CHARACTERIZATION OF ANTIMALARIAL DRUGS RESISTANT 
PLASMODIUM FALCIPARUM ISOLATES, USING DOT BLOT 
HYBRIDIZATION MOLECULAR TECHNIQUE

By
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Characterization of antimalarial drugs
DECLARATION

This thesis is my original work and has not been presented in any other University for an award of a degree or any other award name.

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This thesis is dedicated to my beloved parents, brothers and sisters who have all along allowed themselves to be used of God to see my progress to victory.

Don't be deceived, my dear brothers.

Every good and perfect gift is from above, coming down from the Father of the heavenly lights, who does not change like shifting shadows. He chose to give us birth through the word of truth, that we might be a kind of firstfruits of all he created.

James 1:16-18
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ABSTRACT

One of the major obstacles to the control of malaria is the emergence and spread of parasites resistant to anti-malarial drugs. Resistance by *Plasmodium falciparum* to antifolate chemotherapy is a significant problem where drugs such as Fansidar® (sulfadoxine – pyrimethamine; S/P) have been in use for the treatment of chloroquine-resistant malaria. With the current trend in malaria treatment policies, this study aimed at assessing the level of resistance to one of the artemisinin combination therapy candidate sulfadoxine/ pyrimethamine in Kisii, an epidemic area of Kenya. Standard clinical efficacy testing *in vivo*, as recommended is time consuming and expensive method for establishing the levels of drug resistance, especially for large scale epidemiological surveys. The study also aimed at evaluating the dot-blot hybridization, a radionuclide based technique, for use in laboratory settings and for drug efficacy and resistance monitoring at an epidemiological scale. This was taken up alongside the conventional PCR-RFLP technique. Pre-treatment peripheral blood was collected by lancet prick from a fingertip for direct microscopic diagnosis. Some amount of blood obtained was spotted directly onto Glass Fiber Membrane (GFM) or on 3MM Whatman filter paper. Parasitic DNA was extracted using GFM and Chelex-100® methods respectively. PCR amplifications were done using specific primers for RFLP and blotting. RFLP was by the use of specific restriction enzymes by overnight digests. Single stranded fragments were blotted on nitrocellulose membranes for probing using [Gama-32P] dATP 5' end labeled allele specific probes. The nature and distribution of mutations in the *dhfr* gene (chromosome 4) in codons 51, 59 and 108 were profiled in Kisii, a *P. falciparum* malaria epidemic area in Kenya. Mutation profiles after dot blotting *dhfr* 108 showed that there were mutations in 69 (58.9%) of the study sample with no wild type, *dhfr* 59 showed 14 (11.97%) wild and 67 (59.82%) being mutant type while *dhfr* 51 had 2 (1.7%) wilds and 70 (59.82%) being mutants. Fifty six (47.8%) of the study population carried mutations in the three codons that were studied and these represented 80% of the PCR positive samples. Sixty one (52.1%) of these samples had double mutations in *dhfr* codons 108 and 59 and this was equivalent to 87.1% of the PCR positive isolates. Fifteen (12.82%) isolates which had been characterized as wild type by RFLP turned out to be mixed infections by radio probing. Moreover 29 (24.79%) of the isolates which had been characterized as mutant type actually came out as mixed type by radio probing. These findings would imply a high failure rate on the S/P drugs making them not to be good combination candidates in this epidemic area. Continued use of the drugs would lead to increased resistance due to drug pressure selection. The dot blot technique was found to have similar levels of specificity but more sensitive than PCR/RFLP. Moreover it was found to be suited for large-scale epidemiological surveys of genes associated with antimalarial drug resistance. From the study it is recommended that this technique be adopted even in the monitoring of the efficacy of the new antimalarials which would help in detecting any possible resistance early enough for appropriate policy change.
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LIST OF ABBREVIATIONS

ACT  Artemisinin-Based Combination therapy
AIDS  Acquired immune deficiency syndrome
ATP  Adenosine Triphosphate
CBRD.  Center for Biotechnology Research and Development
CQ  Chloroquine
dd H$_2$O  Double Distilled water
dhfr  Dihydrofolate reductase
dhps  Dihydropteroate synthase
EMP  Erythrocyte membrane protein
DNA  Deoxyribonucleic Acid
GFM  Glass Fibre Membrane
HIV  Human Immuno Deficiency virus
IAEA  International Atomic Energy Agency
KEMRI  Kenya Medical Research Institute
MoH  Ministry of Health
Msp1, 2,  Merozoite surface proteins, 1 or 2
PABA  Para-Amino Benzoic Acid
Pfmdrl  Plasmodium falciparum multi drug resistance gene-1
PfCRT  Plasmodium falciparum Chloroquine resistance gene
PBS  Phosphate buffered saline
PCR  Polymerase Chain Reaction
RBC  Red blood cells
RFLP  Restriction Fragment Length Polymorphism
SP  sulfadoxine-pyrimethamine
WHO  World Health Organization
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Unit</th>
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<tr>
<td>Kg</td>
<td>Kilograms</td>
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<tr>
<td>bp</td>
<td>Basepairs</td>
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<td>µl</td>
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<td>g</td>
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<td>Ci</td>
<td>Curie</td>
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<td>pM</td>
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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

1.1.1 Background information

Malaria remains a leading cause of morbidity and mortality in African and it has been well documented that children under 5 years and pregnant women are at highest risk (WHO 2000a, Bremen, 2001), with the basis for its control being appropriate case management, focusing on prompt treatment with effective antimalarials (Remme et al., 2001, WHO 2000a). Malaria is an insect-borne infection caused by a parasitic organism belonging to the Phylum Apicomplexa of the protozoans. The causative agents are various species of the genus *Plasmodium* that infect a wide range of hosts including reptiles, birds, primates and humans. In the case of human malaria, the disease is transmitted only by female *Anopheles* species mosquitoes, which survive only in tropical and sub-tropical climates with high mean temperatures and high humidity. There are approximately 156 named species of *Plasmodium*, which infect various species of vertebrates. Four are known to infect humans, these include: *P. falciparum* which causes tertian malaria, *P. vivax* for benign tertian malaria, *P. ovale* for quartan malaria and *P. malariae* which causes infrequent mild febrile disease (Paniker, 1989; CDC, 2004).

In Kenya, *P. falciparum*-inflicted malaria accounts for over 90% of all the malaria cases and is transmitted most often by *A. gambia*, with *A. funestus* being a secondary vector. (MOH-Kenya, 1992; Khaemba et al., 1994). Chloroquine (CQ) has been the mainstay of antimalarial treatment, but emergence of *P. falciparum* infections resistant to this drug
has challenged control efforts and has been linked to an increase in childhood mortality (Trape, 2001). The fixed combination of Sulfadoxine and Pyrimethamine (SP) has replaced chloroquine as the first-line therapy for uncomplicated malaria in several countries (White, 1999). In Kenya SP was introduced in 1983 as a second-line treatment for uncomplicated malaria. In 1997, the Kenyan National Malaria Control Program (now Division of malaria control) replaced CQ with SP as first line drug for uncomplicated malaria treatment owing to the increasing number of CQ treatment failure rated at 66-87% (Shretta et al., 2000; EANMAT, 2001). Unfortunately, rapid emergence of parasite resistance to SP has limited its use (White, 1998; Mberu et al., 2000; Omar et al., 2001a, b, c; Takechi et al., 2001) not only in Kenya but also elsewhere where it has been adopted as first-line drug. The level of SP resistance in Kenya has been rated at between 27-40% (MSF, 2002; EANMAT, 2003). The high SP failure rate has prompted the switch of attention to the combination therapy with drugs that have different modes of action to improve antimalarial therapeutic efficacy and delay emergence of drug resistance (White, 1999). Artemisinin-based combination therapy is thought to be the best option. Limited data are available on Artemisinin-based Combination Therapy (ACT) in Kenya.

*Plasmodium falciparum* is the most pathogenic of all *Plasmodium* species infecting humans, and is responsible for over 80% of malaria cases, worldwide (Paniker, 1989). *P. falciparum* malaria has several pathophysiological effects such as depression of immune system and it has been suggested that the immune depression caused by endemic malaria is responsible for burkitt lymphoma seen in African children (Paniker, 1989). Some of the most important complications of *P. falciparum* malaria include:
I. Cerebral malaria, which results from cerebral anoxia due to reduced blood flow rate, caused by sequestration of mature parasite forms in the brain vasculature (WHO, 2000).

II. Blackwater fever as a result of *P. falciparum* parasites causing red blood cells (RBC) to become auto-antigenic and thus produce haemolysis through an antibody–antigen reaction (Charters, 1983).

III. Anemia due to loss of iron as the parasite breaks down hemoglobin and multi-organ dysfunction, like respiratory distress syndrome (Nchinda, 1998).

1.1.2 The malaria problem

Until the late 1960’s, malaria was not only found in tropical areas of Africa, Latin America and Asia but also in large parts of Europe, parts of the USA and in Northern Australia. Malaria now exists in 100 countries but is mainly confined to poorer tropical areas of Africa, Asia and Latin America, with 2,400 million persons, about a third of the world’s population at risk. More than 90% of malaria cases and the great majority of malaria deaths occur in tropical Africa, mainly due to *P. falciparum* infections. Each year an estimated 300 to 660 million clinical cases of malaria occur worldwide and an estimate of between 1.5 and 2.7 million deaths are attributed to malaria, 75% of which are African children below age 5 years (Radloff, 1996; Nchinda, 1998; Snow *et al.*, 1999; Bremen, 2001; Snow *et al.*, 2005). The combined effects of climate change, population growth and urbanization on the population at risk (PAR), the number of people living in areas of climatic suitability for stable *P. falciparum* malaria transmission in Africa, has been stipulated. The results suggest that the PAR will change from approximately 0.63
billion in 2005, to 0.87 billion in 2015 and 1.15 billion in 2030 (Hay et al., 2006; Snow et al., 2006)

Seventy-four percent (74%) of African population lives in highly endemic areas and 5% of the African children are likely to die of malaria before reaching the age of 5 years (WHO, 1999). This childhood mortality constitutes nearly 25% of childhood mortality in Africa and is mainly due to anemia and cerebral malaria (WHO, 2000). When malaria infection is viewed as a risk factor, increasing parasite exposure has been shown to account for over 60% of the variation in all the causes of mortality in young African children (Snow et al., 2004). Malaria is not only a direct cause of death, but also contributes indirectly to death due to respiratory infections, diarrhoea and malnutrition by impairing immunity. Over 70 percent of the Kenyan population (approximately 20 million Kenyans) are at the risk of disease, with approximately 8.2 million clinical cases and 34,000 deaths of children below age five annually (approximately 93 children per day) (Teklehaimanot, 1999; MSF, 2002; EANMAT, 2003). A hidden burden is borne by patients who survive the severe consequences of infection and are left with debilitating sequelae, such as spasticity or epilepsy (Mung’ala et al., 2004), or subtler consequences including behavioral disturbances or cognitive impairment (Holding and Kitsao-Wekulo, 2004).
1.1.3 Malaria burden

Malaria has economic implications also over and above the losses occasioned by the morbidity and mortality of the disease (AID, 1985). A comparison of income in malaria-prone and non-prone countries indicates that average gross domestic product (GDP) in malarious countries in 1995 was US$ 1,526 compared with US$ 8,268 in countries without intensive malaria - more than five-fold difference (Gallup and Sachs, 2001). The annual economic burden of malaria in 1995 was estimated as US$8 billion, for Africa alone (Foster and Phillips, 1998). Households in Africa spend between US$2 and US$25 on malaria treatment, and between US$0.20 and US$15 on prevention, each month (WHO, 1999). Kenyans spend 5% of total household expenditure on malaria treatment (WHO, 1999). Overall production loss in Kenya is estimated at 26% of GDP (WHO, 1999). This figure is mainly made up of estimated productivity losses through premature deaths and spells of sickness. This calls for an urgent need to control this debilitating infection.

One of the major obstacles to the control of malaria is the emergence and spread of parasites resistant to anti-malarial drugs and lack of suitable epidemiological drug resistance monitoring techniques. The emergence and rapid spread of strains resistant to some antimalarial drugs including chloroquine, a cheap and safe drug, has worsened the malaria burden. Resistance of *P. falciparum* to antifolate chemotherapy is a significant problem where combinations such as Fansidar® (S/P) are used in the treatment of chloroquine-resistant malaria. Antifolate resistance has been associated with variant sequences of dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*), the
targets of pyrimethamine and sulfadoxine respectively, but these variations have not been fully studied in Kenya. Resistance to chloroquine is estimated at 66-87% (Shretta et al., 2000), while resistance to Fansidar® is over 40% (Omar et al., 2001 a, b and c; MSF, 2002; EANMAT, 2003). However, this has not been established in all the regions in the country especially in the epidemic areas, implying that successful chemotherapeutic intervention cannot be employed.

1.2 Literature Review

1.2.1 Human malaria parasites

Malaria is a disease which can be transmitted to people of all ages. The distribution of the disease (Figure 1) mirrors the distribution of the vector. Approximately, 40% of the world’s population, mostly those living in the world’s poorest countries, is at risk of malaria (Aultman et al., 2002). The disease is mainly confined to poorer tropical areas of Africa, Asia and Latin America. Malaria is highly endemic in most of sub-Saharan Africa, the Indian sub-continent, Southeast Asia and much of the central and South America. In Africa, P. falciparum is by far the most important species and it is this species that causes almost all malarial deaths. In Asia and S. America, P. vivax is common and is a major cause of malaria morbidity, but rarely fatal (Paniker, 1989; CDC, 2004).
1.2.2 Malaria causing organisms

Malaria is an insect borne infection belonging to the Phylum Apicomplexa of the protozoans. The causative agents are various species of *Plasmodium* that infect a wide range of hosts: reptiles, birds and primates including humans. In the case of human malaria the disease is transmitted only by female *Anopheles* spp. mosquitoes which survive only in tropical and sub-tropical climates with high mean temperatures and high humidity. Human malaria infection is caused by one or more of the four species of intracellular protozoan parasites, *P. falciparum* (causing tertian malaria), *P. vivax* (causing benign tertian malaria), *P. ovale* (causing quartan malaria) and *P. malariae* (causing infrequent mild febrile disease) (Garnham, 1966; Paniker, 1989; Nchinda, 1998).
*P. falciparum* is the predominant species and most virulent (Bruce-Chwatt and Peters, 1979). The species is found worldwide in tropical and subtropical areas. It is the only species that can cause severe, potentially fatal malaria. It is the *P. falciparum* species which has given rise to the formidable drug resistant strains emerging in the world. *P. falciparum* is found throughout tropical Africa, Asia and Latin America; *P. vivax* is mostly found in Asia, Latin America, and in some parts of Africa. While *P. vivax* only exceptionally causes death (most often due to rupture of an enlarged spleen), it can cause symptoms that are incapacitating. Thus, *P. vivax* contributes substantially to the disease morbidity with a resulting social and economic impact. *P. vivax* and *P. ovale* have dormant liver stages ("hypnozoites") that can activate and invade the blood ("relapse") several months or years after the infecting mosquito bite. *P. ovale* is found mostly in Africa, especially West Africa and the islands of the western Pacific. It is biologically and morphologically very similar to *P. vivax*. However, differently from *P. vivax*, it can infect individuals who are negative for the Duffy blood group, which is the case for many residents of sub-Saharan Africa. This explains the greater prevalence of *P. ovale* (rather than *P. vivax*) in most of Africa. *P. malariae*, found worldwide, is the only human malaria parasite species that has a quartan cycle (four-day cycle). The three other species have a tertian, three-day cycle. *P. malariae* causes a long-lasting, chronic infection that in some cases can last a lifetime. In some patients *P. malariae* can cause serious complications such as the nephritic syndrome (Paniker, 1989; Nchinda, 1998).

### 1.2.3 The lifecycle of malaria parasites

The lifecycle of the *Plasmodium* parasite comprises of two phases: the asexual phase and sexual phase (Figure 2). There is an alternation of generations in lifecycle of malaria
parasites, asexual and sexual generations alternatively. There also occurs an alternation of hosts as asexual phase takes place in human followed by the sexual one in the mosquito. The mosquito is considered to be the definitive host of the malaria parasite (the host in which the sexual cycle or reproduction occurs), while human is the intermediate host (Paniker, 1989; WHO, 1987).

In the asexual phase, the parasite multiplies by division or splitting, a process designated schizogony (schizo - split, gene - generations). Since this cycle occurs in the vertebrate host it is also called the vertebrate or intrinsic or endogenous phase. Schizogony occurs in two locations, in the RBC (erythrocytic schizogony), and in the liver cells (exo-erythrocytic schizogony or the tissue phase). The latter is an essential step before the parasite can invade erythrocytes, and is also called pre-erythrocytic schizogony. The products of schizogony, whether erythrocytic or exo-erythrocytic, are called merozoites.

