	510-670 310-360	IC FC	7 6	13
• Kendu Bay	31	470-700 290-410	IC FC	11 6	17
Epidemic					
• Mosoriot	20	510-660 270-390	IC FC	3 5	8
• Mt. Elgon	30	490-680 310-390	IC FC	4 3	7
• Kisii	36	520-670 320-380	IC FC	8 5	13
• Chogoria	24	480-690 300-400	IC FC	8 4	12

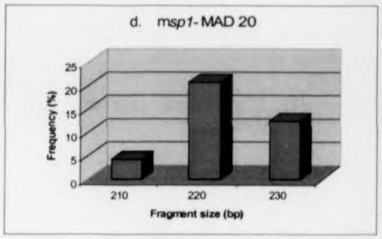
Note: * = the total number of the IC/ FC sequence by size (bp) observed in total isolates examined per site.

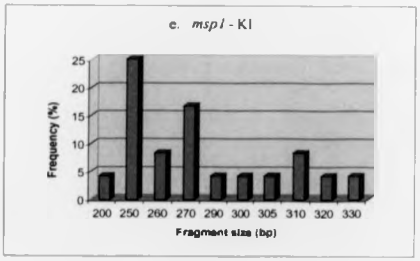
Figure 5.3.3 **A comparative graphical representation of *glurp, msp1* and *msp2* alleles frequency of sample from epidemic Chogoria.**











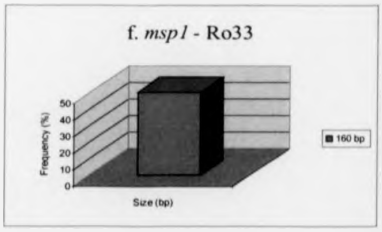


Table 5.4: ***Msp2*** **profile of** ***P. falciparum*** **in endemic /epidemic malarious countries**

Country	Malaria* Endemicity	No. of isolates investigated	*msp2* IC	FC	% Multiple Infections	Author
Brazil	Meso-	20	-	-	30	Creasy *et al.*, 1990
Thailand	Hypo-	56	51.8	48.2	70.4	Paul *et al.*, 1998
Sudan	Meso-	40	55	45	-	Babiker *et al.*, 1995
PNG	Hyper-	116-221	53	47	72	Paul *et al.*, 1995
Senegal	Holo-	135	52**	41**	93	Konate *et al.*, 1999
Kenya:						
Kilifi	Hyper-	42	50	50	71	Kyes *et al.*, 1997
Sokoke	"	95	50	50	69	"
Mwea	Meso-	28	54	46	69	This study
Tiwi	Hyper-	38	54	46	84	This study
Kendu Bay	Holo-	31	65	35	76	This study
Mosoriot	Epi-	20	38	62	55	This study
Mt. Elgon	Epi-	30	57	43	58	This study
Kisii	Epi-	36	62	38	56	This study
Chogoria	Epi	24	67	33	64	(This study; Omar *et al* 2001b)

* = Different malaria transmission intensities
** = 7% were IC/FC hybrids, [Konate *et al.*, 1999]
Hybrid = parasite carrying neither IC or FC allele but has a propotion of sequence from both alleles due to recombination

Figure 5.3.4 Parasite diversity in *Plasmodium falciparum*

Figure 5.3.4a *msp1*-KI

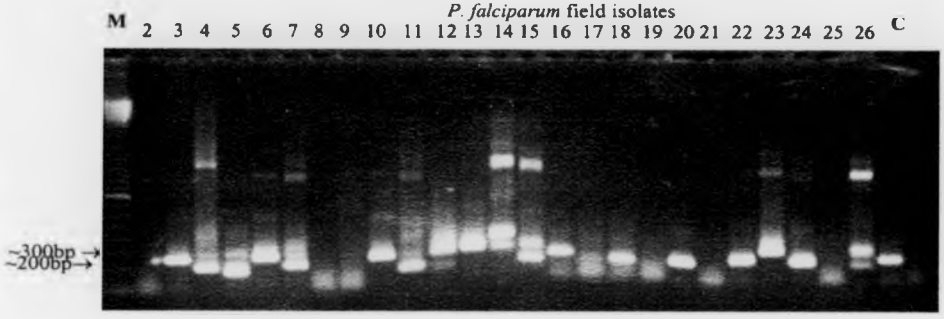
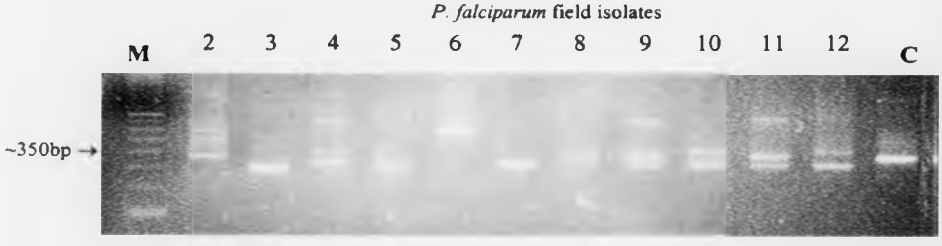


Figure 5.3.4b *msp2*-FC



The diversity of *P. falciparum* was demonstrated through amplification of the polymorphic regions of the 3 genetic markers *glurp*, *msp1* and *msp2* (Snounou *et al.*, 1999). The PCR primers used produce one single PCR fragment per allele, hence detection of more than one-sized band in the gel indicated the presence of more than one allele in that isolate. The detection of different sized bands indicated diverse strains of *P. falciparum*. A representative of field isolates showing the nested-PCR products is shown in figure 5.3.4a (*msp1*-K1) and figure 5.3.4b (*msp2*-FC). The predominant pattern corresponds to 230bp/ K1 family for *msp1* [Figure 5.3.4a] whereas the predominant 350bp fragment of the FC family for *msp2* [Figure 5.3.4b].

5.4 Discussion

Transmission intensity of malaria depends, in addition to climatic factors, on the density and infectivity of the anopheline vector and on variation in parasite rate in the human host (Babiker *et al.*, 1997). In highly endemic areas, stable parasite rates are usually seen all year round. This is observed despite seasonal fluctuations in mosquito densities and entomologic inoculation rates (EIR) (Smith *et al.*, 1993). In contrast in areas of low endemicity the prevalence of infection may be sensitive to changes in transmission intensity, for example due to rainfall variation that causes changes in mosquito density to occur (Babiker *et al.*, 1997). Difference in transmission intensities between endemic regions has been implicated as one of the factors leading to geographical variation and parasite diversity (Creasy *et al.*, 1990; Paul *et al.*, 1995; Babiker and Walliker, 1997). A number of studies have suggested a positive correlation between the mean number of genotypes per infected person and transmission intensities (Babiker *et al.*, 1997; Paul *et al.*, 1995; Babiker and Walliker, 1997). Multiple parasite genotypes have frequently been observed in individuals living in endemic areas, for example multiclonal infections were apparent in 83% of the samples analysed in a study carried out in Zimbabwe (Creasy *et al.*, 1990). Similarly, 82% of samples from Senegal carried more than one clone (Ntoumi *et al.*, 1995). Another study conducted by Babiker and Walliker, (1997) and carried out in Michenga village in Tanzania, (where malaria is holoendemic) and in Asar village in Sudan (where malaria is mesoendemic), indicated that the number of alleles of *msp1* and *msp2* genes was much greater in Michenga than in Asar. Furthermore, studies from hypo-endemic and seasonal malaria areas have shown higher than expected levels of *P. falciparum* parasite diversity, (Babiker *et al.*, 1991; Paul *et al.*, 1998).

Results from Kenyan endemic and epidemic sites described in this chapter indicated presence of multiple genotypes in 7 sites of differing malaria transmission intensities, 4 epidemic and 3 endemic. Results from 2 epidemic sites, Mt. Elgon and Kisii show that the *P. falciparum* genotype number per infected host is an average between 1 and 2 clones, showing significant difference between these 2 epidemic sites and endemic sites of Mwea, Tiwi and Kendu Bay (P=0.03) where an average of 2 to 4 genotypes was observed. In contrast, an average of 2 to

4, and 3 to 4 genotypes per infected human host, was observed in the epidemic sites of Mosoriot and Chogoria, respectively, with no significant difference (P=0.5) with the endemic sites.

The *msp2* profile of the samples examined in this study resembled those of other African countries (Babiker *et al.*, 1995; Konate *et al.*, 1999) suggesting a similar pattern of diversity.

Polyclonality and extensive diversity as seen in endemic infections were not expected in epidemic outbreaks [see Table, 5.3.1.2]. Genetic diversity could be expected to correlate with transmission intensity [see Table 5.4], because human superinfection would result in superimposition of genotypes carried by different vector mosquitoes (Babiker *et al.*, 1995). The reduced parasite diversity in Mt.Elgon and Kisii highlands is similar to the limited diversity in the Amazonian region of Venezuela (Laserson *et al.*, 1999) and supports this contention. These observations contrast with epidemic outbreaks in Mosoriot and Chogoria (P<0.001), Table 5.4. The extensive diversity seen in the epidemic regions of Chogoria and Mosoriot may not be surprising in view of the fact that these areas are bordered by endemic lowlands. Here the outbreaks could be due to parasite transfer from the surrounding endemic areas. Population movement between epidemic and endemic areas is likely to be a major vehicle for the spread of diverse parasite strains. The transfer of infective anopheline mosquitos could also be involved, carrying different parasite lines.

Linkage analysis of the parasite phenotype and allelic variants of markers (*msp1, msp2, glurp*) in the epidemic site of Chogoria was not possible due to polyclonality. The analysis would have investigated the association of severe malarial infection and multi-drug resistance with genetic diversity of the markers studied (see chapter 4 I-II).

CHAPTER 6

Detection and Quantification of *P. falciparum* in blood samples using nucleic acid sequence based amplification

6.1 Introduction

Widespread resistance of *P. falciparum* against standard antimalarial drugs such as chloroquine and Fansidar is leading to increased morbidity and mortality. As well as a need for better techniques to diagnose the infection, there is a need for reliable molecular methods to monitor drug therapy following treatment. Efforts currently focus on the detection of drug resistant determinants and of their association with treatment failure. This places great reliance on the accurate determination of treatment failure especially on the detection of persistent infections following drug therapy.

The availability of a fast, sensitive, reliable and quantitative method for the detection of parasite survival during and after drug treatment will help clinicians to monitor and, if necessary, adjust the treatment regimen. The detection and identification of specific nucleic acids present at very low levels, have been greatly facilitated by the development of *in vitro* target amplification techniques that exploit the enzyme-mediated processes of DNA replication (Mullis and Faloona, 1987), DNA ligation (Wu and Wallace, 1989; Barany, 1991). The main objective of this study was the evaluation and further development of a quantitative nucleic acid sequence based amplification (QT-NASBA) assay for the detection and quantification of *P. falciparum* RNA in blood samples from malaria patients for monitoring viable parasite persistence following treatment, (defined by recrudescing parasitaemia). In this chapter, the QT-NASBA was put into use and evaluated. The second phase involving antimalarial drug treatment efficacy studies is currently underway.

This work was performed in collaboration with Biomedical Research Centre of the Royal Tropical Institute (The KIT), in Netherlands (Appendix K). The field component involving sample collection from patients was conducted in Kenya, (see chapter 2 on *in vivo* aspect of the study) and the NASBA study was performed at The KIT.

6.1.1 Description of Nucleic acid sequence based amplification NASBA

The invention of polymerase chain reaction PCR has revolutionized the ability to amplify and manipulate nucleic acid sequence *in vitro* (Mullis, *et al.*, 1986). The commercial rewards of PCR technology has driven the development of other nucleic acid amplification and detection methodologies and one of these technologies is nucleic acid sequence based amplification NASBA. The company Organon Teknika is the inventor of the NASBA technology.

NASBA technique amplifies a target nucleic acid sequence, amplifying RNA in the presence of homologous genomic DNA, under isothermal conditions. Detection and amplification of RNA target sequences means therefore, NASBA, almost invariably measures persistence of living organisms, because of the unstable characteristics of RNA. It can also be applied to studies of gene expression where required. Smits *et al.* (1997) previously described the development of a qualitative and semi-quantitative NASBA assay for the detection and semi-quantification of *P. falciparum* and in this study further development of this qualitative NASBA into a quantitative NASBA is continued.

6.1.1.1 NASBA amplification process

NASBA operates under isothermal conditions (i.e., without temperature cycling) making it suitable for large scale manual screening. The amplification procedure employs reverse transcriptase (RT), RNAse H, and T7 RNA polymerase, in concert with two primers, to produce an isothermal amplification process, (Sooknanan and Malek, 1995) (see Figure 6.1.1.1). Initially a single stranded RNA (referred here as RNA analyte and denoted as RNA(+)) anneals to primer 1 (P1) which has a 5' T7 promoter tail. This step is followed by extension of P1 with RT to synthesize a single copy of cDNA. The RNA of the RNA-cDNA hybrid is then degraded by RNAse H to allow primer 2 (P2) to anneal to the single-stranded cDNA [DNA (-)]. P2 is then extended with RT to form a double stranded DNA consisting of a template for transcription and a T7 promoter. T7 RNA

polymerase then initiates transcription and generates multiple copies of antisense RNA product [RNA (-)], which terminates at the 5' end of P2.

In the subsequent stage each newly synthesized RNA(-) product then anneals to a P2 primer from which a DNA product is synthesized with RT. The RNA strand of the resulting RNA:DNA hybrid is degraded by RNAse H to allow P1 to anneal to the 3'-end of the DNA product [DNA (+)]. The DNA product is extended with RT, using P1 as the primer, to form a double-stranded DNA template with T7 promoter. The resulting DNA product directs the synthesis of many copies of RNA product [RNA (-)] by T7 RNA polymerase. Each newly transcribed RNA product [RNA (-)] can again be used as a template for synthesis of a DNA product [DNA (+)], which, in turn, can be used as a template to synthesize more of the RNA product [RNA (-)]. The continuous self-perpetuating, repetition of this process results in the exponential amplification of RNA and intermediate DNA products, which can be in excess of 1-trillionfold within 90 minutes, (Sooknanan and Malek, 1995). In addition, DNA template can be used in the NASBA reaction following a pre-treatment step of primer annealing, extension and denaturation. For schematic representation, see Figure 6.1.1.1.

Quantitation of template RNA or DNA, is a useful procedure in many biological fields, including virology. Indeed, the availability of reliable techniques for the absolute quantitation of DNA and RNA species may be the key to a better understanding of the pathogenic steps of most viral or parasitic diseases and for a more precise monitoring of patients treated with specific antiviral or antiparasitic compounds.

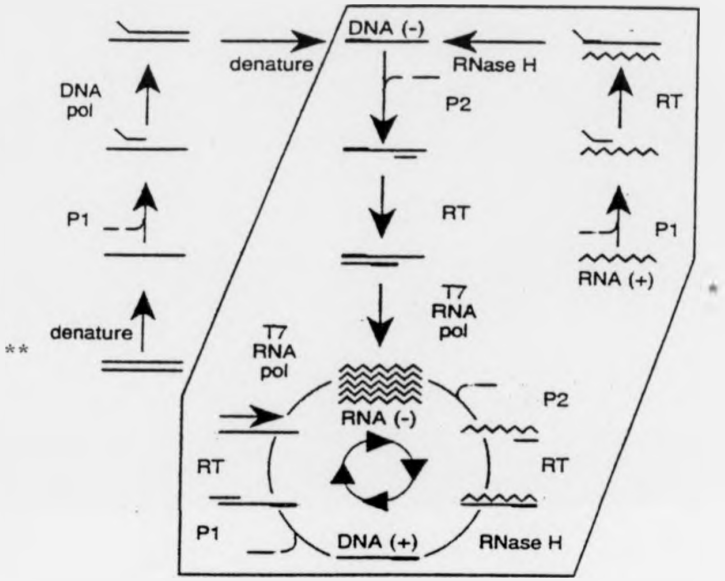


Figure 6.1.1.1 Schematic representation of Nucleic acid sequence based amplification NASBA (adapted from Sooknanan and Malek, 1995).