The sexual phase takes place in the female *Anopheles* mosquito, even though the sexual forms of the parasite (gametocytes) originate in human RBC. Maturation and fertilization takes place in the mosquito giving rise to a large number of sporozoites (sporos - seed). Hence this phase of sexual multiplication is called sporogony or the extrinsic or exogenous or invertebrate phase (Paniker, 1989; WHO, 1987).

1.2.3.1 The human phase

The human host gets infected through bite of a female *Anopheles* mosquito. The sporozoites, which are the infective forms of the parasite, are present in the salivary glands of the mosquito. Sporozoites are transferred into the blood capillaries when the
mosquito feeds on the blood after piercing the skin then pass into the blood stream and some reach the liver where they enter the hepatocytes.

1.2.3.1.1 Exo-erythrocytic (tissue) stage

Within an hour of being injected into the bloodstream, the sporozoites reach the liver and enter the hepatocytes where they start the pre-erythrocytic schizogony. In this stage, they undergo repeated nuclear division and each daughter nucleus is surrounded by cytoplasm to form pre-erythrocytic or primary exo-erythrocytic schizont. In 6 to 16 days, the schizonts mature and burst, releasing thousands of merozoites (WHO, 1987; Paniker, 1989).

Figure 2: The lifecycle of malaria parasites. Source: National Center for Infectious Diseases, Division of Parasitic Diseases (CDC, 2004).
1.2.3.1.2 Erythrocytic stage

The merozoites released by the pre-erythrocytic schizonts enter the red blood cells by endocytosis and the RBC membrane seals itself. The entry process takes about 30 seconds. In the RBC, these develop into forms having annular or 'signet ring' appearance and are therefore called the ring forms. The merozoite feed on hemoglobin of the RBC but do not metabolize hemoglobin completely and so leaves behind as residue a hematin-globin pigment (hemozoin), called the malaria pigment. This iron-containing pigment accumulates in the body of the parasite as dark granules, which become more prominent as the parasite grows. The ring forms enlarge in size as they develop and become irregular in shape and show amoeboid motility. This is called amoeboid form and when it reaches a certain stage of development, its nucleus starts dividing. The parasite within the RBC before its nucleus starts dividing is called the trophozoite form (tropho - growth). The ring form is called early trophozoite and the amoeboid form the late trophozoite. From the time the latter starts dividing, the parasite within the RBC is called the schizont. During its early stage only its nucleus divides but not the cytoplasm. This is followed by the late schizont stage where each daughter is surrounded by cytoplasm. This mature schizont is a fully grown form in which a number of small merozoites are seen, each having a nucleus with surrounding cytoplasm. The mature schizont bursts open releasing the merozoites into circulation. The merozoites invade fresh erythrocytes in which they undergo the same process of development. The process of erythrocytic schizogony is repeated sequentially leading to progressive increase in intensity of parasitemia (WHO, 1987; Paniker, 1989).
1.2.3.1.3 Gametogony

After many cycles of erythrocytic schizogony, some merozoites that infect RBC do not proceed to become schizonts but instead form sexually differentiated forms, the gametes. Gametocyte development generally takes place within the internal organs, such as spleen and bone marrow, and only mature forms appear in peripheral circulation. The mature gametocytes are round in shape, except in *P. falciparum*, where they are crescent shaped. The macrogamates (female) and microgametocyte (male) do not cause any clinical illness in the host but are essential for the transmission of the infection. They do not develop further in the vertebrate host and unless taken up by the vector mosquito, they die in a few days (Paniker, 1989; WHO, 1987).

1.2.3.2 The mosquito phase

The microgametocytes taken with a blood meal by an *Anopheles* mosquito divide into 5 to 8 nuclei in the gut. From each nucleus, there protrudes a long actively motile whip like filament. These filaments, the male gametes (microgametes), lash about for some time and then break free. This process of formation of male gametes from gametocytes is called exflagellation and can be demonstrated *in vitro*. The macrogamete does not divide but matures into a female gamete or macrogamete. It is fertilized by one of the microgametes to produce a zygote. Fertilization occurs in half to two hours after the blood meal. The zygote develops into an ookinete, which in turn develops into an oocyst. The latter matures, increasing in size with the nucleus undergoing multiple divisions.

This sporogony leads to development within the oocyst of about 1,000 sporozoites. The rupturing of a mature oocyst releases sporozoites into the haemocoel. The sporozoites reach the salivary glands situated in the thorax of the mosquito, penetrate the acinar cells
and enter the salivary ducts. The mosquito is now infective and when it feeds on susceptible human hosts the sporozoites are injected into the skin capillaries to initiate human infection, and the cycle is repeated (WHO, 1987; Paniker, 1989).

1.2.4 Mosquito vectors

Mosquitoes of the genus Anopheles are the vector for the four Plasmodium species that cause malaria in humans. Although there are about 400 species of Anopheles mosquitoes only 60 transmit malaria under natural conditions, and only 30 are of major importance (Bruce-Chwatt, 1985; Greenwood et al., 2005). In Africa, the main vectors of malaria include species of the Anopheles gambiae complex (A. arabiensis, A. bwambiae, A. gambiae senso stricto, A. melas, A. merus, A. quadriannulatus), and A. funestus, whose distribution show similarities with patterns of annual rainfall across Africa (Coetzee et al., 2000). A. gambiae breed in temporary water bodies exposed to sunlight, such as pools and puddles that are mostly found during the rain season. They are responsible for most seasonal malaria transmission. A. funestus breeds in permanent vegetation such as swamps and rice fields. A. melas is rare and is adapted to brackish (slightly salty) water (Coetzee et al., 2000).

The most important reason for persistence of malaria in Africa is the presence of vector A. gambiae, but social and economic factors are also important. A. gambiae feeds preferentially on humans and is long lived, making it particularly efficient at transmitting malaria from one person to another. The task of interrupting transmission is daunting because in several parts of sub-Saharan Africa Entomological Inoculation Rates (EIRs) of
over 1,000 have been recorded compared to 5 in Asia and South America per year (Greenwood and Mutabingwa, 2004). EIR is a measure of the frequency with which an individual is bitten by an infectious mosquito. EIR is the product of the number of mosquito bites per human per unit time and the proportion of mosquitoes with sporozoites (MacDonald, 1957). Human infection risks are highly heterogeneous across Africa, and an individual may receive between 0.3 and 1,000-malaria-infected bites per year (Hay et al., 2000). Stable and intense transmissions result from high EIR, with young children and pregnant women being the most vulnerable (McElroy et al., 1997; Beier et al., 1999).

1.2.5 Malaria endemicity

This is the pattern of transmission of malaria in a particular locality and 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 Cater, 1993; Miller et al., 1994). Historically, outbreaks of malaria have occurred in areas of unstable malaria transmission when environmental conditions are favorable (Garnham 1945; 1948). However, there is evidence that the number of outbreaks have increased during the past decades. In eastern Africa, many countries have suffered from serious epidemics with a high number of deaths (Malakooti, Biomndo and Shanks, 1998; Lindblade et al., 1999; Bonora et al., 2001; Etchegorry et al., 2001).
In western Kenya, malaria epidemics have spread from 3 to 15 districts during the past 13 years, often with devastating effects (Githeko and Ndegwa, 2001). Some of these epidemics have been associated with extreme weather events, such as the El Niño Southern oscillation event in 1997-1998, which caused heavy rainfall and flooding in eastern Africa (WMO, 1999). In contrast, the same climatic event in Tanzania was associated with lower malaria incidence (Lindsay et al., 2000), suggesting that no clear-cut answer exists to what the effects of these extreme weather events are on malaria transmission. Increased malaria incidence over the past 30 years in the highland tea estates of western Kenya could not be explained by increased temperatures or a changed climate in general (Shanks et al., 2000; 2002). Similar observations were made for other East-African countries (Hay et al., 2002a), although a re-analysis of climatic data did show a warming trend for the same countries (Patz et al., 2002). This has led to a debate on the potential causes of malaria resurgence in East African highlands, with global warming on the one hand and non-climatic events, such as the emergence of drug resistance during the 1980s on the other (Hay et al., 2002b; Patz et al., 2002). Although climatic changes may not be evident from the recent past, a warming climate in the near future, as predicted by various climate-change scenarios (IMAGE team, 2001), may have severe consequences for malaria transmission.

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 while malaria parasites have developed resistance to the drugs that should control the deadly disease. In addition to this changing geographical 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). Projections on global warming also predict that there will be significant increases in the territory where malaria can occur. The combined effects of climate change, population growth and urbanization on the population at risk (PAR), of \textit{P. falciparum} malaria in Africa is stipulated to change from approximately 0.63 billion in 2005, to 0.87 billion in 2015 and 1.15 billion in 2030 (Hay et al., 2006; Snow et al., 2006). Antimalarial-drug-resistant strains are likely to pose a greater threat in the highlands, where immunity to malaria is low or absent, than in the low lands where exposure to the disease is generally greater. It seems likely that resistant strains of falciparum malaria found in the hills may have spread from the lowlands.

The highlands were considered to be free of malaria through the 1960s but since the 1980s malaria has been increasing (Oloo et al., 1996). It is generally assumed that malaria in the highlands of western Kenya is not due to local transmission but is imported from nearby holo-endemic areas around lake Victoria by the frequent travel of the tea plantation workers and their families (Oloo et al., 1996). This could be the case with the Kisii area where the study was carried out.

\textbf{1.2.6 Malaria control}

Eradication of malaria is desirable but not achievable and thus malaria control is the option available. Malaria control simply implies reducing its health impact as much as possible through employment of resources that are available. According to WHO (1993), malaria control is based on the following interventions:

1. \textit{Case management} (diagnosis and treatment).
II. Vector control (destruction of larval breeding sites, insecticide spraying inside houses and use of insecticide-treated bed nets).

III. Detection, containment and prevention of epidemics at an early stage (Early warning system) and Prophylaxis.

IV. Strengthening of the local capacities in basic and applied research to permit and promote the regular assessment of countries' malaria situation, in particular the ecological, social and economic determinants of the disease.

1.2.7 Diagnosis of malaria

This involves the identification of malaria parasite or its antigens/products in the blood of the patient. The efficacy of the diagnosis is subject to many factors. The different forms of the four malaria species; the different stages of erythrocytic schizogony; the endemicity of different species; the population movements; the inter-relation between the levels of transmission, immunity, parasitemia, and the symptoms; the problems of recurrent malaria, drug resistance, persisting viable or non-viable parasitemia, and sequestration of the parasites in the deeper tissues; and the use of chemoprophylaxis or even presumptive treatment on the basis of clinical diagnosis can all have a bearing on the identification and interpretation of malaria parasitemia on a diagnostic test. The diagnosis of malaria is confirmed by blood tests and can be either microscopic or non-microscopic tests.

1.2.7.1 Microscopic diagnosis

Malaria parasites can be identified by examining under the microscope a drop of the patient's blood, spread out as a "blood smear" on a microscope slide. Prior to
examination, the specimen is stained most often with the Giemsa stain, to give to the parasites a distinctive appearance. This technique remains the gold standard for laboratory confirmation of malaria (WHO, 1985; Dowelts, 1989). However, it depends on the quality of the reagents, the microscope, and on the experience of the microscopist. Its advantages include differentiation of species, quantification of parasite density and ability to distinguish clinically important asexual parasite stages from gametocytes that may persist without causing symptoms.

In many developing countries, microscopy is not reliable because the microscopists are insufficiently trained and supervised. They are also overworked, the microscopes and reagents are of poor quality and often the supply of electricity is unreliable. Conversely in non-endemic countries, laboratory technicians are often unfamiliar with malaria and may miss the parasites (WHO, 1985; Dowelts, 1989). Microscopic laboratory techniques for investigating falciparum malaria include:

I. Examination of stained thick blood films to detect parasites and to examine the cells for malaria pigment.

II. Examination of stained thin blood films to identify the species when this cannot be done on thick film and to give an estimate of the percentage of red cells infected. Heavy parasitemia in falciparum malaria is associated with severe disease, which requires special treatment and follow-up. Other methods of quantifying falciparum parasites can also be used.

III. Examination of the buffy coat and red cells immediately below the coat after centrifugation of blood in a capillary tube. This can sometimes help to detect parasites when they are few in numbers, particularly late-stage trophozoites and
gametocytes. Malaria pigment is often more easily detected in this type of preparation because the white cells are concentrated (WHO, 1985; Dowelts, 1989).

1.2.7.2 Clinical (presumptive) diagnosis

In highly endemic areas particularly Africa, clinical (presumptive) diagnosis is common. In this case diagnosis is based on signs and symptoms. Lack of enough resources and trained health personnel, necessitates the employment of presumptive diagnosis as the only realistic option. Its advantages being ease, speed and low cost but acute lower respiratory tract infection can greatly increase the frequency of misdiagnosis and mistreatment in the case of presumptive diagnosis as the signs and symptoms of the disease closely mimic those of malaria (Redd et al., 1992). This type of diagnosis therefore can identify most patients who truly need antimalarials, but it is also likely to misclassify many who do not need it (Olivar et al., 1991).

1.2.7.2.1 Clinical Features

Uncomplicated (the classical but rarely observed) malaria attack lasts 6-10 hours and presents as cold stage (sensation of cold and shivering), hot stage (fever, headaches, vomiting and seizures in young children) and sweating stage (sweats, return to normal temperature and tiredness). More commonly, the patient presents with a combination of the following symptoms: fever, chills, sweats, headaches, nausea and vomiting, body aches and general malaise. Physical findings may include elevated temperature, perspiration, weakness and enlarged spleen. In P. falciparum malaria, additional findings
may include mild jaundice, enlargement of the liver and increased respiratory rate (Warell, 1992).

Severe malaria occurs when *P. falciparum* infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism. The manifestations of severe malaria (Warell, 1992) include, cerebral malaria, with abnormal behavior, impairment of consciousness, seizures, coma, or other neurologic abnormalities, severe anemia due to hemolysis (destruction of the red blood cells), hemoglobinuria (hemoglobin in the urine) due to hemolysis, pulmonary edema (fluid buildup in the lungs) or acute respiratory distress syndrome (ARDS). This may occur even after the parasite counts have decreased in response to treatment and abnormalities in blood coagulation and thrombocytopenia (decrease in blood platelets), cardiovascular collapse and shock. Other conditions that should raise concern are: acute kidney failure, hyperparasitemia, where more than 5% of the red blood cells are infected by malaria parasites, metabolic acidosis (excessive acidity in the blood and tissue fluids), often in association with hypoglycemia, (low blood glucose). Hypoglycemia may also occur in pregnant women with uncomplicated malaria, or after treatment with quinine (Warell, 1992). Severe malaria occurs most often in persons who have no immunity to malaria or whose immunity has decreased. These include all residents of areas with low or no malaria transmission, young children and pregnant women in areas with high transmission. In all areas, severe malaria is a medical emergency and should be treated urgently and aggressively (Warell, 1992).
Neurological defects may occasionally persist following cerebral malaria, especially in children. Such defects include troubles with movements (ataxia), palsies, speech difficulties, deafness and blindness. Recurrent infections with *P. falciparum* may result in severe anemia. This occurs especially in young children in tropical Africa with frequent infections that are inadequately treated. Malaria during pregnancy (especially *P. falciparum*) may cause severe disease in the mother, and may lead to premature delivery or delivery of a low-birth-weigh baby. On rare occasions, *P. vivax* malaria can cause rupture of the spleen or acute respiratory distress syndrome (ARDS). Nephritic syndrome (a chronic, severe kidney disease) can result from chronic or repeated infections with *P. malariae*. Hyperreactive malarial splenomegaly (also called "tropical splenomegaly syndrome") occurs infrequently and is attributed to an abnormal immune response to repeated malarial infections. The disease is marked by a much enlarged spleen and liver, abnormal immunologic findings, anemia, and a susceptibility to other infections such as skin or respiratory infections (CDC, 2004).

1.2.7.3 Antigen detection tests (rapid or “dipstick” tests)

In this technique, parasite antigens are detected through rapid immuno-chromatographic techniques. Multiple experimental tests have been developed targeting a variety of parasite antigens (Mackey *et al.*, 1982; Fortier *et al.*, 1987; Khusmith *et al.*, 1987). Various kits including ParaSight(R) Beckton-Dickinson, Malaquick(R)ICT are available to perform rapid diagnostic tests and are based on the detection of Histidine-Rich Protein 2 (HRP II) of *P. falciparum*. These tests yield rapid and highly sensitive diagnosis of *P. falciparum* infection as compared to light microscopy (WHO, 1996; Craig and Sharp,
1997). The advantages for this technique are that no special equipment is required, minimal training is needed, the test and reagents are stable at ambient temperatures and no electricity is required. The disadvantages currently are the high cost per test and an inability to quantify the density of infection. In addition for tests based on HRP II, detectable antigens can persist for days after adequate treatment and cure. Therefore, the test cannot adequately distinguish a resolving infection from treatment failure due to drug resistance, especially early after treatment (WHO, 1996). A test based on detection of a specific parasite enzyme (Lactate Dehydrogenase, pLDH) has been developed (optimal\textsuperscript{TM} Flow Inco. Portland, OR, USA) and reportedly only detects viable parasites, eliminating prolonged periods of false positivity post-treatment (Beck, 1999; Piper et al., 1999; Palmer et al., 1999).

1.2.7.4 Molecular diagnosis

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

1.2.7.5 Serological diagnosis

Serological tests detect antibodies against malaria parasites using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA). Specific serological markers have been identified for each of the four species of human malaria. A positive test generally indicates past infection. Serology is not useful for diagnosing acute infections because detectable levels of antimalarial antibodies do not appear until weeks into infection and persist long after parasitaemia has resolved. Moreover, the test is relatively expensive and not widely available (WHO, 1985).

1.2.7.6 Biochemical tests

According to WHO (1985), biochemical tests that find use in diagnosis of malaria include:

I. Measurement of hemoglobin; this is especially important in cases of high parasitemia.

II. Blood urea or serum creatine if renal damage is suspected.

III. Testing of urine for free hemoglobin if water fever is suspected.

IV. Tests for liver functions if liver cell damage is suspected. Measurement of serum bilirubin, albumin and aspartate aminotransferase can provide useful information.

V. A platelet count and measurement of plasma fibrinogen, if disseminated intravascular coagulation (DIC) or other bleeding disorder is suspected.
1.2.8 Malaria and HIV infections

Malaria and HIV are among the two most important global health problems of our time. Together they cause more than four million deaths per year. Malaria and HIV/AIDS are both diseases of poverty and causes of poverty and they share determinants of vulnerability. Given the wide geographic overlap in occurrence and the resulting co-infection, the interaction between the two diseases clearly has major public health implications (WHO, 2004).

The consequences of such interactions are particularly serious for reproductive health. Co-infected pregnant women are at very high risk of anemia and malarial infection of the placenta. As a result, a considerable proportion of children born to women with HIV and malaria infection has low birth weight and is more likely to die during infancy. It is unclear whether malaria during pregnancy increases the risk of mother-to-child transmission of HIV, as studies examining this relationship have shown conflicting results. Among adult men and non-pregnant women HIV/AIDS may augment the risk of malarial illness, especially in those with advanced immunosuppression (WHO, 2004). In areas of unstable malaria transmission, HIV-infected adults with low CD4 cell counts may also be more susceptible to treatment failure of antimalarial drugs. Furthermore, acute malarial episodes temporarily increase viral replication and hence HIV viral load. As an important cause of anemia, malaria frequently leads to blood transfusions, which is a potential risk factor for HIV infection (WHO, 2004).
1.2.9 Malaria chemotherapy

1.2.9.1 Anti-malarial drugs and their mode of action

The effectiveness of early diagnosis and prompt treatment as the principal technical components of the global strategy to control malaria is highly dependent on the efficacy, safety, availability, affordability and acceptability of antimalarial drugs. The effective antimalarial therapy not only reduces the mortality and the morbidity of malaria, but also reduces the risk of resistance to antimalarial drugs. Antimalarial chemotherapy is thus the cornerstone of malaria control efforts. The existing antimalarial drugs are categorized by WHO using two major criteria, namely:

I. How they exert their action on target organism (biological activity).

II. The chemical class to which they belong (chemical class) (WHO, 1984).

Over the past few years, scientific progress in malaria chemotherapy has concentrated on the improvement of existing drugs than the development of new ones. However, the search for new antimalarial drugs has regained importance due to resurgence of malaria in many countries.

1.2.9.1.1 Biological activity;

Based on biological activity, antimalarial drugs fall into the following classes;

Blood schizontocides exert their action primarily on asexual intra-erythrocytic stages of human plasmodia (WHO, 1984). Some of the drugs in this group are shown in Table 1 below:
Table 1: Blood schizontocides

<table>
<thead>
<tr>
<th>Main site of action</th>
<th>Compound</th>
<th>Chemical class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Para-amino benzoic acid (PABA)</td>
<td>Dapsone</td>
</tr>
<tr>
<td>2</td>
<td>Folate metabolism (PABA incorporation blockers)</td>
<td>Sulfadoxine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sulfalene</td>
</tr>
<tr>
<td>3</td>
<td>Folate metabolism (DHFR inhibitors)</td>
<td>Proguanil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyrimethamine</td>
</tr>
<tr>
<td>4</td>
<td>Hemoglobin digestion products</td>
<td>Chloroquine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amodiaquine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quinine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mefloquine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Halofantaine</td>
</tr>
<tr>
<td>5</td>
<td>Protein metabolism</td>
<td>Artemisinin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Artesunate</td>
</tr>
</tbody>
</table>

Tissue schizontocides are of two types, namely primary tissue schizontocides (causal prophylactic drugs) that affect the pre-erythrocytic schizonts and hypnozoitocides, that affect the latent forms, the hypnozoites (dormant forms in the liver that cause relapses in *P. vivax* and *P. ovale* infections) (WHO, 1984). Some of these drugs are shown Table 2 below. Gametocytocides destroy the sexual forms of the parasite in the blood and therefore prevent transmission of the infection to the mosquito. Chloroquine and quinine have gametocytocidal activity against *P. vivax* and *P. malariae*, but not against *P. falciparum*. Primaquine has gametocytocidal activity against all plasmodia, including *P. falciparum*. Sporontocides prevent the development of oocysts in the mosquito and thus abate the transmission. Primaquine and chloroguanide (proguanil) have this action (WHO, 1984).
Table 2: Tissue schizontocides

<table>
<thead>
<tr>
<th>Stage affected</th>
<th>Site of action</th>
<th>Compound</th>
<th>Chemical class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Pre-erythrocytic</td>
<td>Mitochondria</td>
<td>Primaquine</td>
<td>8-aminoquinoline</td>
</tr>
<tr>
<td>schizont</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Pre-erythrocytic</td>
<td>Folate metabolism</td>
<td>Proquanil</td>
<td>Biquanide</td>
</tr>
<tr>
<td>schizont</td>
<td>PABA incorporation</td>
<td>Pyrimethamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sulfadoxine</td>
<td>Pyrimidine</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sulfonamine</td>
</tr>
<tr>
<td>3 Hypnozoite</td>
<td>Unknown</td>
<td>Primaquine</td>
<td>8-aminoquinoline</td>
</tr>
</tbody>
</table>

1.2.9.1.2 Chemical classification

1.2.9.1.2.1 4-Aminoquinoline

Examples are chloroquine (7-chloro-4-[4’-diethyl-amino-1’-methylbutyl-amino]-quinoline) and amodiaquine (7-chloro-4- [3’-diethyl-aminomethyl-4’-hydroxyamino] quinoline). Chloroquine is the most effective blood schizontocide against *P. vivax*, *P. malariae* and susceptible forms of *P. falciparum*. It also has the germicidal activity against *P. vivax*, *P. malariae*, *P. ovale* and immature *P. falciparum*. Amodiaquine is a blood schizontocide and more active than chloroquine against resistant strains of *P. falciparum*. However, amodiaquine is not recommended for chemo-prophylaxis because of agranulocytosis and toxic hepatitic reactions (Thomas *et al.*, 2004). The therapeutic value of this group of drugs in many countries has been seriously reduced in some *P. falciparum* malaria areas due to the development of resistance (Alder, 1992).
1.2.9.1.2.2 8-Aminoquinolines

The most commonly used is primaquine (6-methoxy-8-[4'-amino-1-methylbutylamino] quinoline). Tissue schizontocides such as primaquine are thought to act by inhibiting mitochondrial respiration in the parasite. Primaquine is used to treat liver stages of *P. vivax* and *P. ovale* infections (anti-relapse drug) and is also a gametocytocidal drug against *P. falciparum* especially in areas with chloroquine or multi drug resistance (Wernsdorfer and Trigg, 1988).

Both 4- and 8-aminoquinolines have two highly electronegative nitrogen atoms while the related aryl alcohols have one. These drugs act on the growing intra-erythrocytic stages, actively digesting hemoglobin. 4-aminoquinolines further have a rapid effect on the hemoglobin-containing digestive vacuole of intra-erythrocytic parasites causing fusion of adjacent vesicles followed by sequestration of the fused vesicles into a large autophagic vacuole (Warhurst, 1986).

1.2.9.1.2.3 Quinolinemethanol

These include quinine (6-methoxy-α-[5-vinyl-2-quinuclidinyl]-4-quinolinemethanol, and mefloquine  α-[2-pineridyl-2,8-bis[tri-fluoromethyl]-4-quinolinemethanol]. Quinine isolated from cinchona tree is a blood schizontocide. It is effective against the asexual blood forms of all plasmodia. In addition it has activity on sporozoites and primary erythrocytic stages. Several quinolines including chloroquine, mefloquine and primaquine were developed using quinine as a template.
Mefloquine is a blood schizonticide. It has been used to treat *P. falciparum* malaria resistant to chloroquine and SP. In combination with sulfadoxine and pyrimethamine (MSP), it was used to treat malaria in Thailand but resistance to MSP has been reported (Nosten *et al.*, 1991). Chloroquine and related quinoline drugs exert their antimalarial activity by interfering with detoxification of ferriprotoporphyrine IX (FP IX), a hemoglobin-digestion product (WHO, 1984). This is by complexing with FP IX in the acid food vacuoles and the toxic FP IX-drug complex poison the food vacuole thus killing the parasite (WHO, 1987).

### 1.2.9.1.2.4 Lumefantrine

Lumefantrine (2-dibutylamino-1-[2,7-dichloro-9-(4-chlorobenzylidene)-9H-fluoren-4-yl]-ethanol), a racemic fluorine derivative, was synthesized originally by the Academy of Military Medical Sciences in Beijing, China. It conforms structurally, physicochemically, and in mode of action to the aryl amino alcohol group of antimalarial agents including quinine, mefloquine, and halofantrine. Preliminary studies of the pharmacokinetic properties of lumefantrine are reminiscent of those of halofantrine, with variable oral bioavailability (augmented considerably by fats), a large apparent volume of distribution, and a terminal elimination half-life for malaria estimated initially at approximately 4 to 5 days (Ezzet, 2000).

A four-dose regimen of artemether-lumefantrine has proved highly effective in studies conducted in Africa, India, and China, but in Thailand, which harbors the most drug-resistant *P. falciparum* in the world, cure rates were inferior to those seen with the 3-day artemesunate-mefloquine combination (Ezzet, 2000).
1.2.9.1.2.5 Antifolate drugs

These drugs inhibit the synthesis of folic acid (Jawetz, 1989). They include dihydrofolate reductase (dhfr) inhibitors and para-aminobenzoic acid incorporation inhibitors (PABA blockers). These drugs are various combinations of dhfr inhibitors (Proguanil, Chlorproguanil, Pyrimethamine, Trimethoprim) and sulfa drugs (Dapsone, Sulfalene, Sulfamethaxazole, Sulfadoxine). These compete with dihydrofolate (Ferone et al., 1969; Zolg et al., 1989; Siriwarporn et al., 1990, 1997) and interfere with the action of dihydropteroate synthase (dhps) (Zhang and Meshnick, 1991) respectively. Typical combinations include sulfadoxine-pyrimethamine (SP, Fansidar®), sulfalene-pyrimethamine (Metakelfin®), sulfamethoxazole-trimethoprim (Co-Trimoxazole®), and chlorproguanil-dapsone (LapDap®). Fully reduced folate cofactors are essential for the key one-carbon transfer reactions needed for nucleotide biosynthesis and amino acid metabolism (Sherman, 1998).

1.2.9.1.2.5.1 Dihydrofolate reductase (DHFR) inhibitors

Examples include proguanil (N’[p-chrolophenyl-n-isopropyl-diguanidine), cycloguanil, pyrimethamine (2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine) and trimethoprin (2,4-diamino-5-[3’,4’,5’-trimethoxybenzyl]. The use of dihydrofolate reductase inhibitors as chemoprophylactic agents is not recommended due to widespread resistance of P. falciparum. Dihydrofolate reductase is a key enzyme in folate metabolism since it converts the pteridine ring to the tetrahydro reduction state required for reactions in which the folate cofactors are synthesized. DHFR inhibitors competitively inhibit DHFR by competing with dihydrofolic acid. Their affinity for plasmodial enzyme is 100-1000
times more than the host enzyme, hence their selective toxicity (Wernsdorfer and Trigg, 1988).

1.2.9.1.2.5.2 Dihydropteroate synthase inhibitors (PABA blockers)

These drugs compete with para- amino benzoic acid (PABA) and include both sulfonamides and sulfones. Sulfonamides include sulfadoxine (N’-[5,6-dimethoxy-4-pyrimidinyl]-sulfanilamide and sulfalene (N-3-methoxy-2-pyracilyl]-sulfanilamide). The pyrimethamine and sulfadoxine are used in combination (Fansidar), since they exhibit potentiating effect (synergism) (Alder, 1992). The combination is effective against chloroquine-resistant strains of *P. falciparum*.

Sulfones include dapsone (4,4-diamino diphenylsulfone), which is a blood schizontocide with no activity against sporozoites and trophozoites and works like sulfonamides (Peters and Richards, 1984). Activity against human parasites by these PABA blockers appears to be restricted to an effect on the asexual blood cycle. No effect is observed on the gametocyte stage of any species. These have selective toxicity as they compete with para-amino benzoic acid (PABA) for the binding site of the enzyme dihydropteroate synthase which catalyses the condensation of PABA with phosphorylated pteridine to form dihydropteroate. This in turn is converted to dihydrofolate, which is used as a cofactor in the formation of precursors of purines required for nucleic acid synthesis. Mammalian cells can produce their dihydrofolate directly from dietary folic acid (Wernsdorfer and Trigg, 1988).
1.2.9.1.2.6 Antibiotics

Antibiotics are used in combination with other antimalarials to augment their activity. These include tetracyclines, doxycycline and minocycline. Tetracycline has potent but slow action against asexual erythrocytic stages of all plasmodia, active against the primary intrahepatic stages of *P. falciparum* and is used for both treatment and prophylaxis. Doxycycline and minocycline have similar activities (Wernsdorfer and Trigg, 1988).

In areas where response to quinine has diminished, tetracyclines are often used in combination with quinine to improve cure rates. Clindamycin offers limited advantage as compared to other available antimalarial drugs. Parasitological response is slow to clindamycin and recrudescent rates are high (Kremsner *et al.*, 1989; 1994). It has been postulated that the antimalarial effects of antibiotics are as a result of inhibition of mitochondrial protein synthesis by direct action in the mitochondrial ribosomes. This explains the relatively slow clinical effects of antibiotics as antimalarials (Wernsdorfer and Trigg, 1988). Antibiotics are also thought to inhibit parasite growth through the inhibition of "prokaryote-like" protein synthesis in the apicoplast, an organelle unique to the apicomplexa parasites (Waller, 2000).

1.2.9.1.2.7 Phenanthrenemethanols

These include halofantrine (1,3-dichrolo-\-[2-dibutylaminol-ethyl]-6-(trifluoromethyl)-9-phenanthrene methanol). This is effective against *P. falciparum* strains that are resistant to chloroquine, pyrimethamine and quinine as well as against *P. vivax*. Its mode of action is similar to that of quinine and chloroquine (Peters, 1987; Blauer, 1988).
1.2.9.1.2.8 Sesquiterpene lactones

These include qinghaosu (artemisinin), artemether (methylene derivative of artemisinin, artesunate – sodium salt (sodium succinyl salt of artemisinin) and arteether.

Artemisinin was originally isolated from the Chinese herb, Artemisia annua, which was used as a febrifuge for many centuries. Artemisinin is effective against chloroquine-resistant and sensitive strains of *P. falciparum*. The water-soluble derivatives of artemisinin, artemether, and arteether are more effective antimalarial agents than the parent compound (Peters, 1987). It has been suggested that the mode of action of artemisinins and its derivatives may be on parasite protein synthesis. Since inhibition of incorporation of H-isoleucine into proteins of *P. falciparum* in vitro was observed within one hour of drug administration.