Key:

P1 primer 1 incorporating T7 promoter

P2 primer 2

RT: Reverse Transcriptase

T7 RNA pol: T7 RNA polymerase (requires T7 promoter sequence on DNA)

RNA-ase H breaks down SSRNA strand when bound to c-DNA product

* start of reaction

** double stranded DNA can be used in NASBA reaction following a pre-treatment step.

6.2 Materials and methods

6.2.1 Sample collection and storage

Blood samples were collected from malaria patients visiting two health centers, Oyugis Bay (60) and Kendu Bay (60) in Kenya, located in an area where malaria is holoendemic. Fifty µl of EDTA blood was mixed with 950 µl of guanidinium isothiocyanate (GuSCN) L6 lysis buffer and stored at –70°C until processed for RNA isolation. At the same time thick blood films were prepared from these blood samples for counting the parasites microscopically.

Lysis buffer (L6) was made by dissolving 120 g of GuSCN in 100 ml 0.1 M Tris-HCl pH 6.4 after which 22 ml 0.2 M EDTA pH 8.0 and 2.6 g Triton X-100 were added (Boom *et al.*, 1990).

6.2.2 Microscopy

The microscopic examination of thick blood smears was used as 'gold standard' for comparison with the quantification of parasites in the QT-NASBA. Thick blood films were stained with Giemsa and microscopically examined. Parasites and white blood cells were counted to a total of 200 white blood cells. The number of parasites/ µl of blood was calculated assuming that there are 8,000 white blood cells per µl blood.

6.2.3 Cloning and production of *in vitro* RNA for standards

Primers Plas-1F (5' TCAGATACCGTCGTAATCTTA 3') and Plas-2R (5' AACTTTCTCG CTT GCGCGAA 3') were used to amplify by PCR a 170 base pairs (bp) region of the *P. falciparum* asexual phase 18S rRNA gene. The amplified fragment was cloned into the plasmid pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and large quantities of *in vitro* RNA were produced using the SP6/T7 transcription system (Boehringer, Mannheim, Germany).

6.2.4 Site-directed mutagenesis for quantitation standard

In order to quantify the number of parasites in the blood samples by QT-NASBA, an internal standard RNA was co-amplified as a competitor using the same amplification primers as the wild type RNA (WT-RNA). For this construct,

20 bases in the centre of the WT target sequence were randomly rearranged using a specialized computer program. The resulting sequence was checked by computer analysis for the presence of secondary structures and possible homology with the original 170bp sequence. Next, the modified sequence was constructed using site directed mutagenesis (Ciceron *et al.*, 1999) and cloned into plasmid pCR2.1-TOPO. *In vitro* RNA was transcribed as described above. This modified *in vitro* RNA is referred to as "quantification RNA" (Q-RNA). For the quantification of *in vitro* WT-RNA, 10^6 molecules of *in vitro* Q-RNA were added to each 50µl sample; for the quantification of parasites in patient blood, 10^7 molecules of *in vitro* Q-RNA were added to each 50µl sample.

6.2.5 Nucleic acid isolation

To verify if the limit of parasite detection by the QT-NASBA is below 40 parasites/µl of blood detected by microscopy, 10^8 parasites/ml (10^5 parasites/µl of blood) was diluted down to 10^1 parasites/µl of blood, using *in vitro* culture as parasite source. These were used as controls (standard). Each control was individually spiked with 10^6 molecules of *in vitro* Q-RNA as competitor (note that pilot studies indicated that 10^6 Q-RNA molecules are the optimal amount that could be quantitatively measured, hence this amount was used to spike the controls). Similarly, all samples to be examined were spiked with this amount of Q-RNA. Nucleic acid from both samples and controls was isolated using GuSCN-silica procedure described by Boom *et al.*, (1990). Briefly each sample/control was mixed with L6 lysis buffer (see 6.2.1) and activated silica (30µl) was added to bind nucleic acid [activated silica was prepared as follows: 60g silica particles (SIGMA, S5631) was suspended in a total volume of 500 ml H_2O. The particles were left to sediment for 24 hours. After removal of 430 ml of the supernatant, another 500 ml of double distilled water was added. Upon sedimentation 440 ml of the supernatant was removed. Finally, 600 ul 32% HCl was added and the silica was suspended and dispensed in 4 ml portion and autoclaved to destroy contaminating nucleic acids].

The nucleic acid-silica complex was then washed twice with wash buffer (10 M GuSCN, 100 mM Tris-HCl, pH 6.4), and then once with 70% ethanol and

finally washed once with 30µl of acetone. Then nucleic acid was eluted from the silica into 100µl of double distilled water. This thus contained both Q-RNA and parasite [WT] RNA.

6.2.6 QT-NASBA

Primers and probes were selected based upon published sequences of the 18S rRNA genes of *P. falciparum* (McCutchan *et al.*, 1988). For the NASBA, primers Plas-1F (5' TCAGATACCGTCGTAATCTTA 3') and Plas-2R T7 (5'AATTCTAATACGACTCACTATAGGGAGAGAACTTTCTCGCTTGCGC GAA 3') were used. RNA from *P. falciparum, P. malariae, P. vivax and P. ovale* are all amplified in the NASBA using these primers.

6.2.7 Detection

The NASBA amplification products were hybridized to a biotinylated capture probe (5'ACCATAAACTATGCCGACTAGG 3') which was bound to streptavidin-coated magnetic beads. The samples were then separately hybridized to ruthenium-labeled WT- (5' CCTTATGAGAAATCAAAGTC 3') and Q- (5' AATAACTGCACCAGTGTATA 3') -detection probes followed by electrochemiluminescence (ECL) detection. The light emitted by ruthenium is detected by a photoelectric cell, thus offering a precise measurement for quantification.

6.2.8 Calculations and statistical analysis

The ECL provided for each sample a WT-RNA and a Q-RNA signal generated from hybridization to the WT- and Q-detection probes, respectively. The WT-RNA signal is proportional to the number of parasites present in the sample, whereas the Q-RNA signal is inversely proportional. The WT-RNA signal was divided by the Q-RNA signal for the calculation of the number of *in vitro* WT-RNA molecules per sample. Using linear regression, the raw data were then compared to the graph drawn from the values for the calibration series of blood samples containing known numbers of parasites. Below are the calculation steps used in determining the unknown parasite density (count).

1. The ECL signal was obtained for WT and Q RNA.
2. Then $\log_{10}$ [WT-RNA signal/ Q-RNA signal] was calculated.

Parasite count for each sample was then obtained from the standard curve [$y = \mathbf{a}(x) + \mathbf{b}$; y = is the $\log_{10}$ number of parasites, **a** = slope, $x = \log_{10}$ [WT-RNA signal/ Q-RNA signal], and **b** = intercept on the y-axis (e.g Fig. 6.3a).

6.3 Results

6.3.1 Quantitative ribonucleic acid Q-RNA

The detection limits of *in vitro* WT- and Q- RNA with their homologous, ruthenium-labelled detection probes were determined. After amplification in the NASBA followed by ECL detection amounts as low as 200 copies of *in vitro* RNA per 2µl sample could be detected for both RNAs. No cross hybridization between the two probes and target RNAs could be observed. This made the *in vitro* Q-RNA ideally suited for use as competitor RNA since Q-RNA and WT-RNA were amplified with equal efficiency and did not mutually interfere.