The semi-synthetic derivatives artesunate, artemether and arte-ether are all metabolized to dihydroartemisinin, the main active agent in the body. These compounds have shown very rapid parasite clearance times and faster fever resolution as opposed to quinine and thus are anticipated to reduce chances of development of drug resistance to partner drugs (White, 1999). These drugs kill growing parasites including young rings by interacting with Heme to produce carbon-centered free radicals that alkylate proteins and damage parasite micro-organelles and membranes (Meshnick et al., 1996). Artemisinins concentrate in the food vacuole and are thought to exert their activity through the interaction with heme. They undergo oxidoreductive cleavage of their peroxide bond in the food vacuole, most probably through interaction with Fe (II) heme (Meshnick et al.,
1996; Meshnick, 2002). This generates fatal free-radical-induced damage to the parasite. The exact mechanisms by which free radicals are generated and the mechanism of parasite death are still matters of debate (Olliaro et al., 2001).

Different workers have suggested that SERCA-pfATPase is the target for artemisinins (Krishna et al., 2004; Jambou et al., 2005; Uhlemann et al., 2005). It has been demonstrated that artemisinins inhibit the sarcoendoplasmic reticulum Ca$^{++}$ ATPase ortholog of *P. falciparum* with marked specificity. A single amino acid in trans membrane segment 3 of SERCA can determine susceptibility to artemisinins. An L263E replacement of a malarial by a mammalian residue abolishes inhibition by artesminin.

Introducing residues found in other *Plasmodium spp* also modulates artesminin sensitivity, suggesting that the artesminins interact with the thapsigargin-binding cleft of the susceptible SERCAs (Eckstein-Ludwig et al., 2003; Uhlemann et al., 2005). The short half-life of both the parent semi-synthetic derivatives and the dihydroartesminin metabolite necessitate treatment over 5-7 days when these compounds are used alone.

They are therefore being used with long half-life drugs to reduce treatment time and increase individual compliance (WHO, 2001).

### 1.2.9.1.2.9 Acridines and other compounds

Acridines include mepcarine (2-methoxy-6-chrolo-9-[4-diethylamino-1-methylbutyl amino]-acridine) and miscellaneous compounds that include dabequine (WHO, 1984; AID, 1985). Pyronaridine, an acridine derivative, has shown 100% efficacy in Cameroon (Ringwald et al., 1996) and between 63% and 88% in Thailand (Looareesuwan et al., 1996).
1.2.9.1.2.10 Atovaquone

Atovaquone (hydroxy napthoquinone) is a novel drug developed by Wellcome Trust research laboratories. It is a new antimalarial agent that has undergone advanced stages of clinical trials (Canfield et al., 1995). Its combination with proguanil has been shown to have a synergistic effect in vitro (Canfield et al., 1995), and in vivo (Looareesuwan et al., 1996; Radloff et al., 1996). Atovaquone interferes with mitochondrial electron transport chain. It is effective against CQ-resistant *P. falciparum*. When used alone resistance to it develops rapidly, thus it is used in combination with Proguanil (Looareesuwan et al., 1996; Radloff et al., 1996).

1.2.10 Global distribution and mechanisms of antimalarial drug resistance

Drug resistance has been 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. Such resistance may be relative, i.e. yielding to increased doses of the drug tolerated by the host or complete, i.e. withstanding maximum doses tolerated by the host (Wernsdorfer and Trigg, 1988). Drug resistance in micro-organisms may be due to a variety of mechanisms including non-genetic physiologic adaptations of the organism to the drug, general changes such as mutational events followed by drug selection, non-adaptive changes or a combination of all these mechanisms (Wernsdorfer and Trigg, 1988; Kun, 1999).
Resistance to antimalarials has been documented for *P. falciparum*, *P. vivax* and recently, *P. malariae*. In *P. falciparum*, resistance has been observed to almost all currently used antimalarials (amodiaquine, chloroquine, mefloquine, quinine and sulfadoxine–pyrimethamine) except for artemisinin and its derivatives. The geographical distributions and rates of spread have varied considerably. *P. vivax* has developed resistance rapidly to sulfadoxine–pyrimethamine in many areas. Chloroquine resistance is confined largely to Indonesia, East Timor, Papua New Guinea and other parts of Oceania. (White, 1999; WHO, 2006). Chloroquine resistance by *P. falciparum* was first suspected in South America in the late 1950’s and confirmed in 1959 in Thailand. More countries in East Asia and South America became affected in the following years. In 1978, East Africa became affected and it was initially detected in Kenya and Tanzania (WHO, 1984). *P. falciparum* CQ-resistance was first reported from the eastern region in Kenya (Fogh *et al.*, 1979) and Tanzania (Campbell *et al.*, 1979) in the late 1970s and it spread from east to west. A few years later, the number of countries with CQ-resistance had increased considerably in Africa (WHO, 1984). By 1989, the distribution of CQ-resistance was almost identical to that of *P. falciparum* (Wernsdorfer and Payne, 1991). This has made CQ to be dropped by many countries where falciparum malaria is a problem.

1.2.10.1 Resistance to 4-aminoquinolines

It has been suggested that chloroquine-resistant parasites produce no haematin. This is thought to be as a result of more efficient proteolytic activity of such parasites compared to the chloroquine-sensitive ones. It has also been reported that the protease activity in resistant parasites is 700-800 times compared to the sensitive ones (Macreadie *et al.*, 2000). Red cells infected with chloroquine resistant *P. falciparum* accumulate less
chloroquine than chloroquine-sensitive strains. It was thus suggested that ferriprotoporphyrin IX is more efficiently sequestered in resistant parasites so that it is not available for chloroquine binding. Another suggestion is that resistance to this class of compounds is a multigenic phenomenon which involves mutations of the genes which code for transport proteins involved in the uptake of these drugs (Macreadie et al., 2000).

1.2.10.2 Resistance to antifolate drugs

The combination of sulfadoxine and pyrimethamine is being extensively used against *P. falciparum* malaria. In Kenya, the MoH had declared a ban on the use of chloroquine as the first-line drug against malaria, replacing it with SP drugs like Fansidar®. Fansidar® has also been widely used for prophylaxis (suppression) and this might have accelerated the occurrence of resistance. Resistance has been reported in Thailand, Vietnam, Burma and Malaysia. Failures of prophylaxis and treatment with SP have also occurred in persons who had contracted *P. falciparum* malaria in East Africa, especially in Kenya and Tanzania. Resistance to other drugs such as mefloquine has also been reported and cross-resistance of *P. falciparum* to drugs is not a rare occurrence (Wernsdorfer and Trigg, 1988; EANMAT, 2001; 2003).

1.2.10.2.1 Mechanisms of resistance to the *dhfr* inhibitors

It has been shown that resistance to *dhfr* inhibitors (type II antifolates) such as pyrimethamine can be attributable to mutants showing alterations in the enzyme, alterations in the transport of the drug across the cell membrane and gene amplification, i.e. an increase in the number of genes which result in the production of larger amounts of
the enzyme in resistant cells. Pyrimethamine-resistant mutants show various degrees of cross reaction to sulfonamides and sulfones. Mechanisms through which parasites may survive the action of antifolates include use of alternative pathways, modifications of drug-transport mechanisms, increase in drug-inactivating enzymes, production of mutant enzymes which have low affinity to the drug and gene amplification leading to increased synthesis of blocked enzymes (WHO, 1984; Michelle, 2004).

The evidence suggesting that the major mechanisms of resistance to pyrimethamine in *Plasmodium* spp, was due to altered drug binding to *dhfr* is consistent with most work that has analyzed the enzyme kinetics of *dhfr* in the rodent malaria parasites, *P. bergei*, *P. vinckei* and *P. chabaudi*, as well as studies in *P. falciparum*. The most striking feature in this work is the almost universal finding that the inhibition constant (Ki) for pyrimethamine of the *dhfr* enzyme had increased in pyrimethamine-resistant strains and isolates (Ferone, 1970; McCutchan *et al.*, 1984; Walter, 1986; Siriwaraporn and Yuthavong, 1984; Chen *et al.*, 1987; Zolg *et al.*, 1989). One exception to this finding was the observation that the *dhfr* activity from pyrimethamine-resistant *P. falciparum* isolates had the same Ki 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 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 coding for the \textit{dhfr} enzyme, from \textit{P. falciparum} (Bzik \textit{et al.}, 1987; Cowman \textit{et al.}, 1988) and \textit{P. chabaudi} (Cowman and Lew, 1989a,b) allowed a detailed analysis of alteration in the enzymes and the role in mechanism of resistance. The \textit{dhfr} enzyme had previously been shown to co-purify with \textit{thymidylate synthase (ts)} in \textit{Crithidia fasculata} (Ferone and Roland, 1980), and the \textit{dhfr} and 'ts' genes were confirmed for \textit{P. falciparum} when it was shown that a single reading frame (Bzik \textit{et al.}, 1987; Cowman \textit{et al.}, 1988) encoded both activities (Siriwaraporn \textit{et al.}, 1990), a property that is shared by most protozoan organisms (Garrett \textit{et al.}, 1984; Grumont \textit{et al.}, 1986).

Analysis of alterations in the \textit{dhfr} gene of experimentally induced pyrimethamine-resistant \textit{P. chabaudi} and \textit{P. falciparum} strains has shown that both amplification and mutation of the gene can occur (Inselburg \textit{et al.}, 1987; Cowman and Lew, 1989; 1990; Tanaka \textit{et al.}, 1990a, b; Watanabe and Inselburg, 1994). Induction of pyrimethamine resistance in \textit{P. chabaudi} and \textit{P. falciparum}, by slowly increasing the level of anti-folate drug, resulted in increased expression of \textit{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 to 15 chromosomes. Increased selection for high levels of pyrimethamine resulted in mutated \textit{dhfr} gene from serine to asparagine at position 108 (Cowman and Lew, 1990). This is equivalent to mutation that has been found to be important in resistance to pyrimethamine in field isolates of \textit{P. falciparum}.

Analysis of genetic cross between pyrimethamine-resistant and pyrimethamine-sensitive \textit{P. falciparum} parents demonstrated that the \textit{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 high levels of drug resistance (Sirawaraporn et al., 1997). These studies provide strong suggestive evidence that the amino acid differences found between pyrimethamine-sensitive and pyrimethamine-resistant *P. falciparum* are 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 conferring *in vitro* pyrimethamine resistance (de Pecoulas et al., 1996), although genotype without this mutation has been described (Wang et al., 1997b). An asparagine to isoleucine change in position 51 and cystine 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 asparagine 108 and arginine -59 mutations have been found in *P. falciparum* lines that are highly resistant to both pyrimethamine and cycloguanil (Basco *et al.*, 1995; 1998; Reeder *et al.*, 1996). Ala-16 to val-16 and ser-108 to Thr-108 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.*, 1990; 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.

### 1.2.10.2.2 Mechanism of resistance to sulfonamides and sulfones

The parasite may survive the action of these drugs (type I antifolates) by bypassing the step at which PABA is incorporated into dihydropteroate and it is possible that they may be able to achieve this by utilizing host-cell folates. (Wernsdorfer and Trigg, 1988). It has been shown that some *P. falciparum* lines can make efficient use of exogenous folates either as folic acid or as folinic acid. This salvage of folates can by-pass the blockade of endogenous folate biosynthetic pathway by the sulphur-based drugs, such as sulfadoxine...
and dapsone. Analysis of the genetic-cross progeny revealed that the folate-utilization allele was mapped to a region of chromosome 4 close to *dhfr* gene but was distinguishable from the gene itself (Macreadie *et al.*, 2000).

The sulphur drugs are an important group of anti-malarial compounds and have generally been used in combination with *dhfr* inhibitors such as pyrimethamine. Sulfadoxine, the most commonly used sulphur drug, has been used extensively in combination with pyrimethamine. A sulphur-based drug dapsone (a sulfone) is used in combination with chlorproguanil and registered as Lapdap (Winstanley, 2001; Mutambingwa *et al.*, 2001). The sulphur drugs are known to inhibit the enzyme *dhps* from murine malarial parasites *P. bergei* (Ferone, 1973), and the enzyme from *P. falciparum* (Zhang and Meshnic, 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 covert the drug to its sulfa analogue, which would inhibit dihydropteroate synthase, the next enzyme in the folate biosynthetic pathway. The gene encoding the enzyme *dhps* has been cloned, and is on chromosome 8 and encodes for a bi-functional enzyme with 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (PPPK) at the 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). 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 five highly conserved positions within the *dhps* gene (codons 436, 437, 540, 581 and 613) have been found in sulfadoxine-resistant parasites (Triglia *et
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 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 changes from Ala to Gly-437 gave the parasite a 5-fold increase in sulfadoxine resistance and accumulation of further mutations raised the levels of resistance 24-fold (Triglia *et al.*, 1998).

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).

**1.2.10.3 Resistance to sesquiterpene lactones**

Resistance of *P. falciparum* field isolates to *in-vitro* artemether has been reported (Jambou *et al.*, 2005). Different workers have suggested that SERCA-pfATPase is the target for artemesinins (Krishna *et al.*, 2004; Jambou *et al.*, 2005; Uhlemann *et al.*, 2005). A single amino acid in trans-membrane segment 3 of SERCA can determine susceptibility to artemesinins. An L263E replacement of a malarial by a mammalian
residue abolishes inhibition by artemisinins. Introducing residues found in other *Plasmodium* spp also modulates artemisinin sensitivity, suggesting that the artemisins interact with the thapsigargin-binding cleft of the susceptible SERCAs (Eckstein-Ludwig *et al.*, 2003; Uhlemann *et al.*, 2005).

### 1.2.11 Drug combinations and resistance.

Combination therapy (CT) is defined as "the simultaneous use of two or more antimalarial drugs with different biochemical targets in the parasite or in the tissue hosting the parasite, which are synergetic, additive, or complementary in their effect" (WHO, 2001). CT can be either fixed combination where all components are co-formulated in a single tabulate/capsule or free combination where the components are separate tablets/capsules but are co-administered. Non-artemisinin-based CT available include: chloroquine plus SP, amodiaquine plus SP; atovaquone plus proguanil, mefloquine plus SP and chlorproguanil (Lapudrine®) plus dapsone. CTs involving an artemisinin derivative which have been recommended include: mefloquine, SP and amodiaquine with a derivative of artemisinin (White N., 1999). Lumefantrine in combination with artemether (currently available as Coartem®) is another such combination (Olliaro and Trigg, 1995; Van Vugt *et al.*, 1999). Other CTs are in the pipeline for example lap dap with artesunate, metakelfin with artesunate (Cor-arinate®) and artesunate with amodiaquine (Amornate®) (WHO, 2001).

In response to prevailing CQ resistance, Malawi, Kenya, Botswana and South Africa changed the first-line drug from CQ to SP in 1997 and other countries have followed.
However, the Useful Therapeutic Life (UTL) predicted for SP has been very short, probably because of its prolonged half-life, causing a higher probability of selecting resistant strains and consequent rapid development of resistance (Nzila et al., 2000). This is why new alternative drugs or new approaches, such as drug combinations, are urgently needed.

Drug pressure has been identified as one of the key factors in the emergence of resistance to antimalarial drugs. Selection of resistant strains can occur when a drug is misused (Wernsdorfer et al., 1991; Wernsdorfer 1994) or used alone extensively. Readily absorbed drugs with a long half-life, such as mefloquine and SP, can permit effective single-dose treatment of malaria and the following chemoprophylactic period prevents infection for several weeks. However, these drugs are likely to exert undesirable drug pressure for a long time once their concentrations drop below the critical threshold and may select resistant parasites. The contribution of monotherapy and misuse of antimalarial drugs to the emergence of drug resistance became particularly evident during the Global Malaria Eradication Campaign launched in 1955 by WHO (Bruce-Chwatt, 1956; 1986). This has been shown in Kenya where potent selective pressure for resistance operates even under conditions of supervised drug administration and optimal dosage (Watkins et al., 1997). In order to address this problem drug combinations have recently been proposed to delay the emergence and spread of drug resistance, an approach already used for highly drug-resistant infectious diseases such as tuberculosis, AIDS and leprosy (White and Olliaro, 1996; White, 1998; 1999).
The first consequence of using drugs in combination is that the starting frequencies of malaria parasites resistant to all the components are much reduced, such that the evolution of resistance is delayed compared with when its components are used alone. The second consequence is that the resistant combinations will be broken down much more frequently during recombination in meiosis: the greater the number of genes required to encode resistance, the greater the rate of loss (Hastings et al., 2000; 2001). In practice, the emergence and spread of resistant strains should be delayed and the useful therapeutic life of the combination should be much longer than its single components. A technical consultation of experts (WHO. Antimalarial Combination Therapy) organised by WHO, Geneva, in April 2001, identified the following options (in priority order) as currently available antimalarial combination therapies with a potential for introducing into the treatment policy in Africa if cost was not an issue:

I. Artemether-lumefantrine (currently available only as Coartem)

II. Artesunate (3 days) + amodiaquine

III. Artesunate (3 days) + SP (in areas where SP efficacy remains high)

IV. SP + amodiaquine (in areas where efficacy of both amodiaquine and SP monotherapy remain high; limited to countries in West Africa) (WHO 2001).

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

The efficacy of combination therapy with 4-aminoquinolines (CQ, Amodiaquine) with SP has been reviewed (McIntosh and Greenwood, 1998) which showed improved parasitological clearance compared to SP alone. Few studies have evaluated CT in Kenya (MSF, 2002; Obonyo et al., 2003) and yet it seems a viable option in fighting *falciparum* malaria. It is important to further investigate combination therapy in Kenya in order to ascertain conclusively that ACT can be employed to effectively fight *P. falciparum* malaria and clearly define the best options for use in the combination.
1.2.12 Tests for drug resistance

There are four basic methods for testing malaria parasites for drug resistance: human *in vivo* tests, *in vitro* tests, molecular characterization, and animal models (WHO 2001). Of these, only the first 3 are routinely done.

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

**In vitro tests**: In these tests, blood samples from malaria patients are obtained and the malaria parasites are exposed to different concentrations of antimalarial drugs in the laboratory. Some methods call for adaptation of parasites to culture first, while others put blood directly from patients into the test system.

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

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

Apart from increasing sensitivity of detection over ethidium bromide staining and confirming the specificity of the amplicon, hybridization with specifically designed complementary probes, can be used to detect point mutations in its nucleotide sequence. The radioisotope Phosphorus–32 is used to label the probes. Hybridization with radiolabelled probes is more sensitive than non-isotopic probing (Mifflin et al., 1987; Valentine et al., 1991). Nylon membranes can be reused repeatedly for dot blot hybridization. The probe can be stripped off the membrane after autoradiography, and re-probing carried out with another radiolabelled probes. In addition the cost of radio isotopic hybridization is less than non-radio isotopic probe based methods (Cockerill, 1999). Radioisotopes are also simple, quick and convenient for labeling probes (Sinclair, 2000). Even the short half-life of P-32, which is 14 days, has its advantages, as its disposal is less problematic.

The atom phosphorus –32 \((^{32}\text{P})\) is an unstable isotope of phosphorus-31 \((^{31}\text{P})\), with a nucleus containing 15 protons and 17 neutrons i.e. has excess neutrons. It regains stability by transforming the excess neutron into a proton to form the stable daughter isotope sulfur -32 \((^{32}\text{S})\). This is by emission from the parent nucleus of a negatively charged electron and a chargeless, virtually mass less particle called antineutrino. The ejected electron is the Beta (Minus) particle \((\beta^-)\).

\[
^{32}\text{P} \rightarrow ^{32}\text{S} + \beta^- + \gamma^-
\]

In reaching stability there is a surplus of energy that is shared in varying amounts between the beta \((\beta^-)\) particle and the antineutrino \((\gamma^-)\), thus the beta particle is ejected.
from the parent nucleus with a range of energies from zero to a well defined maximum.

The atom phosphorus $-32$ ($^{32}P$) has been used in many studies with great success (Haliassos et al., 1989; Beecham et al., 1991, Conway et al., 1999; Allouche et al., 2000; Abdel-Muhsin et al., 2002).

The use of radioactive isotopes has had a profound effect on the practice of medicine. (Parodi et al., 1986; Knoche, 1991). Radioisotopes were first used in medicine in the treatment of cancer. This treatment is based on the fact that rapidly dividing cells, such as those in cancer, are more adversely affected by radiation from radioactive substances than are cells that divide more slowly. Radium-226 and its decay product radon-222 were used for cancer therapy a few years after the discovery of radioactivity. Today gamma radiation from cobalt-60 is more commonly used. Cancer therapy is only one of the ways in which radioactive isotopes are used in medicine. The greatest advances in the use of radioactive isotopes have been in the diagnosis of disease. Radioactive isotopes are used for diagnosis in two ways. They are used to develop images of internal body organs so that their functioning can be examined. They are also used as tracers in the analysis of minute amounts of substances, such as a growth hormone in blood, to deduce possible disease conditions (Knoche, 1991). The high degree of specificity has been exploited for the detection of point mutations, within genomic DNA, associated with many genetic diseases (Haliassos et al., 1989). In *P. falciparum*, this approach has recently been used for the analysis of single-base polymorphisms in the merozoite surface protein-1 (MSP-1) (Conway et al., 1999), the circumsporozoite protein (CSP), (Allouche et al., 2000), and the dihydrofolate reductase (dhfr) gene (Abdel-Muhsin et al., 2002).
In these procedures autoradiography comes in hand. The photographic emulsion can be exposed by light photons, X-rays, gamma rays, or, in the case of autoradiography, by moving charged particles such as electrons or positrons (Knoche, 1991). Intensifying screens are used to reduce the exposure time or increase the sensitivity in the detection of radiolabeled samples (Knoche, 1991; Carlton et al., 2001). The process of development transforms the latent image into a visible image by reducing silver ions into twisted strands of metallic silver. After development, the image is fixed with a thiosulfate solution, which forms complexes with undeveloped silver halide and hardens the gelatin within the photographic emulsion. The image is then ready for qualitative or quantitative analysis. (Knoche, 1991; Carlton et al., 2001).
1.3 Problem Statement and Justification

Unless new strategies are deployed to combat malaria, the already enormous health and economic burden related to the disease in tropical countries is bound to worsen. Malaria is a major public health problem placing almost half of the World population at risk (PAR), with estimated mortality of one million in Africa alone annually and the PAR expected to change from approximately 0.63 billion in 2005, to 0.87 billion in 2015 and 1.15 billion in 2030 (Hay et al., 2006; Snow et al., 2006).

The main obstacle to malaria control is the emergence of drug-resistant strains of *P. falciparum*. Similar to the case in HIV/AIDS and tuberculosis, the use of combinations of antimalarial drugs reduces the risk of selecting for resistant mutants of the plasmodial parasites. Massive economic assistance will be needed to detect and treat adequately the estimated 515 million cases of malaria per year, but without radical action there is no prospect of ‘Rolling Back’ malaria (Nosten and Brasseur, 2002; Snow et al., 2005). In Kenya an estimated 8.2 million cases of malaria are reported every year, in a total population of 30 million. Malaria is responsible for the greatest number of consultations (30% of new cases in medical centers within the public health service) and is most common reason for hospital admission (over 22,000 cases per year in public hospitals) (Anon, 1997; Omar et al., 2001b; MSF, 2002). Every year, malaria kills 34,000 children younger than five years -93 children a day. Over 70% of the population is at risk with 54% at endemic risk (MSF, 2002; EANMAT. 2001;2003).
There is need to assess the mutation profiles of the parasites resistant to SP drugs from the endemic area to enhance the prediction of treatment outcomes in a given treatment including in the use of artemisinin based combination therapy (ACT). Standard clinical efficacy testing in vivo is a time-consuming and expensive method to survey levels of drug resistance; thus the need to adopt a technique that is best suited for epidemiological survey. There are currently no optimal conditions for use in mutation detection by dot blot technique suited for the application.

The purpose of this study was to get the mutation profiles of the parasites from an epidemic site, stipulate the level of drug resistance in this area and hence conclude on the usefulness of the SP drugs either individually or in combination. By the end of the study, optimal conditions for similar work were to be established and evaluate the usefulness of this technique for epidemiological applications.

1.4 Null Hypothesis

I. The prevalence of point mutations in the dhfr gene responsible for resistance to antifolate drugs used in malaria treatment in Kisii is low.

II. There is no difference in the sensitivity and specificity when dot-blot hybridization and PCR-RFLP techniques are used for detecting point mutations.
1.5 Objectives

1.5.1 General objective

To determine point mutations profiles in the dhfr gene of *P. falciparum* isolates from Kisii and laboratory clones, using dot blot hybridization and PCR-RFLP techniques, and relate their use for epidemiological studies on antimalarial drug resistance.

1.5.2 Specific objectives

I. To detect mutations in the in the dhfr gene of *P. falciparum* parasites from Kisii using dot blot hybridization technique.

II. To detect mutations in the in the dhfr gene of *P. falciparum* parasites from Kisii PCR-RFLP technique.

III. To determine the population diversity of *P. falciparum* parasites in Kisii by genotyping the polymorphic MSP-1 gene by PCR.

IV. Compare the mutation profiles of the dhfr gene for sensitivity and specificity of the two techniques in I and II above, as tools for epidemiological studies on antimalarial drug resistance

V. To stipulate the effect of a given point mutation profile with the treatment outcome when SP drugs are in use singly or in combination therapy at Kisii.
CHAPTER TWO
MATERIALS AND METHODS

2.1 Study Site

This study was carried out in Kisii district in Nyanza Province about 320 km from the Nairobi City (Figure 3). The area is mostly green throughout the year with an annual average rainfall of over 1500 mm. Temperatures range from 24 to 26°C though there are variations. The area is about 1500 m above the sea level. During the rainy season, there are ample breeding sites for *A. gambiae*, *A. arabiensis* and *A. funestus*, the important malaria vectors in the lowland areas surrounding this highland. Malaria transmission in the area is considered epidemic. The predominant malaria parasite in the area is *P. Falciparum*.

Figure 3: Map showing the study site.
Source: http://geography.about.com/library/cia/blkenya.htm
2.2 Ethical Considerations, Inclusion and Exclusion Criteria

The study was approved by Kenya Medical Research Institute (KEMRI) Nairobi, Ethical Steering Committee (ESC). Informed consent was obtained from adults or parents of children aged between six months and ten years before they were enrolled in the study. Patients visiting the outpatient clinics were enrolled in the study if they met the following criteria: consent, mono-infection of *P. falciparum* with parasitemia between 1,000 and 200,000 parasites/μL of blood, auxiliary temperature ≥ 37.5 °C or with a history of fever and no history of antimalarial drug intake during the previous week. Patients were excluded from the study if: there was administration of any additional antimalarial drugs, emergence of any non-malarial febrile illness that would interfere with the classification of the treatment outcome, patient relocation from the study site and withdrawal from the study. After sampling, treatment with SP-artesunate was carried out whereby 25mg Sulfadoxine plus 1.25 mg pyrimethamine per kg body weight was administered as a single dose with 4 mg/kg bodyweight artemate administered for 3 days. Samples were collected from all the patients who met the stated criteria without any regard to gender or age.

2.3 Sample Size

The sample size was estimated using the Double Lot Quality Assurance Methodology (DLQAM) as described by WHO in the "Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated Falciparum malaria in areas with intense transmission" protocol (WHO, 1996). The minimum number for N was 63 samples, as laid down in Annex 6 of the protocol but 117 samples were collected. P₀ defined as the
upper threshold level of clinical failure beyond which replacement of the drug under study is deemed necessary, was assumed to be 25% and $P_1$, defined as the lower threshold level of clinical failure below which it would be acceptable to continue the utilization of the drug, was assumed to be between 10 – 12.5%. With the above assumptions, the minimum sample size ($N$) derived according to the lot quality assurance method with confidence level of 95% and power of 90% was taken.

2.4 Sample Collection

Peripheral blood was collected by lancet prick from a fingertip for direct microscopic diagnosis. Some amount of blood was spotted directly onto Glass Fiber Membrane (GFM) (Whatman®) (Warhurst et al., 1991) or on 3MM Whatman filter paper (Whatman®) (Sambrook et al., 1989). Blood spots were allowed to air-dry, then placed individually in plastic bags and stored at 4°C with a silica gel (Sigma) desiccant to prevent dampness until DNA extraction was carried out.

2.5 Plasmodium falciparum Clones and Isolates

To optimize for the specificity and sensitivity of the technique, $P. falciparum$ clones (3D7, HB3, T9-94, Dd2, SL/D6, T9-96, W2, HB3) from Dr. Lisa Cartwright of University of Glasgow, which contain known $dhfr$ and $dhps$ alleles, were used. These were also used as controls in the scoring of the sample results on the autoradiograph after radio probing.
2.6 Preparation of the DNA Template

2.6.1 Glass fiber membrane samples

Preparation of the DNA template was as described by Warhurst et al. (1991). Blood spots on GFM were outlined with a pencil to clearly mark the spot. Each GFM was supported on a fresh 2.5 cm Whatman filter paper disc (Whatman®) and placed in a sintered glass vacuum filter (Millipore®) then washed with 2-3 ml, sterile distilled water to lyse blood. Blood proteins were removed by washing with 2-3 ml of sterile normal saline. The prepared GFM was then air dried at room temperature for 1-2 hours and stored in fresh-labeled self-seal bags at 4°C with desiccant to maintain dryness.

2.6.2 Filter paper samples

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

The brown solution was then removed and discarded into a beaker containing 10% bleach after microfuging the tubes for 5 seconds. This was then replaced with 1 ml of PBS, the tube inverted again, and then placed at 4°C for 15-30 minutes. Most of the PBS was then removed and discarded into the 10% bleach after microfuging the tubes for 5 seconds. Then, 50 μl of stock 20% (W/V) chelex-100® (Bio-Rad) solution and the 100 μl of DNase-free water (Sigma) were added directly to the tube containing the filter paper, after removal of the PBS wash. These were placed immediately into the heated block (Bio-Block), at 98–100 °C for 10 minutes with frequent vortex treatment at 2 minutes intervals (Ginie vortexer®) and once after incubation. After incubation the tube was spun at 10 000 x g in a microfuge (Eppendorf-5414D®) for 2 minutes and the supernatant removed to a fresh tube. This was then spun for a further 2 minutes at 10 000 x g, and then the supernatant transferred into another fresh tube. During the final transfer, care was taken not to transfer any of the chelex matrix. The DNA solution was then stored at -20°C (short term) or at -70°C (long term) in a freezer (Revco®).

2.7 Polymerase Chain Reaction (PCR)

2.7.1 PCR amplification of the dhfr gene for dot blotting

The DNA templates for PCR amplification were obtained from all clones and samples. For DNA template on GFM, a piece of the membrane was cut for the outer amplification
while for DNA extracted using chelex method, 2-5 ul of the extract was used in this amplification as the template. The DNA extracts from chelex method were thawed and the GFM cut then put in new labeled 0.2 or 0.5 ml eppendorf tubes taking care not to cause cross contaminations. The PCR pre mix was prepared while always working on ice and the amplifications done in 0.10 µM of each nest 1 primer (See appendix I), 1 X standard PCR buffer [1.5 mM MgCl₂, 50 mM KCl, 10mM Tris HCl (pH 8.3), 0.5% DMSO], 200 µM of each of the dNTPs, 1U of Taq polymerase (Roche, Promega), in a final reaction volume of 25µl or 30µl for chelex and GFM DNA extract respectively. The PCR amplification reaction volume made to 30 ul consisted of the reagents shown in Table 3 below.

Table 3: *dhfr* gene primers AMP1/2 PCR premix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final conc in PCR</th>
<th>Volume (µl) per 30µl tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X PCR Buffer (Roche, Germany)</td>
<td>1 X</td>
<td>3.0</td>
</tr>
<tr>
<td>2 mM Mixed dNTPs (Promega, USA)</td>
<td>200 µM</td>
<td>3.0</td>
</tr>
<tr>
<td>25 mM MgCl₂ (Roche)</td>
<td>1.5 mM</td>
<td>1.8</td>
</tr>
<tr>
<td>10 µM Amp1 (MWG Biotech, USA)</td>
<td>100 nM</td>
<td>0.3</td>
</tr>
<tr>
<td>10 µM Amp2 (MWG Biotech)</td>
<td>100 nM</td>
<td>0.3</td>
</tr>
<tr>
<td>Taq DNA polymerase(5 u/µl) (Roche)</td>
<td>1 U/PCR</td>
<td>0.2</td>
</tr>
<tr>
<td>DNase-free water (Sigma, USA)</td>
<td>-</td>
<td>21.4</td>
</tr>
</tbody>
</table>
The samples were run on My Cycler® (Bio Rad, UK) PCR machine under the following programme:

<table>
<thead>
<tr>
<th>Programme</th>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary denaturation</td>
<td>95°C</td>
<td>3 Min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>92°C</td>
<td>30 Sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>45°C</td>
<td>45 Sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>45 Sec</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>3 Min</td>
</tr>
<tr>
<td>Hold at</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>

Cycles 45

After this 2-5 µl of the outer amplification products were used as the template in the nested amplification. The PCR pre mix was also prepared while working on ice and the amplifications done in 0.10 µM of each nested primers (See appendix I), 1 X standard PCR buffer [1.5 mM MgCl₂, 50 mM KCl, 10mM Tris HCl (pH 8.3), 0.5% DMSO], 200 µM of each of the dNTPs, 1U of Taq polymerase (Roche, Promega), in a final reaction volume of 25µl or 30µl. The PCR pre-mix consisted of the reagents as described in Table 4 below.