6.3.2 Detection limit

Figure 6.3.a shows the relationship between the number of parasites present in the sample and the amount of RNA or number of parasites as calculated from the QT-NASBA results. Using a simple mathematical formula a wide range of both *in vitro* RNA molecules and parasite numbers can be accurately quantified. Two hundred up to 10^8 molecules of *in vitro* RNA can be detected in a 2µl of sample. However, the control quantitation was carried out on parasitised cells in culture and thus does not entirely reflect conditions using actual patient samples, which usually have a restricted parasite stage representation with mainly small ring stages.

6.3.3 Reproducibility

RNA isolates from thirty different individual blood samples from cultures were tested in the QT-NASBA on 2 different days. Analysis of these data shows a high reproducibility. No significant day-to-day variation could be observed. When duplicate blood samples from the same patient were tested, only 1 out of 15 samples showed a more-than-tenfold (14-fold) difference between duplicate samples, whereas all other samples showed a less-than-tenfold difference.

6.3.4 Comparison with microscopy

Comparison of the microscopy count obtained by examination of thick blood smears with the results obtained with the QT-NASBA on 120 blood samples

from patients with clinical malaria revealed in 112/120 cases (93%) a less-than-tenfold difference between the results obtained with both methods, see Figure 6.3.b. The detection limit of infected patient blood by the QT-NASBA, was <10 parasites/ µl of blood. The differences for the other 8 samples were a factor 11(2x), 12 (3x), 34(1x), 100(1x) and 166(1x). Fifteen samples had a relatively low (less than 500 parasites/µl) parasitaemia. Four out of seven samples with a higher-than-tenfold difference between microscopy and QT-NASBA belonged to this group of samples from patients with a low parasitaemia. Four blood samples that were negative by microscopy showed low parasite numbers (0.01, 0.2, 1.1 and 1.2 parasites/ µl blood) when tested in the QT-NASBA.

Figure 6.3a **Graphical representation showing log values of the ECL signal against standard (cultured) parasite count (antilog value gives true parasite count).**

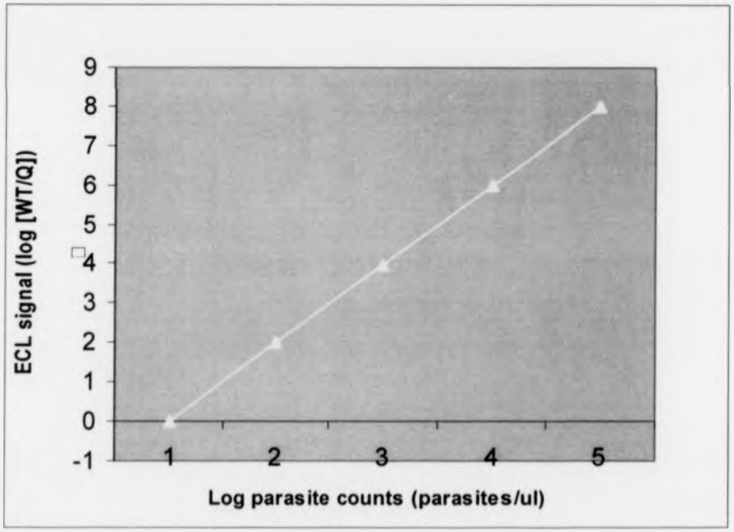
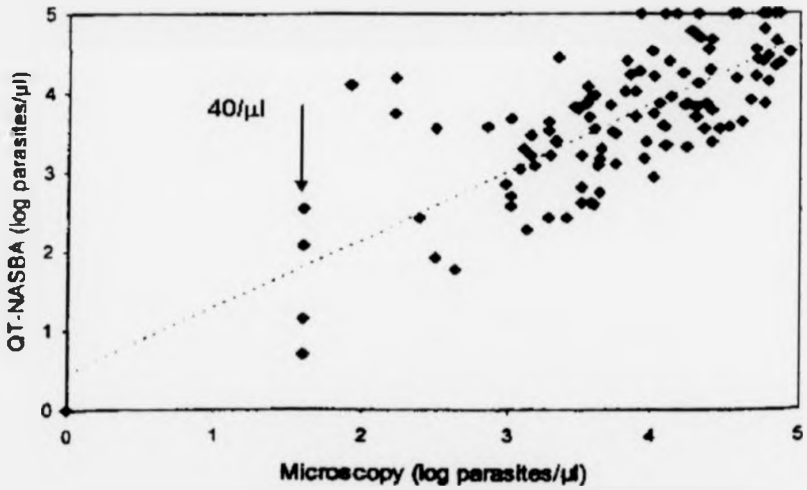


Figure 6.3b Comparison between field (microscopy count) and QT-NASBA



6.4 Discussion

Microscopic examination of Giemsa stained thick blood films is for many good reasons still the most widespread method for the diagnosis of malaria (Kain *et al.*, 1994). The method is cheap, relatively fast and allows species identification as well as quantification of parasites. However, for non–immune individuals such as small children and for pregnant mothers a detection threshold which is as low as possible is desirable. Especially in the early stages of malaria, when patients have a low parasite burden, the microscopic examination does not always fulfil this requirement. Early detection of low viable parasite counts in treated persons would also be valuable for clinical evaluation of drugs and, possibly, for following routine therapy.

Many alternative approaches for the diagnosis of malaria have been developed and applied, such as antigen detection tests, acridine orange (a fluorescent dye for staining parasite DNA/RNA) microscopy, PCR and serology, all with more or less success (Kain *et al.*, 1994). The NASBA technique has proved to be a sensitive and specific assay in diagnostic microbiology. In addition, there is extensive evidence, especially from the field of virology, that NASBA can be used for the quantification of infectious agents (Chan *et al.*, 1999). Smits *et al.*, (1997) previously described the development of a qualitative NASBA assay for the detection and semi-quantification of *P. falciparum*. The further development of this qualitative NASBA into a quantitative NASBA (QT-NASBA) makes use of modified Q-RNA which is added to each sample. The addition of *in vitro* Q-RNA, which serves as a competitor, allows for the simple and reproducible quantification of parasites in a single tube assay.

The QT-NASBA, as observed in this study, allows for quantification of *P. falciparum* over a wide range of parasitaemia (10 to 10^8 parasites per ml blood). The threshold of detection of 10 parasites per ml (0.01 parasites per µl) blood in cultured samples is at least 2,000 times more sensitive than routine microscopy, which has a detection limit of approximately 20 parasites per µl (Bruce Chwatt *et al.*, 1984). The detection limit of the QT-NASBA is <10parasites/ µl blood for patient blood. The results of routine microscopy and QT-NASBA gave an excellent correlation in 112/120 (93%) of the samples. However, parasite detection

from patient samples of 40 parasites/ µl, (see figure 6.3.b) varied widely between 10 to 10^3 parasites/µl blood when detection by QT-NASBA was employed. Only 8 out of 120 blood samples from patients with clinical malaria showed a discrepancy between microscopy count and QT-NASBA of more than a factor 10. Four of these eight samples were from patients with low parasitaemias. It is well known that the microscopy count is not very exact especially with respect to low parasitaemias (Carballo *et al.*, 1996; Kain *et al.*, 1994). In addition, QT-NASBA was capable of detecting low parasitaemias in four blood samples which were originally scored as "negative" by routine microscopy. Since all samples were from patients with clinical signs of malaria, this suggests that the QT-NASBA indeed has a lower threshold of detection than routine microscopy.

The QT-NASBA will in all probability not replace routine microscopy at high parasitaemias, especially in highly endemic areas where most clinical malaria cases have parasitaemias ranging between 1000 to 20,000 parasites per µl. In addition the QT-NASBA test is expensive, relative labour-intensive and requires a high level of expertise and a well-equipped laboratory. However, QT-NASBA, in contrast to routine microscopy, is excellently suited for detecting and quantifying low parasitaemias, making it a valuable tool for both the diagnosis of malaria in those patients who have low parasitaemias and for the monitoring of the efficacy of drugs or vaccines in clinical trials. The usefulness of QT-NASBA in antimalarial efficacy studies may be in the detection or quantification of low level parasitaemia in patients failing therapy.