Table 4: *dhfr* gene primers SP1/2 PCR premix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final conc in PCR</th>
<th>Volume per 30ul tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X PCR Buffer (Roche)</td>
<td>1 X</td>
<td>3.0</td>
</tr>
<tr>
<td>2 mM Mixed dNTPs (Promega)</td>
<td>200 µM</td>
<td>3.0</td>
</tr>
<tr>
<td>25 mM MgCl₂ (Roche)</td>
<td>1.5 mM</td>
<td>1.8</td>
</tr>
<tr>
<td>10 µM Sp1 (MWG Biotech)</td>
<td>100 nM</td>
<td>0.3</td>
</tr>
<tr>
<td>10 µM Sp2 (MWG Biotech)</td>
<td>100 nM</td>
<td>0.3</td>
</tr>
<tr>
<td>Taq DNA polymerase(5 u/µl) (Roche)</td>
<td>1 U/PCR</td>
<td>0.2</td>
</tr>
<tr>
<td>DNase-free water (Sigma)</td>
<td>-</td>
<td>16.4</td>
</tr>
<tr>
<td>Outer PCR product</td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

The samples were also run on My Cycler® (Bio Rad, UK) PCR machine under the following programme:
Primary denaturation 95°C 3 Min
Denaturation 92°C 30Sec
Annealing 45°C 30Sec
Extension 72°C 30Sec
Cycles 35
Final Extension 72°C 3 Min
Hold at 4°C

Nested PCR products were obtained from all clones and isolates. The primers used were based on conserved sequences flanking each codon in the \textit{dhfr} (See Appendices 1) as described by Plowe \textit{et al.} (1995). Controls used were as described in Table 5 below.

\textbf{Table 5: Controls for \textit{dhfr} dotblotting which were on each blot}

<table>
<thead>
<tr>
<th>Control No</th>
<th>Parasite</th>
<th>Dhfr 51</th>
<th>Dhfr 59</th>
<th>Dhfr 108</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>3D7</td>
<td>Asn</td>
<td>Cys</td>
<td>Ser</td>
</tr>
<tr>
<td>C2</td>
<td>Dd2</td>
<td>Ile</td>
<td>Arg</td>
<td>Asn</td>
</tr>
<tr>
<td>C3</td>
<td>T9-94</td>
<td>Asn</td>
<td>Cys</td>
<td>Thr</td>
</tr>
<tr>
<td>C4</td>
<td>ddH2O</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

\textbf{2.7.2 PCR amplification of the \textit{dhfr} gene for RFLP}

Parasites were examined for antifolate-resistance associated point mutations in the \textit{dhfr} (chromosome 4) gene using a nested PCR/RFLP method described by Duraisingh \textit{et al.}, (1998). The nested PCR approach was used to amplify regions of \textit{dhfr} gene containing antifolate associated point mutations. The nest 1 or outer amplification reaction volume was 30μl containing a sector of prepared GFM or 5 μl DNA from chelex extraction,
0.25μM of each nest 1 primer (See appendix I), 1 X standard PCR buffer [1.5 mM MgCl2, 50 mM KCl, 10 mM Tris HCl (pH8.3), 0.5% DMSO], 200 μM of each of the dNTPs, 1 U of Taq polymerase (Roche, Promega).

The reaction was allowed to proceed for 1cycle at 94°C for 3 mins, then 40 cycles at 94°C for 1 min, 50°C for 2 min, 72 °C for 2 mins and finally 1 cycle at 72°C for 10 min. The nest II reaction consisted of 0.25μM of each nest II primers (See appendix I), standard PCR buffer, 200 μM of each of the dNTPs, 1 U of Taq polymerase, and 5 μl of nest 1 PCR products as template. Tubes were briefly centrifuged and placed in a thermocycler, (Bio-Rad / MJ Research). The reaction was cycled 40 times at 94°C for1 min, 45°C for 1min, 72°C for 2min and a final extension step of 72°C for 10 min. The amplification product for the M3-F/ and F-M4 primers were analysed for the expected 522 and 326 base pairs nested PCR gene fragments respectively.

2.8 Restriction Digests of the Dihfr Gene

These were done as described by Duraisingh et al. (1998). PCR amplification of the regions flanking the mutations was done using mutation specific primers as outlined above. Restriction digestion were carried overnight at optimum temperatures for each enzyme (Table7) in the presence of 1X buffer, 1X BSA (where required) 1-2 units of the specific enzyme to a total volume of 15μl and at optimal conditions as indicated by the suppliers (New England Biolabs, UK). The volumes used in the restriction digest are shown in the Table 6 below.
Table 6: Restriction digests of the *dhfr* gene premix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final conc.</th>
<th>Volume per tube V=15ul</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X NEB Buffer</td>
<td>1X</td>
<td>1.5</td>
</tr>
<tr>
<td>100X NEB BSA</td>
<td>1X</td>
<td>0.15</td>
</tr>
<tr>
<td>Restriction Enzyme Eg.20,000u/ml XmnI</td>
<td>1U</td>
<td>0.05</td>
</tr>
<tr>
<td>DNase free water</td>
<td></td>
<td>8.3</td>
</tr>
</tbody>
</table>

Restriction enzymes used for specific codons and the expected fragments are as in Table 7 below. A volume of 5 μl of the PCR product was used without purification and when the incubation temperature was above 50°C, samples were overlaid with few drops of mineral oil.

Table 7: Restriction enzymes for the *dhfr* gene

<table>
<thead>
<tr>
<th>Codon</th>
<th>Amplification fragment</th>
<th>Restriction enzyme</th>
<th>Incubation temp (°C)</th>
<th>Product in base pairs (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dhfr 51</td>
<td>M3-F/</td>
<td>Tsp509I</td>
<td>65</td>
<td>154 &amp; 64 for wild</td>
</tr>
<tr>
<td>Dhfr 59</td>
<td>F-M4</td>
<td>XmnI</td>
<td>37</td>
<td>163,137 &amp; 26 Mutant 189 &amp; 137 Wild</td>
</tr>
<tr>
<td>Dhfr 108</td>
<td>F-M4</td>
<td>AluI  BsrI BstNI</td>
<td>37  65  60</td>
<td>180 &amp; 119 for Ser allele 180 &amp; 146 for Asn allele 181 &amp; 145 for Thr allele</td>
</tr>
</tbody>
</table>

2.9 Genotyping

Molecular genotyping of the isolates was done to identify cases of mixed strain infections. This technique was used to assess the genetic diversity and complexity of the parasite population. This was done by targeting some polymorphic genes of the *P.*
*falciparum* parasites, such as; Msp-1, Msp-2 or Glurp using specific primers as described by Ranford–Cartwright *et al.* (1997). Specific primers are as listed (see appendix II). The PCR analysis of each gene involved 2 rounds of amplification, using nested-primers in the second round. This approach increases the sensitivity of PCR detection and allows DNA extracted from samples with low parasitaemia to be amplified successfully. Following electrophoresis, sizes (in terms of numbers of base pairs [bp]) were calculated according to their mobility relative to molecular size standards run on the gel.

### 2.10 Product Analysis

Target gene amplification products for either dot-blotting, restriction or genotyping were confirmed by analysis using agarose gel electrophoresis. Agarose gel was prepared at a concentration of 1.5-2 % (w/v), depending on the fragment being analyzed, using molecular grade agarose (Sigma) in 1X TAE or TBE buffer. For DNA product visualization 0.5 μg/ml of ethidium bromide (Promega) was included in the gel preparation for staining. On to the wells of the gel 3μl loading buffer (Promega) mixed with 5 μl of the amplified product, were loaded. This was then left to run for 30 minutes at 80 Volts electrode potential on horizontal electrophoresis tank (Bio Rad®) submerged with 1X TAE buffer. DNA amplification products were visualized under ultraviolet light on a transilluminator (Vilber Lourmat®) and the results photographed using Polaroid® camera and Polaroid® instant films, or stored as a soft copy on the gel documentation system (Vilbert Lourmat®: France).
2.11 Restriction Digests Data Recording, Processing and Analysis

After restriction digest, the restriction fragments that resulted were used to score the isolates based on the controls used. Isolates were scored either as wild type or mutants compared to the specific enzyme used in a given digest and as indicated by the controls. Data recording was done on MS Excel sheets, MS Access database and also on the laboratory workbook. Statistical computer software were used for data processing and analysis.

2.12 Dot Blot Membrane Preparation

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

Table 8: Dhfr gene amplification products denaturation mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Conc.</th>
<th>Volume per 30 ul Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>0.1 M EDTA</td>
<td>10 mM</td>
<td>3</td>
</tr>
<tr>
<td>4 M NaOH</td>
<td>0.4 M</td>
<td>3</td>
</tr>
<tr>
<td>DNase Free water</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>
A piece of nitrocellulose membrane (Millipore®) was cut to the exact size to fit the
manifold. This was then pre-wetted by soaking in TE buffer (10 mM Tris-Cl: 1 mM
EDTA) or 2X SSC for 10 minutes. The nitrocellulose membrane was fitted on to the dot
blotting apparatus (Bio Rad®) by layering it on the apparatus avoiding bubbles in
between the layers, and the apparatus assembled as per the manufacturers instructions
then the screws tightened in a diagonal crossing. The membrane was then re-hydrated by
pipetting 200 µl of TE buffer into each well and this was then drained. Each denatured
sample was then loaded into two non-adjacent positions on the blot by loading 30 µl in
the two positions as illustrated below.

Figure 4: Duplicate loading of sample and controls
The solution was then allowed to stand for 30 minutes before releasing the vacuum. The membrane was then removed and neutralized by washing it in 2X SSC for 30-60 seconds followed by a 30-60 seconds wash in 0.4M NaOH to ensure complete denaturation of immobilized DNA and a final rinse in the neutralizing solution (1M Tris-HCL, 1.5M NaCl, pH 8) for 30 seconds. UV cross-linking in an UV cross linker (Vilber Lourmat®) for 5 minutes was carried out to fix the PCR products onto the membrane. This was by placing the membrane on a clean glass plate and putting in the cross linking chamber set at 0.120 joules for 5 minutes. The membrane was then wrapped using cling film and stored at -20°C until it was needed.

2.13 Labeling of Oligonucleotide Probes

Probes for all the possible alleles in each codon were labeled; for example: Thr, Ser and Asn probes (MWG Biotech) for codon 108 and Arg and Cys probes for codon 59 of the dhfr gene. Ten picomoles of each probe were labeled with 10 µCi of [γ-32P] dATP using 10 units of polynucleotide kinase. This was prepared by adding 25 µl water to the tube containing the Ready To-Go T4- Polynucleotide kinase (T4 PNK) (Amersham Biosciences, UK, Cat 27-0737-01) then the tube was incubated at room temperature for 2 to 5 minutes, as recommended by the manufacturer. The contents of tube were then mixed by pipeting up and down briefly. 1 µl of each 10 µM 5'-ends oligonucleotide was added and the reaction volume made to 49 µl using sterile water (23 µl). From this stage all procedures were carried out in a radiation containment room, using beta-shields (e.g. 1
cm acrylic) for protection from the radiation, and wearing appropriate personal radiation monitors (e.g. film badges). Solid and liquid wastes were disposed of according to the advice of the local Atomic Energy Agency/radiation protection advisors. To the reaction mix of 1 μl of [γ-32P] dATP (Amersham Biosciences, UK: Redivue [γ-32P] ATP, 3000 Ci/mmol: Cat No. AA00068) was added and mixed gently. These were then spun briefly in a microfuge to collect the contents at the bottom of the tube followed by incubation at 37°C for 30 minutes in a programmable heating block (Eppendorf®, USA). To stop the reaction, 5 μl of 250 mM EDTA was added into the tube.

2.14 Removal of Unincorporated [γ-32P] ATP

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

The prepared blot was unwrapped and placed into a rotor bottle, making sure that there were no overlapping areas, into which 20 mls hybridization buffer was added (0.25-0.125 ml per cm² of membrane). The hybridization buffer consisted of 5X SSPE, 5x Denhardt’s reagent, 0.5% SDS, 0.02 mg/ml sonicated salmon sperm DNA (Gibco) in DNAse-free water. This was then incubated at the appropriate temperature for the different probes as listed in Table 9, in a hybridization oven for 30 minutes with agitation making sure that the bottle was closed properly and the buffer did not leak. 20 μl of the labeled oligonucleotide probe (1 μl for every 1 ml of the hybridization buffer) was then added into the bottle contents. Hybridization at appropriate temperature for at least 5 hours with agitation followed. Overnight hybridization was preferred especially when the labeled probe was 2-3 weeks old.

The hybridization solution was poured and disposed off accordingly. An excess (at least 1ml/cm² blot) of wash buffer 1 (2 X SSC) was added at the corresponding temperature and incubated with agitation in the oven for 20 minutes at the same temperature as shown in Table 9. The washes were repeated once or twice using excess (at least 1 ml/cm² blot) wash buffer 2 (1 X SSC/0.1% SDS) at the same temperature and incubated with agitation for 10 minutes each. The washing solutions were poured and disposed off accordingly then the blot sealed by wrapping in cling film without allowing the blot to dry out.
Table 9: Hybridisation and washing conditions for *dhfr* probes

<table>
<thead>
<tr>
<th>Locus</th>
<th>Probe name</th>
<th>First wash</th>
<th>Stringent washes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHFR-108</td>
<td><em>dhfr</em>108Ser-specific</td>
<td>2XSSC, 20 min @ 48°C</td>
<td>[1XSSC/0.1%SDS) 10 min x 2 @ 48°C</td>
</tr>
<tr>
<td></td>
<td><em>dhfr</em>108Asn-specific</td>
<td>2XSSC, 20 min @ 48°C</td>
<td>[1XSSC/0.1%SDS) 10 min x 2 @ 48°C</td>
</tr>
<tr>
<td></td>
<td><em>dhfr</em>108Thr-specific</td>
<td>2XSSC, 20 min @ 47°C</td>
<td>[1XSSC/0.1%SDS) 10 min x 2 @ 47°C</td>
</tr>
<tr>
<td>DHFR-51</td>
<td><em>dhfr</em>51Ile-specific</td>
<td>2XSSC, 20 min @ 53°C</td>
<td>[0.5XSSC/0.1%SDS) 10 min x 2 @ 53°C</td>
</tr>
<tr>
<td></td>
<td><em>dhfr</em>51Asn-specific</td>
<td>2XSSC, 20 min @ 53°C</td>
<td>[0.5XSSC/0.1%SDS) 10 min x 2 @ 53°C</td>
</tr>
<tr>
<td>DHFR-59</td>
<td><em>dhfr</em>59Arg-specific</td>
<td>2XSSC, 20 min @ 51°C</td>
<td>[0.5XSSC/0.1%SDS) 10 min x 2 @ 51°C</td>
</tr>
<tr>
<td></td>
<td><em>dhfr</em>59Cys-specific</td>
<td>2XSSC, 20 min @ 51°C</td>
<td>[0.5XSSC/0.1%SDS) 10 min x 2 @ 51°C</td>
</tr>
</tbody>
</table>

2.16 Auto-radiography

The sealed blot was taped right side up (DNA-side up) into an autoradiography cassette (Kodak) with intensifying screens. To avoid problems with autorad orientation, a marker strip was used or the film folded at bottom right corner and this allowed for accurate positioning of the autorad after developing. Blots were exposed on Kodak® (Rochester, NY, USA) X-Omat film for 12-24 hours at −70°C in the freezer (Revco®, UK). The films were then removed from the cassette and developed to score the sample against the controls. A second piece of film could also be exposed to the membranes for longer, to assist in the detection of weak bands. If any of the controls showed non-specific
hybridization, an extra stringent wash was carried out. The autorad was obtained by developing the image in a developer solution (Kodak, USA) for 5 minutes followed by a brief rinse in clean water and finally fixing the developed image in a fixative solution (Kodak) for 5 minutes and the fixative rinsed off with clean tap water. These processes were done in a dark room as the films are light sensitive. The film was then air dried and scored.

2.17 Stripping the Probe from the Membrane

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

2.18 Radio-Probing Data Recording, Processing and Analysis

After radio-probing, parasite clones and isolates were scored either as wild type or mutants relative to the specific probe used in a given blot and as indicated by the controls. Data recording was done on MS Excel sheets, MS Access database and also on the laboratory workbook. Statistical computer software were used for data processing and analysis.
CHAPTER THREE

RESULTS

3.1 Laboratory Microscopy on Samples Collected

A total of 267 samples were collected from the study site. These were all pre-treatment blood samples collected on glass fiber membranes (GFMs). These were all positive cases according to the microscopists at the study site. A total of 117 (43.8%) of the sampled population were analyzed, 55 (47%) of which were females, while 61 (52%) were males. One isolate (1.3%), which was lab microscopy positive, was not recorded for the age and sex. Seventy-nine (66.6%) of the study samples were children under five years while 37 (31.6%) were patients over five years.

After repeating microscopy at the KEMRI laboratory, 74 of the isolates were confirmed as positive, whereas 42 turned out to be negative. Microscopy for one of the samples was not done. Among the lab microscopy positive isolates, 56 were from patients less than five years while 17 were from the over 5 years group.

Table 10: Microscopy of samples from patients aged 5 years and below

<table>
<thead>
<tr>
<th>Sex</th>
<th>Positive(%)</th>
<th>Negative(%)</th>
<th>Not Rec(%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>32 (27.3)</td>
<td>11 (9.4)</td>
<td>0(0)</td>
<td>43(36.7)</td>
</tr>
<tr>
<td>Females</td>
<td>24(20.5)</td>
<td>11(9.4)</td>
<td>1(1.3)</td>
<td>36(29.9)</td>
</tr>
<tr>
<td>Total</td>
<td>56(47.8)</td>
<td>22(18.8)</td>
<td>1(1.3)</td>
<td>79(66.6)</td>
</tr>
</tbody>
</table>
Table 11: Microscopy of samples from patients aged 5 years and above

<table>
<thead>
<tr>
<th>Sex</th>
<th>Positive (%)</th>
<th>Negative (%)</th>
<th>Not rec (%)</th>
<th>Totals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>9 (7.6)</td>
<td>9 (7.6)</td>
<td>0 (0)</td>
<td>18 (15.4)</td>
</tr>
<tr>
<td>Females</td>
<td>8 (6.8)</td>
<td>11 (9.4)</td>
<td>0 (0)</td>
<td>19 (16.2)</td>
</tr>
<tr>
<td>Totals</td>
<td>17 (14.5)</td>
<td>20 (17.1)</td>
<td>0 (0)</td>
<td>37 (31.6)</td>
</tr>
</tbody>
</table>

3.2 Amplification of the Dhfr Gene by PCR for Dot Blotting

Gene fragments for dot blotting were obtained from both the quality control samples from and the field isolates using using AMP/SP primers by nested PCR. Analysis of the amplification products was done by agarose gel electrophoresis using a 100 base pair molecular weight marker (Roche). This gave expected product size was 720 base pairs of the dihydrofolate reductase (dhfr) gene using AMP/SP primers.

Positive controls and a water negative control were used in all the runs. Quality control samples and clinical specimens PCR products are shown in Figure 5 below. Eighty five-eight (72.6%) of the study samples gave positive amplification by PCR while 32 (27.4%) of the samples had no detectable amplification. Comparing microscopy and PCR (Figure 6), 71 (60.68%) of the study samples were both PCR and microscopy positive while 29 (24.78%) were negative. 3 (2.58%) of the samples, which were microscopy positive, turned out to be PCR negative while 13 (11.1%) of the samples, which were microscopy
negative, were PCR positive. One of the samples was not recorded for microscopy results but was PCR positive

Figure 5: Agarose gel electrophoresis of the quality control samples

Mk is the 100 base pair molecular weight marker (Roche) which gives an intense band at 500 base pair. Fragment. Wells 1, 3, 5, 10, 11, 12, 13, 14, 16, 17, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 36, 37, 38, 39, 45, 46 and 47 show positive amplifications.
Wells 2, 4, 6, 7, 8, 9, 12, 15, 19, 34, 35, 48 and 49 had no detectable amplification.
Well 40 was a water negative PCR control.
Wells 41 to 44 were gel electrophoresis negative controls, ie no PCR products were loaded.
Figure 6: Comparison between microscopy and PCR

<table>
<thead>
<tr>
<th>No</th>
<th>Microscopy</th>
<th>M+VE</th>
<th>M-VE</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>71</td>
<td>PCR +VE</td>
<td></td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>3</td>
<td>PCR -VE</td>
<td></td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>74</td>
<td>TOTALS</td>
<td>42</td>
<td></td>
<td>116</td>
</tr>
</tbody>
</table>

M +VE – Microscopy positive
M -VE – Microscopy negative
PCR +VE – Molecular positive
PCR -VE – Molecular negative

3.3 Amplification of the Dhfr Gene by PCR for RFLP Analysis

M3-F/ and the F-M4 primers nested PCR products obtained were 522 and 326 base pairs respectively (Figure 7). Standard laboratory lines of *P. falciparum* were used as controls (negative and positive control) for wild type and mutant type for each polymorphic site on *dhfr* gene. Both the quality control samples and the field samples (N=117) were amplified for the 326 bp M3-F PCR products. All the quality control samples, (Figure 7 and 8), gave the expected results. For the field samples (Figure 9), 89 (76.07%) isolates had an amplification product while 28 (23.93%) of them did not amplify
Figure 7: M3 F/ and the F M4 primers nested PCR products

Mk - 100 base pair marker
Wells 1-8 the 326 bp M3- F/ primers PCR products
Wells 9-13 the 522 bp F-M4 primers PCR products

Figure 8: F-M4 primers nested PCR products of the control samples

Mk – 100 base pair marker
Wells 14-26 the 522 bp F-M4 primers PCR products on the quality control samples
Figure 9: The F M4 primers nested PCR products for the field samples

Wells 1, 2, 3, 4, 7, 13, 14, 16, 17, 18, 19, 20, 22, 23 and 25 on 2% agarose gel on a potential difference of 80V for 30 minutes, had amplification products. Wells 6, 8, 9, 10, 12, 15, 21 and 24 gave no amplification products. Well 5 had the negative water control.

3.4 Restriction Digests

Digestion of the 326 bp F-M4 product with XmnI restriction enzyme gave three fragments for the mutant strains and two fragments for the wild types. These were 163, 137, 26bp for the mutant and 189, 137 bp for the wild type strains. Restriction digest of the 326bp fragment of the F M4 primers was done on the quality control samples from Glasgow (Figure 10), and also on the clinical field samples (Figure 11).

After restriction digestions of the dhfr 59 using XmnI, 27 (23.1%) of the sample were wild types Cys 59 (TGT) while 55 (47.0%) were mutant types Arg 59 (CGT). Seven (5.9%) of the total samples showed mixed clones of both Cys 59 and Arg 59, while 28 (22.2%) of the samples were not determined. (Figure 12).
Figure 10: Restriction fragment length polymorphisms - control samples

These were run on 2% agarose gel on a potential difference of 80 Volts for 30 minutes. 
Well 1 - water negative control for the digest, 
Well 6 - mutant type control (Dd2) restriction fragments sized at 189 and 137bp 
Well 7 contains an un-cut (non restricted nest II product) control. 
Wells 2,3,4,5,8,9,10,11 and 12 had the wild type controls.

Figure 11: Restriction fragment digests on the clinical samples

Mk -100bp ladder 
Well 1 had a mutant type control (Dd2) with restriction fragments sized at 189 and 137bp 
Well 2 had a wild control (3D7) with fragments sized at 163,137 and 26bp. 
Well 11 contains an un-cut (non restricted nest II product) control. 
Well 22 had the water negative control.
Figure 12: Restriction digestions of *dhfr 59* using *Xmn1* clinical samples

<table>
<thead>
<tr>
<th>ALLELE</th>
<th>wd</th>
<th>mt</th>
<th>xd</th>
<th>na</th>
<th>np</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>27</td>
<td>55</td>
<td>7</td>
<td>26</td>
<td>2</td>
</tr>
</tbody>
</table>

wd- Wild type isolates  
mt- Mutant isolates  
xd- Mixed infections isolates  
na- non amplified samples  
np- restriction digest not done

3.4.1 Restriction Digests; Resolution of the Digestion Products

To allow for clear separation of the digestion fragments, the agarose gel was run and visualized at different periods. Prolonged run times led to fading away of some restriction fragments and complete disappearance of others as observed in Figures 13 A-C.
Figure 13: RFLP for clinical samples; effects of prolonged gel electrophoresis in enhancing resolution

These were run on 2% agarose gel on a potential difference of 80Volts. Well 10 had the un-cut control
A-after 30 minutes,
B-after 50 minutes and
C-after 60 minutes
3.5 Mutation Profiles by Radio Probing of the *Dhfr* Gene

All the quality control samples and the field isolates were hybridized with the relevant probes for the specific codons in the *dhfr* gene. This was at the specific hybridization conditions after optimization. Figure 14 and 15 show the effect of using unoptimized and optimal conditions. Figures 16 to 21 show the hybridization conditions used for the different probes in the specific codons. Mutation profiles after dot blotting *dhfr* 108 showed that there were mutations in 69 (58.9%) of the study samples with no wild type, *dhfr* 59 showed 14 (11.97%) wild and 67 (59.82%) being mutant type while *dhfr* 51 had 2 (1.7%) wild and 70 (59.82%) being mutants as summarized in figure 22 below.

On further evaluation as shown in table 12 below, it was observed that 56 (47.8%) of the study population carried mutations in the three codons that were studied and these represented 80% of the PCR positive samples. Sixty-one (52.1%) of these samples had double mutations in *dhfr* codons 108 and 59 and this was equivalent to 87.1% of the PCR positive isolates.

Looking into codon *dhfr*108 which has three possible alleles, (Figure 24), the observed mutations were found to be *Asn* in 57 (83.3%) of the mutant samples, *Thr* in 4 (5.88%) of the samples while 7 (10.29%) isolates had both *Asn* and *Thr* alleles. These represented 48.72%, 3.42% and 5.98% of the study population respectively.
Figure 14: Autoradiograph showing unoptimized conditions
Autoradiograph showing unoptimized thus giving non specific hybridization by the Cys59 probe (dhfr59Cys:GAAATATTTTTGTCAGTTAC). Sample position A1, A2, A3 and A4 consist of *P. falciparum* clones alleles, i.e. 3D7, Dd2, T9/94 and HB3.

Figure 15: Autoradiograph showing optimized conditions
Autoradiograph showing optimized conditions thus giving specific hybridization by the Arg59 probe (dhfr59Arg:GAAATATTTTCGTGCAGTTAC). Sample position A1, A2, A3 and A4 consist of *P. falciparum* clones alleles, i.e. 3D7, Dd2, T9/94 and HB3. The blot was hybridized for 10 hours at 52.5°C stringent washes were as described and exposed for 14 hours.
Figure 16: Autoradiograph showing hybridization using Ser 108 specific probe
Hybridization using Ser 108 specific probe (dhfr108Ser: AACAAAGCTGCGAA AGCATTCCAA) showing specific hybridization with the wild type control 3D7 that has S108 allele-AGC. The blot was hybridized for 10 hours at 53°C stringent washes were as described and exposed for 14 hours.

Figure 17: Autoradiograph showing hybridization using Asn 108 specific probe
Hybridization using Asn 108 specific probe (dhfr108Asn: AACAAACTGGG AAAAACTGCGAA AGCATTCCAA) showing specific hybridization with the mutant type control Dd2 that has N108 allele-AAC. The blot was hybridized for 10 hours at 48°C stringent washes were as described and exposed for 14 hours.
Figure 18: Autoradiograph showing hybridization using \textit{Thr} 108 specific probe
Hybridization using \textit{Thr} 108 specific probe (\textit{dhfr}108\textit{Thr}:AACAACCTG
CGAAACCATTCCAA) showing specific hybridization with the mutant type control
T9/94 that has T108 allele-ACC. The blot was hybridized for 10 hours at 48°C stringent
washes were as described and exposed for 14 hours.

Figure 19: Autoradiograph showing hybridization using \textit{Cys} 59 specific probe
Hybridization using \textit{Cys} 59 specific probe (\textit{dhfr}59\textit{Cys}:GAAATATTTTT
GTGCAGTTAC) showing hybridization with reduced specificity the wild type control
T9/94 that has C59 allele-TGT. The blot was hybridized for 10 hours at 51°C stringent
washes were as described and exposed for 24 hours.
Figure 20: Autoradiograph showing hybridization using Asn 51 specific probe
Hybridization using Asn 51 specific probe (dhfr51Asn:CCATGGAAATGT AATCGCTAG) showing specific hybridization with the wild type control 3D7 that has N51 allele- AAT. The blot was hybridized for 10 hours at 51°C stringent washes were as described and exposed for 14 hours. Autorad 353 shows the fainting signals due to reduced activity of the radiomarker after its first halflife.

Figure 21: Autoradiograph showing hybridization using Ile51 specific probe
Hybridization using Ile51 specific probe (dhfr51Ile:CCATGGAAATGTATTTCGCTAG ) showing specific hybridization with the mutant type control Dd2 that has C59 allele- ATT. The blot was hybridized for 10 hours at 51°C stringent washes were as described and exposed for 14 hours
Figure 22: Mutation profiles of the *dhfr* gene
No- sample number
NA- no amplicons
N/OP- Dot blotting not done

Table 12: The prevalence of double and triple mutations in *dhfr* by probe hybridization

<table>
<thead>
<tr>
<th>Allele</th>
<th>MUTANT</th>
<th>NA</th>
<th>WILD</th>
<th>N/OP</th>
</tr>
</thead>
<tbody>
<tr>
<td>108</td>
<td>69</td>
<td>47</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>59</td>
<td>67</td>
<td>36</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>51</td>
<td>70</td>
<td>44</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type</th>
<th>On Total (%)</th>
<th>(%) of PCR +Ves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triple (108+59+51)</td>
<td>56 (47.8)</td>
<td>(80)</td>
</tr>
<tr>
<td>Double (108+59)</td>
<td>61 (52.1)</td>
<td>(87.1)</td>
</tr>
</tbody>
</table>
3.6 Mutation profiles of the dhfr gene by radioprobing compared to RFLP

Results of the two methods in detection of the point mutation in the dhfr codon 59 were compared, (Table 13). Fifteen (12.82%) isolates which had been characterized as wild type by RFLP turned out to be mixed infections by radioprobing. In addition 29 (24.79%) of the isolates which had been characterized as mutant type were actually mixed. Five (4.27%) samples were characterized as wild strains by both techniques while 12 (10.26%) samples were characterized as mutants. Only one (0.85%) isolate that was wild type by RFLP was characterized as mutant by radioprobing but 5 (4.27%) RFLP mutant isolates were characterized as wild type. One isolate (0.85%) that was wild type by radioprobing was mixed by RFLP but 3 (2.56%) isolates were characterized as mixed by both techniques. Two (2) samples (1.7%) were not processed for RFLP though they had been characterized as mixed strains by radioprobing. Further categorization is as in Table 13
below. The proportions in parenthesis show percentage to the total of the radioprobing outcome.

Table 13: Dot blotting Vs RFLP on codon 59

<table>
<thead>
<tr>
<th>RFLP</th>
<th>wd (%)</th>
<th>mt (%)</th>
<th>xd (%)</th>
<th>na (%)</th>
<th>TOTALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wd(%)</td>
<td>5(35.7)</td>
<td>1(7.69)</td>
<td>15(27.27)</td>
<td>6 (17.14)</td>
<td>27(23.07)</td>
</tr>
<tr>
<td>mt(%)</td>
<td>5(35.7)</td>
<td>12(92.3)</td>
<td>29(52.72)</td>
<td>9(25.71)</td>
<td>55(47.0)</td>
</tr>
<tr>
<td>xd(%)</td>
<td>1(7.14)</td>
<td>0(0)</td>
<td>3(5.45)</td>
<td>3(8.57)</td>
<td>7(5.98)</td>
</tr>
<tr>
<td>na(%)</td>
<td>3(21.42)</td>
<td>0(0)</td>
<td>6(10.91)</td>
<td>17(48.57)</td>
<td>26(22.22)</td>
</tr>
<tr>
<td>Totals</td>
<td>14</td>
<td>13</td>
<td>53</td>
<td>35</td>
<td>115</td>
</tr>
</tbody>
</table>

Wd – Wild type isolates  
Mt – Mutant isolates  
Xd – Mixed isolates  
Na – Non amplified

Comparing these with previous work in the area where 25.9% of the population (n=54) had point mutations of the *dhfr* gene, while 74.07% of the population bore the wild genotype (Figure 24). There were 12.96% triple mutants (isolates carrying mutations in the three codons) in the previous study compared to 56% triple mutants in the present study (Table 12). The double mutants accounted for 3.7% in the 1999 study. The results were as summarized in Figure 24 below.
3.7 Genotyping of the Field Samples

A sample of 37 isolates was genotyped to confirm multiclonality among the infections. Agarose gel photos of inner-PCR products (Figure 25), of the \textit{mspl} gene using O1/O2 and N1/N2 primers. The observed products size was 400-600bp. One of the genotyped sample (2.7%), presented three clones, seven (18.91%) had two clones each while 15(40.54%) of the samples had a single strain as shown in table 14 below. In total, eight (34%) samples that amplified had polyclonality (Table 14).
Figure 25: Genotyping the polymorphic \textit{msp}1 gene on the field samples

The gels were run on a 1.5\% agarose gel for 30 minutes.