The use of nucleic acid amplification techniques within the medical microbiology laboratory is becoming more and more accepted. The ability of PCR and NASBA assays to target and to amplify RNA specifically has led to several clinical applications. Some of these are already available in the form of commercial kits. NASBA technology has been adapted for application in routine diagnostic setting with a wide range of application in diagnosis of pathogens. NASBA's *in vitro* application has enabled the detection and quantitation of viruses such as Human Immunodeficiency virus (HIV) with a high degree of sensitivity, (van Gemen, *et al.*, 1993; van Gemen *et al.*, 1994). Quantification of viruses is useful in obtaining information about prognosis of patients and monitoring of

antiviral therapy. Other applications that have been described are the development of an assay for the bacterial pathogen responsible for tuberculosis, and a genetic aberration leading to cancer, (Sooknanan *et al.*, 1993). Clinical utility and relevance of these assays has been reported, though in some studies these techniques are limited because of poor reproducibility, especially for low copy numbers and unequal amplification dynamics for different genotypes, (Bremer *et al.*, 2000; Schmitt, 2001). Other studies have confirmed reproducible and sensitive performance of NASBA (Witt *et al.*, 2000; Loeffler *et al.*, 2001; Simpkins *et al.*, 2000).

Each of these areas of clinical application employing the use of self-sustained sequence replication reaction systems, ideally requires absolute rather than relative quantitation. To achieve these requirements critical challenges including determination of reaction conditions, control of potential carryover contamination, and simplification of the detection systems are important and require standardization in order to improve the reliability of quantitative assays.

CHAPTER 7

Overall Discussion and recommendation

Malaria treatment needs to be prompt, effective and affordable. The situation in Kenya demands critical evaluation of the standard antimalarial drugs in use.

Data presented in this thesis, from susceptibility studies indicated the presence of SP and CQ resistant *P. falciparum* infections *in vivo* in 5 endemic sites. In Mwea, central Kenya, by 1997, parasitological failure rate was 37% to SP and 33% to CQ. Similarly, in Busia, western Kenya, parasitological failure rate to SP was 18% by 1998; whereas in Oyugis (1998) and Kendu Bay (1999) southwest, SP parasitological failure rate was 13% and 19%, respectively. In the coastal region of Tiwi, SP parasitological failure was 18% by 1998. Results from other malarious areas of Kenya have shown similar failure rates for the antifolates. For instance van Dillen *et al.,* (1999) reported parasitological failure rates of 9.5% and 34.5% to SP by 1993, in northern and western Kenya, respectively.

SP resistance in epidemic sites in Kenya varied from 11% in Kisii to 48% in Mosoriot. Indeed in the recent past, morbidity and mortality is rising. Over 300 fatal cases were recorded during the epidemic outbreak in Rift Valley in 1997 (unpublished observations).

Antimalarial drugs play an exceedingly important role in preventing and interrupting transmission, (Garfield and Vermund, 1983). Antimalarial drug prophylaxis offered in populations residing in epidemic-prone areas of Kenya is important in curbing mortality and morbidity associated with the malarial disease but also raise concerns on emergence of drug resistance. This could perhaps partly explain the relatively high antimalarial drug resistance rates recently observed in the Kenyan Highlands, in this study [as observed in Chogoria, Kisii, Mt. Elgon, Mosoriot and elsewhere (Shanks *et al.*, 2000)].

Influence of patient immunity clearly impacted on treatment outcome as seen from endemic and epidemic studies presented here. The influence of host immunity in clearing resistant infections was found to be strongest in high transmission settings. Any immune response of the host, which in one way or another inhibits parasite growth or suppresses the development of clinical symptoms, will supplement the effects of the drug even if the parasite population is partially resistant. Semi-immunity develops with age within the first 5-15 years of

life in areas with stable and intensive malaria transmission, (Gilles and Warrell, 1993) thence the increased ability to clear resistant parasites will be a function of age. Findings from Tiwi (endemic) and Mosoriot (epidemic site), for instance emphasized that age of patient was an important and independent predictor of treatment failure and that resistance in endemic areas will affect drug efficacy in young children and less-immune patient before it affects older patients.

Data in *in vitro* results presented here clearly show that the *P. falciparum* isolates tested from Kenya are resistant to CQ and also in many cases to SP; The observation that strains were sensitive to MQ and QN supports the use of QN in treatment of refractory cases. Sensitivity to MQ and QN would suggest that either of these drugs can be used in treating cases resistant to CQ, SP or AQ. At present, QN is reserved as third-line drug used particularly in the treatment of severe malaria infection. MQ which is also effective against Kenyan *P. falciparum* is costly and may not be affordable by that proportion of Kenyans who need it most. Sensitivity studies on the current second line drug AQ were not performed in this work but reports from national surveys show high sensitivity of more than 90% in Kenyan sentinel sites (Rapouda *et al.*, 1997). This finding must be tempered by the reflection that the tests would have been better carried out using desethylamodiaquine, which is the active species present in the blood (Churchill *et al.*, 1986). However, *in vivo* studies done in 1993 by van Dillen *et al.*, (1999) reported parasitological failure rates to AQ of 18.5% and 35.1% in northern and western Kenya, respectively.

It will be useful for Kenyan government, in the light of findings reported in this thesis, to speedily look for alternatives, possibly a combination or combinations of antimalarials. Such drug combinations should be assessed for efficacy against Kenyan isolates, and if effective should be reserved as "back-up" treatment regimen. Effective drug policy should be in place and antimalarial drug use, handling and outlets should be well controlled and monitored to avoid drug pressure and mis-use of the drugs. Surveillance or drug efficacy monitoring, as has been practised in Kenya should continue, perhaps sentinel sites should be carefully chosen for greater representation and should include epidemic or highland areas.

The resistance profile seen here is mirrored by the presence of resistance-associated mutations. Antifolate combinations of which Fansidar® (S/P) is the new first-line drug, are failing as observed in the study areas examined. This is consistent with other reports (Nzila *et al.*, 2000). Characterisation of the isolates for *dhfr/dhps* mutations revealed a prevalence paralleling the observed *in vivo/ in vitro* resistance. This is a warning signal that this first-line drug may not last very long, stressing the urgent need to look for alternatives. Triple mutations in *dhfr* gene ($dhfr^{51, 59, 108}$) or quintuple mutations in *dhfr/dhps* ($dhfr^{51, 59, 108}dhps^{437/540}$) are highly associated with Fansidar® resistance. This finding would suggest that Fansidar® resistance in Kenya is probably a function of both pyrimethamine and sulfadoxine resistance (Omar *et al.*, 2001a). Further studies will confirm this observation. The antifolate combination co-trimoxazole was as effective in this study as Fansidar®. We are bound to suspect that resistance to both antifolate combinations has similar determinants. Although it is less easy to give co-trimoxazole treatment because its half-life is short, this feature means that "prophylactic" selection for resistance in new infections will not be as common as in Fansidar® (Mberu *et al.*, 2000). Control of co-trimoxazole use is as important as control of the use of Fansidar, but may be more difficult since the former drug combination is widely used in prophylaxis for opportunistic infections in AIDS. It would be ideal to control the use of all drugs and attempt to avoid resistance development but this must be balanced by the need for ready access for life-saving purposes..

Results presented here on CQSP versus SP showed no significant difference in relation to haematocrit and parasite clearance between the two drugs, perhaps explained by the high (>60%) parasitological failure to CQ (MOH, 1997; Shretta *et al.*, 2000). However, a clear improvement was observed in fever clearance times in CQPS treatment group suggesting an advantage over alternative antipyresis. Chloroquine in combination with SP, therefore, has obvious benefits but may not be a good option for routine therapy.

Fear of future development of resistance to newly introduced antimalarial drugs should allow for caution when dictating drug policies and use patterns. Drug pressure is probably the leading factor in development of resistance, as soon as a

drug becomes widely used, the parasite is placed under increasing selective pressure. Fear of resistance to potential alternative drugs leading to an endless cycle of policy changes, each requiring the use of more expensive drugs, has produced an inertia or passivity, which has allowed, at least in part, continued use of ineffective treatment. Findings in this thesis may perhaps lead to reforms in strategies for the management of drug resistance. It may be advisable to discontinue the use of drug early, when resistance is encountered in a parasite population, deviating from the current practice of continuing to use the first line drug for as long as possible. A strategy of switching to alternative drugs earlier would almost certainly reduce the morbidity due to protracted infections during the development of drug resistance. More importantly, it might even improve the chances of being able to return to the use of the first line drug at a later date (Thaithong *et al.*, 1988), if drug pressure is promptly withdrawn and the expansion of the resistant reservoir is arrested. In the Kenyan situation where CQ is no longer utilized as the first-line drug, monitoring the frequency of CQ drug resistant determinants on *pfmdr1*, and *pfcrt* will be important. Recent findings in Malawi suggest that mutant *pfcrt* is deleterious to the parasite, ie renders it unfit and the mutant diminishes in prevalence when CQ pressure is reduced, with a consequent rise in CQ efficacy (Kublin *et al.*, unpublished observations) as also shown in Thailand (Thaithong *et al.*, 1988). Perhaps, after proper and absolute withdrawal of CQ, it may be possible to re-introduce CQ in Kenya, maybe this time in combination with an "appropriate" antimalarial.

Measures have to be put in place to prolong the useful life of the new first and second line drugs and postpone development of drug resistance in Kenya. This is particularly important in view of the fact that currently no new effective and cheap antimalarial drug is in the market for immediate use as a first line treatment. Such protection can be achieved by improving compliance with drug regimens, increasing malaria diagnostic capacities of health facilities and implementing strict guidelines for laboratory confirmation of suspected treatment failures wherever possible before a second line drug is given. The latter will be as important for the government health services as for the private sector. As seen in this study, home treatment by carers is a common practice as the urine study indicated. Certainly

treatment initiated from home appears to be crucial in preventing the attainment of dangerous parasitaemic levels especially in the under 5 years who are mostly at risk of severe complications. Home treatment is also encouraged due to the relative inaccessibility of health care facilites. There are, however, potential problems with the diagnosis and administration of antimalarials at home. These include (1) under-dosage which may contribute to drug resistance, (2) danger of over-dosage especially in children, although this has been found to be uncommon in areas with a high rate of self medication, (Deming *et al.*, 1989). Early and appropriate treatment of malaria detected in children by carers (Hamel *et al.*, 2001) will prevent complications that arise as a result of persistence of symptoms and attainment of high parasitaemic levels leading to severe malaria or anaemia. A strong and effective educational campaign will have to be instituted to address the problem of inadequate and inappropriate treatment especially for children. Increasing the carers' awareness of the signs and symptoms of simple and potentially life-threatening malaria and their appropriate management (Hamel, *et al.*, 2001) might have the much needed impact in controlling malaria morbidity and mortality.

Parasite diversity was determined by use of molecular markers including *msp1, 2* and *glurpII* genes. Allele of *msp2* seen would suggest highly diverse isolates in both malaria endemic and epidemic regions. The diversity observed in the epidemic sites may be due to importation of different parasite strains from the surrounding malaria endemic areas. These highland areas have fertile land and there is intensive agricultural activity that attract migrant workers. Therefore, parasite transfer from the surrounding malaria endemic areas is a possibility. Visits between lowland and highland areas could be a contributing factor. The high prevalence of the "mutated" *dhfr, dhps, cg2,* and *pfmdr1* genes, may be partly due to importation of diverse resistant strains to epidemic areas. Use of mosquito nets (treated/ untreated with insecticides) among highland dwellers should be encouraged to limit transmission of malaria infection.

In this thesis a quantitative nucleic acid sequence-based amplification (QT-NASBA) assay for the detection and quantification of viable *P. falciparum* in blood samples was developed and evaluated. The detection limit of the parasites in

patient blood was demonstrated to be between 0.1 parasites/μl and 10 parasites/μl blood. Wide variations that exist between the QT-NASBA and microscopy, in particular at low level parasitaemia, should be investigated and this is currently the focus of future studies. Application of QT-NASBA will be useful when monitoring parasite clearance following treatment and perhaps for monitoring vaccine efficacy.

It is hoped that the findings of this thesis have enhanced knowledge of antimalarial drug efficacy and the role of point mutations (in genes believed to code for drug targets) in determining drug resistance. The understanding of this subject is important in view of the need to facilitate monitoring for drug resistance and to search for clinically useful antimalarials. While this thesis has contributed to general knowledge it is obvious that similar studies are required. The QT-NASBA is a useful diagnostic protocol, however additional evaluation is necessary in view of improving the sensitity and cost-effectiveness in order to make it user friendly. By this, QT-NASBA will become a useful tool in the epidemiological monitoring of antimalarial drug efficacy of infected malaria patients.

Additional studies in the Kenyan highlands including epidemic sites should be conducted even in the absence of malaria outbreak: monitoring infections in symptomatic and asymptomatic patients; and observations on treatment efficacy will provide essential parasitological and clinical information on parasite strains. These will be useful in view of selecting appropriate regimens for prompt treatment and prophylaxis. Studies addressing population dynamics in endemic and epidemic or, between endemic and epidemic areas of Kenya should be carried out. This will provide further information on the frequency and distribution of resistance-associated gene polymorphisms. The results will provide an early warning signal of "emerging" drug resistance prompting appropriate measures.

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APPENDICES

APPENDIX A

Site	Year	Drug studied	Endemicity	Location
Tiwi	1997	SP	Hyper-endemic	Southeast
Tiwi	1998	Co-tri, SP	"	
Mwea	1997	CQ, SP	Meso-endemic	Central
Busia	1997	CQSP, SP	Holo-endemic	Northwest
Oyugis	1998	Co-tri, SP	Holo-endemic	Southwest
Kendu Bay	1999	SP	Holo-endemic	Southwest
Mosoriot	1997	SP	Epidemic	Western Highlands
Chogoria	1997	SP, CQ, AQ, QN	"	Central (Mt. Kenya)
Kisii	1999	SP	"	Western Highlands
Mt. Elgon	1999	SP	"	West(Kenya-Uganda border)

APPENDIX B

Participation information: You have been asked to participate in a medical research study. It is very important that you understand the following general principles which apply to all participants in our studies.

(1) Your participation is entirely voluntary
(2) You may withdraw from participation in this study any time.
(3) Please feel free to ask any questions that will allow you to understand clearly the nature of the study.

Summary of the project:

Malaria is a major public health problem in many developing countries. Estimates of malaria mortality range from 1.5 to 2.7 million deaths worldwide per year (WHO, 1994), and approximately 1 million of these deaths occur among children under five years of age (WH0, 1993). In tropical Africa alone, 2 million children die from malaria annually.

The emergence and spread of chloroquine resistant *Plasmodium faliparum* in many endemic areas has complicated further the control of malaria. *P.falciparum* resistance to CQ/SP is intense and widespread and maybe responsible for the resurgence of the disease with an inreased death rate. In Kenya, high level resistance to CQ requires the application of second line drugs such as SP (for CQ resistance) and AQ (for SP resistance) for the treatment of uncomplicated malaria. When used inappropriately, resistance to these drugs may rapidly emerge and spread. Accurate assessment of the effectiveness of antimalarial drugs and treatment regimens and of the prevalence and spread of drug-resistant strains is essential to allow public health authorities to develop and evaluate adequate treatment strategies as stated in the strategy for "Global Malaria Control" formulated by the World Health Organization. This study will concentrate on resistance to CQ, SP, CQSP or co-trimoxazole.

Medical care:

You will be entitled to medical care for malaria (non-severe or severe). You will be treated at no cost to you. All recruits with an Hb less than 5.0g/dL will be given iron (ferrous sulphate) tablets 10mg/kg per day single dose for 1 month. Recruits with a temperature >37.5°C will be given paracetamol 6 hourly and the carers/ mothers encouraged to keep the patient cool and drinking plenty. Other conditions which require treatment or admission will be dealt with on individual basis. Care must be taken not to prescribe any other drug with antimalarial properties (eg tetracycline, co-trimoxazole, trimethoprim, proguanil) unless the clinical conditions necessitates such treatment in which case the recruit will be withdrawn from the trial.

I agree to participate in the study whereby fingerstick blood will be obtained.

Name _____

Signature/ Thumb print_____

CASE RECORD FORM							Contact (home) address
Record Number	Study site	Full Name					Guardian's Name
Age (months)	Weight (kg)	Hb/Ht Day 0	Hb/Ht Day 14	Drug name			Total drug dose (mg base)
Previous antimalarials (Y/N/Unknown)	Drug	Dosage		Urine Test (drug)	(concentration)		

	DAY0	DAY1	DAY2	DAY3	DAY4	DAY5	DAY6	DAY7	DAY8	DAY9	DAY10	DAY11	DAY12	DAY13	DAY14
Date															
Danger signs															
History of fever (last 24 hrs)															
Previous medication															
Axillary temperature															
Parasite count															
Treatment (no. tabs)															
Concomitant treatment															
Reasons for exclusion or loss to f/up															
Observations															

Overall assessment ETF LTF ACR Exclude Loss to f/up

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APPENDIX C

FIELD PERSONNEL

Name	Designation	Station	Duty	Sites	Year
SabahA.Omar	Research Officer (RO)	KEMRI	Team Leader PhD student	All	1997-9
Recording					
Sabah A Omar	RO	KEMRI	All	1997-9	
Frederick Kiarie	Technicians	"	All	1997-9	
David Maina	Technician		Oyugis	1998	
Kipruto arap Too	Technician	"	Kendu	1999	
		"	Oyugis	1998	
Binti Omar	Nurse	Tiwi	Tiwi	1997-9	
Grace Ochieng	Nurse	Kendu Bay	Kendu	1999	
Microscopy					
Sabah A Omar	RO	KEMRI	All	1997-9	
Stephen Kniaru	Technician	KEMRI	All	1997-9	
Ayala Ouma	"	"	All	"	
Gachare George	"	Ministry of Health DVBD	Tiwi	1997	
"	"	"	Busia	1998	
Kimani Maina	"	Ministry Of Health DVBD	Mwea	1997	
Urine tests					
Sabah A Omar	RO	KEMRIAll	1997-9		
Daniel Kariuki	Technician	KEMRIAll	1997-9		

Treatment Name	Designation	Station	Sites	Year
Patrick Gichuki	Clinical Officers	Mwea	Mwea	1997
Bakari A Saumu	"	Tiwi	Tiwi	1997-8
Morris Wekesa	"	Busia	Busia	1998
John Namale	"			
Owiti Awour	"	Oyugis	Oyugis	1998
Mark Ouma	"			
John Oduor	"	Kendu Bay	Kendu	1999
Prof. Dieter Gump	Dr.	Chogoria Mission Hospital	Chogoria	1997
Peter Ndaru	Technician"	"	"	1997
Gachihi Goerge	KEMRI	Mosoriot	Mosoriot	1997
"	"	Mt. Elgon	Mt.Elgon	1999
"		Kisii	Kisii	1999
GFM, NASBA, Hb				
Sabah A Omar	RO	KEMRI	All	1997-9
Joseph Mwangi	Assistant research Officer (ARO)	"	Tiwi	1998
			Oyugis	1998
			Kendu Bay	1999
Fatma Shamte	ARO	KEMRI	Busia	1998
			Tiwi	1998
			Oyugis	1998
			Kendu Bay	1999
Peter Ndaru	Nurse	Chogoria Mission Hospital	Chogoria	1997

Name	Designation	Station	Sites	Year
In vitro *test*				
Sabah A Omar	RO	KEMRI	Mwea, Tiwi, Busia, Oyugis	1997-8
Daniel Kariuki	Technician	"	"	1997-8
Maourice Adoyo	"	"	Oyugis	1998

Patient Follow-up

Mary Waithera	Demographers	Mwea	Mwea	1997
Ramadhani Kazumbe	"	Tiwi	Tiwi	1997-8
Mary Nafula Wanyama	"	Busia	Busia	1998
Vivian Akoth	"	Oyugis	Oyugis	1998
Serinah Anyango	"	Kendu Bay	Kendu	1999
Wycliffe Simuyu	"	Mosoriot	Mosoriot	1997
Stephen Migwi	"	Mt. Elgon	Mt.Elgon	1999
Mercy Ogola	"	Kisii	Kisii	1999

Incentive organizer |Lunch and return fare (transport)|

Lydia Chip	Technician	KEMRIAll	1997-9

Drivers

Arap Too Wangunyu Michael Wainaina	Drivers	KEMRIAll	1997-9

APPENDIX D
Calculation of wash media for WHO *in vitro* Mark II drug test (WHO, 1990)

For each isolate 958μL of the RPMI1640/Hepes solution and 42μL is required. This volume adds to 1000μL (ml of wash media). Only 900μL is needed per isolate and into this 100μL of patient blood is added. Fifty microlitres of the washmedia/patient blood is then added into each WHO pre-dosed well. From row A to H requires a total volume of 50μL X 8 = 400μL for a single column, in duplicate, the volume will be 400μL X 2 = 800μL.

To calculate for 50 isolates therefore the wash media required will be:

To perform in vitro analysis in duplicate and per sample:

1640 RPMI/ Hepes:	958μL	X 50 = 47,900μL (47.90mls)
Sodium Bicarbonate:	42μL	X 50 = 2,100μL (2.10mls)
Volume of wash media		*50,000μL (50mls)*

Volume per drug test:
900μL (wash media) + 100μL (patient blood) = 1000μL (1ml),

Aliquot 50μL into column A and B (in duplicate)

APPENDIX E
Concentration of drug tests, WHO Mark II (1990a)

Wells **Drug concentration (pmol/well)**

	CQ	MQ	QN	SP	
A	0	0	0	0	Sensitive
B	1	2	4	10	
C	2	4	8	30	
D	4	8	16	100	
E	**8**	16	32	300	Resistance
F	16	32	64	1000	
G	32	**64**	128	3000	
H	64	128	**256**	10000	

Bold and underlined = cut-off point for resistance; For SP threshold of schizont growth was taken to be 8 nuclei or more normal nuclei. Concentration of SP is at a ratio of 80:1. Dose shown for SDX [sulfadoxine].

APPENDIX F

Saker Solomon Test

This tests describes an adaptation of a method developed by E.G.Saker and E.T.Solomons for assay of drugs of abuse in urine which permits facile qualitative or quantitative determination of CQ and metabolites in the field. The CQ is partitioned from buffered urine (pH8.0) into a chloroform solution of tetrabromophenolphthalein ethyl ester (TBPEE) to give a purple colour, the intensity of which is proportional to the sum of the concentrations of CQ and its metabolites.

Reagents

1) Chloroform
2) Tetrabromophenolphthalein ethyl ester (TBPEE)
3) $K_2HPO_4.3H_2O$
4) KH_2PO_4

The pH8.0 buffer contained 324g of $K_2HPO_4.3H_2O$ and 10g of KH_2PO_4 in 1 litre of water. The solution of 50mg TBPEE dissolved in 100ml of chloroform was shaken with 10ml of 2mol/l aqueous HCl. After the phase separation, the aqueous phase was aspirated by Pasteur pipette, leaving 0.05% TBPEE-in-chloroform reagent solution. Solution was kept in the dark and and refrigerated. Fresh solution was made during every field trip.

APPENDIX G

Bratton-Marshall (BM) Test

The technique employed in this thesis is a simple modification of the BM test, described and adopted from De Almeida and De Souza, (1983).

Reagents

Reagent 1: sodium nitrite solution, 0.03mmol/l or 0.2mg/100ml or 0.2(w/v) in distilled water.

Reagent 2: hydrochloric acid (concentrated)

Reagent 3: Bratton Marshall solution (BM); dissolve 20mg N-(1-napthyl)-ethylene diamine dihydrochloride in 20ml distilled water, adding 3 drops of concentrated HCl acid.

Reagents 1 and 2 are stable at room temperature. Reagent 3 is stable for at least 2 months and was kept 5-10°C in a brown bottle (as described).

APPENDIX H

Oligonucleotide primers:

dhfr and dhps polymorphisms

NESTII primer sequences

dhfr

M3	5'TTTATGATGGAACAAGTCTGCGACGTT3'
F/	5'AAATTCTTGATAAACAACGGAACCTttTA3'
F	5'GAAATGTAATTCCCTAGATATGgAATATT3'
M4	5'TTAATTTCCCAAGTAAAACTATTAGAgCTTC3'

dhps

K	5'TGCTAGTGTTATAGATATAGGatGAGcATC3'
K/	5'CTATAACGAGGTATTgCATTTAATgCAAGAA3'
J	5'TGCTAGTGTTATAGATATAGGTGGAGAAagC3'
L	5'ATAGGATACTATTTGATATTGGAccAGGATTcG3'
L/	5'TATTACAACATTTTGATCATTCgcGCAAccGG3'

NESTI primer sequences

dhfr

M1	5'TTTATGATGGAACAAGTCTGC3'
M5	5'AGTATATACATCGCTAACAGA3'

dhps

R2	5'AACCTAAACGTGCTGTTCAA3'
R/	5'AATTGTGTGATTTGTCCACAA3'

pfmdr1 polymorphisms

Codon	Primer sequence	(5'-3')
184	Pfmdr1-184F:	ATGATAATAATCCTGGATCT
	Pfmdr1-184M:	AGTTCTTTTTAGGTTTACT
	Pfmdr-184R:	AGTTCTTATTCCCATTAAAG
	Pfmdr-184W:	AGTTCCTTTTTAGGTTTACA
1034	Pfmdr-1034W:	AGCTTTATGGGGATACA
	Pfmdr1-1034F:	CATACTGTTATTAATTATGG
	Pfmdr1-1034M:	AGCTTTATGGGGATTGT
	Pfmdr1-1034R:	TCAAATGATAATTTTGCAT
1042	Pfmdr1-1034F:	CATACTGTTATTAATTATGG
	Pfmdr1-1034R:	TCAAATGATAATTTTGCAT
	Pfmdr1-1042M:	AGCGCTCAATTATTTATAG
	Pfmdr1-1042W:	GCGCTCAATTATTTATGA
1246	Pfmdr1-1246F:	GGAAAATCAACTTTTATGA
	Pfmdr1-1246R:	AGGTTCTCTTAATAATGC
	Pfmdr1-1246M:	TGTGATTATAACTTAAGCT
	Pfmdr1-1246W:	TGTGATTATAACTTAAGCG
	P3/184 fragment	
	P3	ATGGGTAAAGAGCAGAAAGAG
	1034F/ P2 fragment	
	P2	TTAGGTTCTCTTAATAATGCT

APPENDIX I

Restriction enzyme	Incubation temperature (°C)	Point mutation	Restriction site	Restrction pattern (bp)
N1aIII	37	$^{GCA}16^{GTA}$	5'.....CATG...3' 3'.....GTAC..5'	376, 146
Tsp5091	65	$^{AAT}51^{ATT}$	5'.....AATT....3' 3'.....TTAA....3'	154, 120
XmnI	37	$^{TGT}59^{CGT}$	5...GAANNNNTTC..3' 3'..CTTNNNNAAG..5'	163, 137
AluI	37	$^{AGC}108^{AAC}$	5'..AGCT...3' 3'..TCGA..5'	326, 196
BsrI	65	$^{AGC}108^{AAC}$	5'...ACTGGN...3' 3'...TGACCN...5'	180, 146
BstNI	60	$^{AGC}108^{ACC}$	5'...CC A/T GG..3' 3'..GG T/A CC..5'	181, 145
DraI	37	$^{ATT}164^{ITA}$	5'.....TTTAAA..3' 3'...AAATTT..5'	245, 143, 107

APPENDIX I

Restriction enzyme	Incubation temperature (°C)	Point mutation	Restriction site	Restriction pattern (bp)
MnlI	37	$^{TCT}436^{GCT}$	5'...CCTC $(N)_7$.....3' 3'...GGAG $(N)_6$.....5'	317, 121
MspA1I	37	$^{TCT}436^{GCT}$	5'...C GC G...3' 3'.....G CG C...5'	406
MwoI	60	$^{GCA}437^{GGA}$	5'..GCNNNNNNGC...3' 3'..CGNNNNNNCG...5'	387

APPENDIX I

Restriction enzyme	Incubation temperature (°C)	Point mutation	Restriction site	Restriction pattern (bp)
AvaII	37	$^{GCA}437^{GGA}$	↓A 5'.....GG CC....3' T T 3'..CC GG.....5' A ↑	404
FokI	37	$^{AAG}540^{GAG}$	↓ 5'.....GGATG(N)$_9$...3' 3'.....CCTAC (N)$_{13}$...5' ↑	320, 85
BstUI	60	$^{GCG}581^{GGC}$	↓ 5'.....CGCG...3' 3'.....GCGC...5' ↑	105
BslI	55	$^{GCG}581^{GGC}$	↓ 5'.....CCNNNNNNNGG..3' 3'...GGNNNNNNNCC..3' ↑	128
MwoI	60	$^{GCC}613^{TCC}$	↓ 5'.....GCNNNNNNNGC..3' 3'.....CGNNNNNNNCG..5' ↑	128
BsaWI	60	$^{GCC}613^{TCC}$	A↓ A 5'.... CCGG ....3' T T T T 3... GGCC ....5' A ↑A	131
AgeI	25	$^{GCC}613^{ACC}$	↓ 5'...ACCGGT..3' 3'...TGGCCA..5' ↑	128

APPENDIX I

Restriction enzyme	Incubation temperature (°C)	Point mutation	Restriction site	Restrction pattern (bp)
AflIII 267	37	$^{AAT}86^{TAT}$	5'...ACPu Py GT...3' 3'...TGPyPuCA.....5'	503,
VspI 300,	37	$^{AAT}1042^{GAT}$	5'...ATTAAT..3' 3'..TAATTA...5'	130, 410,
EcoRV 290	37	$^{GAT}1246^{TAT}$	5'...GATATC...3' 3'...CTATAG...5'	810,

APPENDIX J

Mwea Study 1997: Molecular characterization of *pfcrt* gene

Institute: University of Maryland

Personnel

Joseph Cortese
Christopher Plowe
Jean-Claude Akpa

APPENDIX K

Quantitative Nucleic acid Sequence-based amplification, QT-NASBA: Development and application

Institute: The Royal Tropical Institute

Personnel
Dr. Henk Schallig
Dr. Linda Oskam
Gerard Schoone
Nel Kroone

LIST OF PUBLICATIONS

Omar, S.A., Adagu, S.I., Gump, D., Ndaru, N.P. and Warhurst, D.C. High prevelance of drug resistance-associated alleles in hospital admissions in an epidemic malaria zone of Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1999;**92**: 122-123. (Abstract).

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Omar SA, Owiti O, Bakari A, Adagu SI Warhurst DC. Efficacy of Co-trimoxazole in the treatment of *P. falciparum* infections in Kenyan children. Co-trimoxazole compared with sulfadoxine-pyrimethamine in the treatment of uncomplicated malaria in Kenyan children *Trans R Soc Trop Med Hyg.* 2001c;**95**: 657-660.