\textit{mk} - 100bp marker

Wells 1-24 Msp1 gene products on field samples 400-600bp.

Arrows show the different clones in a sample

Table 14: Genotyping of the \textit{msp} 1 gene in 37 of the study samples

<table>
<thead>
<tr>
<th>Multiplicity</th>
<th>&lt;5</th>
<th>&gt;5</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triple</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Double</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Single</td>
<td>10</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Na</td>
<td>10</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Totals</td>
<td>28</td>
<td>13</td>
<td>37</td>
</tr>
</tbody>
</table>

<5 – patients with five years or less
>5 – patients with over five years
Na – \textit{msp} 1 gene not amplified

In the study, a total of 117 blood samples were collected and analysed from the study site.

These were all microscopy positive as per the field microscopists. On re-reading the slides, that were collected, in the laboratory by two qualified microscopists, 42 (35.9\%) of the samples turned out to be negative (Table 10 and 11).
CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

A number of studies have investigated the association between mutations in \( dhfr \) and \( dhps \) genes and the parasitologic and/or clinical response to SP (Fansidar®) treatment at an individual level (Wang et al., 1997a; Jelinek et al., 1998; Basco et al., 2000; Nagesha et al., 2001; Omar et al., 2001a). Whilst the distribution of mutations is quite different across the regions surveyed, it broadly mirrors the understanding of relative SP usage. In samples taken from individual patients before and after drug treatment, there was an association between the more highly mutated forms of \( dhfr \) and/or \( dhps \) and parasites that were not cleared by antifolate therapy (Wang et al., 1997a). In vitro analyses have clearly demonstrated that resistance to pyrimethamine (Pyr) is associated with the mutation of the amino acid Ser to Asn at codon 108 of \( dhfr \) gene. Ancillary point mutations of asparagine (Asn) to isoleucine (Ile) at codon 51 and of cystine (Cys) to arginine (Arg) at codon 59 are associated with an increase of resistance. A higher level of PYR resistance occurs in the presence of the point mutation isoleucine (Ile) to leucine (Leu) at codon 164 (Cowman et al., 1988; Snewin et al., 1989; Foote et al., 1990; Peterson et al., 1990; Basco et al., 1995; Reeder et al., 1996; Khan et al., 1997; Wang et al., 1997a,b; Nzila et al., 1998).

In this study a total of 117 blood samples were collected and analysed from the study site. These were all microscopy positive as per the field microscopists. On re-reading the slides, that were collected, in the laboratory by two qualified microscopists, 42 (35.9%) of the samples turned out to be negative (Table 10 and 11).
This is in line with previous observations that though microscopy remains the gold standard in malaria diagnosis, the results are subjective (WHO, 1985; Payne, 1988). These may depend on a number of factors including quality of the reagents, the microscope and on the experience of the microscopists. In many developing countries, microscopy in a rural setting may not be reliable because the microscopists are insufficiently trained and supervised. They are also overworked, the microscope and reagents are of poor quality and often electricity supply is unreliable or non-existent. Moreover in nonendemic countries they are often unfamiliar with the malaria parasite, differentiation of species, quantification of parasite density, inability to distinguish clinically important asexual parasite stages from gametocytes that may persist without causing symptoms and may miss them or at the same time pick dust particles or other artifacts to be parasites (WHO, 1985; Payne, 1988).

This pattern also suggests that some of those who may have been treated as microscopy negative in the field could have been actually positive. This implies that a good proportion of the patients (35.9%) may have received a wrong treatment and at the same time most probably a similar or a greater portion may have gone untreated for malaria due to misdiagnosis. Of the false positives, 18.8% were from patients who were <5 years and these are the non-immune group who are at a higher risk of infection. This also creates an avenue for increased drug pressure to the infecting parasite thus selection of the resistant strains. This problem was further expounded after running PCR on the study samples (Figure 5). From this, 11.1% of the study samples that were microscopic negative turned out to be positive with an addition of three more samples that were laboratory microscopy positive being negative showing that even microscopy with a
higher level of quality control is still subjective. PCR also confirmed 29 samples that were diagnosed as negative in the laboratory were true negatives. Indicating a higher level of sensitivity when applying molecular techniques in clinical diagnosis. Taking microscopy as the gold standard the results showed 95.95% sensitivity, 69.05% specificity, a false positive rate of 30.95%, a false negative rate of 4.05%, positive predictive value of 84.52% and negative predictive value of 90.63%, while accuracy of the test was 43.1%. From these observations, it therefore implies that only 85 (72.64%) patients received the right treatment with 32 (27.36%) being treated without infection and many more possibly missed the treatment while in the real sense they may have been infected. Others may have missed a chance for further investigation on other diseases, which have similar clinical presentations as malaria, due to misdiagnosis. This shows a case of drug misuse which is a great threat in malaria control and a great contributor to drug resistance. PCR would go far in reducing this problem if it was to be used in routine diagnosis, but unfortunately it may not be available and affordable to many in the remote countries.

Analysis of the study samples by PCR-RFLP of *dhfr* 59 using *XmnI* restriction enzyme, indicated that there were parasites with the mutant genotype, which is indicative of drug resistance. Amplification with the primers F and M4 yielded a 326-bp PCR product. Primer-F was engineered to create a restriction site, which allows the detection of the polymorphic forms of codon-59 following restriction digestion with *XmnI* restriction enzyme. The PCR product also contains another *XmnI* site, which serves as an internal control. Restriction of a wild type isolate resulted into two fragments of 189 and 137 base pairs while a mutant isolate had three fragments of 163, 137 and 26 base pairs.
(Duraisingh et al., 1998). Presence of 47.0% mutant isolates implies that a higher number has a mutation in dhfr 108 as this follows a sequential order. The level observed in the study was higher than what had been previously observed (Figure 24) in this area (Omar, 2002). This implies that the level of drug resistance has been on the increase. Isolates harbouring both the wild and mutant parasites were also picked in the study, amounting to 5.9%. This suggested that there could be mixed infections of different parasite strains in the study area. A small random sample of 37 isolates helped in understanding this clearly after genotyping using a polymorphic gene and msp1. In these, both triple and double parasite clones over and above single clone infections were picked. This was consistent with the previous findings where multiclonoality was observed in malaria infections in Africa and other parts of the world (Babiker et al., 1997; Thaithong et al., 1984) in Sudan and Thailand and also in Kenya (Omar, 2002). The observations here further compound to the problem of using microscopy in malaria diagnosis since this cannot distinguish between single and multiple infections of the same species though species differentiation is possible (Tham et al., 1999). Moreover, in cases of multiple infections drug sensitivity varies between the infecting strains and hence more selection due to drug pressure thus aggravating the problem of drug resistance. Therefore, this technique comes in hand in such cases where multiple infections are suspected. However, being a molecular technique it’s limited for routine diagnosis in developing countries due to non-availability and affordability. The technique requires the use of expensive equipments and highly trained personnel. Moreover, even in a research setup the technique can also be subjective as seen in this study where it was observed that to be able to score and characterize the isolates easily and specifically longer run time on the gels was nessesary and this resulted in loss of some restriction fragments (Figure 12).
There were difficulties in visualizing and scoring the digest fragments on agarose gels and in complete digestion, even with increased digestion time (14-18 hours) and by increasing the amount of enzyme used (1-1.5 units per sample). Prolonged electrophoresis time aimed at increasing resolution on the gel led to loss of some fragments. This makes the method a less specific and sensitive technique as observed even in other similar studies (Ranford-Cartwright et al., 2002). The PCR/RFLP technique has also the disadvantage of requiring expensive temperature-labile restriction enzymes (Ranford-Cartwright et al., 2002).

These kinds of drawbacks mitigate for the need of a revised and robust technique that is sensitive, specific and affordable that can be adopted for a study. Specific oligonucleotide probing of the study samples gave a clear picture of the nature of the profiles of mutation of the genes of interest (Figure 22). The results showed a significant shift in a rising direction of mutations in three target codons from what had been observed previously in similar study in the study area 6 years previously (Omar, 2002). The previous work in the area showed that 25.9% of the population (n=54) had point mutations of the \textit{dhfr} gene, while 74.07% of the population bore the wild genotype \textit{I} (Figure 24). The present work showed a totally different picture with an almost threefold increase in the mutant genotypes in each of the analysed codons. In fact in codon \textit{dhfr} 108, there was 100% prevalence in the mutant strains compared to 25.92% in the previous study, which shows that there has been continuous selection over time. Of interest here is that there was 12.96% triple mutants (isolates carrying mutations in the three codons) in the previous study and 56% triple mutants in the present study, showing a fivefold increase. This represented 80% of the PCR-positive samples. This was coupled
with 52.1% prevalence of the double mutants, as opposed to the 3.7% observed previously. This indicates a significant change that cannot be ignored. The situation could have arisen due to the fact that drug pressure has been applied or has been in play over time and the selection has led to the elimination of the sensitive strains, hence the predominance of the mutant strains. The presence of these mutant strains has been associated with resistance to antifolate drugs (Wang et al., 1997a; Jelinek et al., 1998; Basco et al., 2000; Nagesha et al., 2001; Omar et al., 2001a).

Observed allele prevalence in codon 108 of the dhfr gene (Figure 23) was consistent with previous studies that have reported high prevalence in the Asn allele (Peterson et al., 1988) and low prevalence in the Thr allele in the African isolates (Wang et al., 1997a). Of interest here is the sharp rise in the mutant isolates over this short time period. Also the slight rise in the prevalence of the Thr allele and especially in samples from an epidemic area, since antifolate resistance has also been linked with paired mutations of dhfr Val-16 and Thr-108 (Biswas et al., 2000). These observed prevalences indicate that SP drugs are failing in this area and continuation of their use may lead to an even greater problem. This effect may be felt even if these drugs were used as proposed, in combination with artemisinin-based drugs, hence SP may not be a good candidate for ACT. SP drugs have all along been an affordable option in malaria treatment due to their relatively low cost, with the above observations and due to the fact that the cost of ACT may have to remain high, then the cost of treating malaria is bound to worsen with phasing out of cheap drugs like SP.
The use of dot blot hybridization technique in this study provided a specific and more sensitive way of profiling the mutation types of a study population than even the PCR-RFLP. Of interest here is the ability by dot blotting to pick some mixed parasite strains in isolates that were characterized as either wild or mutant when using PCR-RFLP (Table 13). Sensitivity in picking some minor populations, for example, in cases of mixed infections as it was seen by msp1 genotyping is critical during diagnosis. In such mixed populations the presence of a mutant strain in a previously diagnosed wild type strain may contribute further to the problem of drug pressure hence selection. Early detection of mixed populations is important as it will assist in changing treatment policies to curb the spread of resistance. With the use of this technique large number of samples would be analysed over a relatively short time and thus per-sample cost may be relatively low hence possibility of adapting this at all levels of clinical diagnosis. However, some special facilities for this procedure are necessary for accurate results and this would be a limitation. The use of a radioactive marker may increase the risk associated with the technique due to exposure to radioactive emissions. Waste disposal can also be a drawback in this technique as all radioactive wastes, both solid and liquid, need special disposal. However, with proper and well established facilities, this is not a major concern as waste disposal is safe and easy. The short half-life of isotopes such as $^{32}$P makes it even easier and safer. Whereas non-radioactive labels can be adopted to minimize the risk involved, this may compromise the degree of sensitivity, specificity and reproducibility. The technique as used in this study may thus be adopted at an epidemiological scale in drug efficacy and resistance monitoring.
Comparison between dot blot and RFLP in detection of the point mutation in the \textit{dhfr} codon 59 showed variations in sensitivity and specificity of the techniques. Fifteen isolates that had been characterized as wild type using PCR-RFLP were actually mixed after radioprobing. Moreover, 29 isolates, which had been characterized as mutants by RFLP, contained the wild-type isolates, thus mixed clones (Table 13). This shows that dot blotting could detect some minor populations in the samples that could not have been detected by restriction digests. It is possible that these isolates contained a mixture of sensitive and resistant clones (Babiker \textit{et al.}, 1997), with one existing at a low parasitemia undetectable by the PCR-RFLP. Multiple clones are frequently found in natural \textit{P. falciparum} infections and drug-sensitive and resistant parasites often co-exist in the same infection (Thaithong \textit{et al.}, 1984). In this study this was demonstrated by genotyping the \textit{Msp1} polymorphic marker.

This work on \textit{dhfr} mutations in the light of SP treatment reveals the utility of simple, relatively rapid method and their application in surveillance programmes to detect drug-resistance mutants (Plowe \textit{et al.}, 1997). The parasite genotypes linked to SP-resistance are abundant in various parasite populations around the world (Biswas \textit{et al.}, 2000). In this study, \textit{dhfr} mutant and thus most probably SP-resistant parasites were detected in more than 47-60\% of the samples. The drug-induced selection of SP resistant genotypes is very efficient, as reported in earlier studies (Wang \textit{et al.}, 1997a), and thus the situation is bound to worsen. The association of the phenotypic resistance of the parasite and the genotype is linked to a common mutation of \textit{Asn} at position \textit{dhfr} 108 (Peterson \textit{et al.}, 1991; Biswas \textit{et al.}, 2000; 2004). Mutation at site 59 along with 108, contributes resistance against higher levels of the drug as observed in the drug sensitivity profiles in
vitro. The antifolate resistance has also been linked with paired mutations of \textit{dhfr} Val-16 and Thr-108 (Biswas \textit{et al.}, 2000; 2001). In a study based on \textit{in vitro} pyrimethamine sensitivity, a predictive hypothesis has been given for characterizing \textit{P. falciparum} isolates with various \textit{dhfr} mutations and their antifolate resistance (Watkins \textit{et al.}, 1999). Parasites exhibiting \textit{in vitro} Pyr IC50 of above 1000 nM may carry mutations of Asn-108; Ile-51 and / or Arg-59 and denote resistance to SP. Like \textit{dhfr}, there is a correlation between the frequency and number of \textit{dhps} mutations with the history and level of Fansidar\textsuperscript{R} usage in different geographical areas, which shows evidence to select \textit{dhps}-mutant strains after SP treatment. The mutations in \textit{dhps} over and above those of \textit{dhfr} would be predicted to cause SP failure (Plowe \textit{et al.}, 1997; Wang \textit{et al.}, 1997a,b; 1999; Omar \textit{et al.}, 2001a). It is thus clear that point mutations in the \textit{dhfr} and \textit{dhps} genes confer resistance to pyrimethamine and sulfadoxine, respectively, with decreasing \textit{in vitro} \textit{P. falciparum} susceptibility related to the number of mutations in each gene (Cowman \textit{et al.}, 1988; Triglia \textit{et al.}, 1997; Wang \textit{et al.}, 1997a,b). The same mutations have been linked to treatment failure in the clinical setting (Basco \textit{et al.}, 1988; Wang \textit{et al.}, 1997a,b; Vasconcelos, 2000; Nagesha \textit{et al.}, 2001; Eskandarian \textit{et al.}, 2002). However, presence of mutations in \textit{dhfr} appears to be more important in causing treatment failure than \textit{dhps} mutations (Basco \textit{et al.}, 1998). The factors promoting the development and transmission of these mutants are also clear (Gatton \textit{et al.}, 2004).

As the world waits for a malaria vaccine, case management with prompt and effective treatment remains the mainstay of malaria control (Winstanley, 2001). Most countries in resource-poor malaria-endemic areas lack current and comprehensive information on
antimalarial drug efficacy, particularly for epidemic areas, resulting in sub-optimal antimalarial treatment policies. Many African countries continue to use chloroquine and SP drugs despite very high rates of resistance, and others changed policies based on limited data with mixed success (Plowe, 2003). This study focused on a malaria epidemic site in Kenya, where SP had been proposed as a candidate for combination therapy for malaria treatment. This was to assess the failure rate on the drugs and thus predict on wheather or not SP drugs are good combination candidates in this epidemic area. Use of a failing option would lead to higher resistance due to selection by drug pressure. The results of this study indicate that the development of drug resistance is on the increase in the study area and further use of these drugs could lead to the selection of existing parasites with genetic mutations in the dihydrofolate reductase or dihydropteroate synthetase gene. This selection may also be driven by the long half-life of the SP combination. Use of SP could also result in the selection of parasites with allelic types of higher resistance within the host during an infection. The timing of treatment, relative to initiation of a specific anti-\textit{P. falciparum} erythrocyte membrane protein 1(EMP1) immune response, is an important factor during this stage, as is the treatment dosage. The end result will be clinical treatment failure becoming prevalent as the parasites develop sufficient resistance mutations to survive therapeutic doses of the drug combination (Gatton \textit{et al.}, 2004; Hastings \textit{et al.}, 2005). These results reaffirm the importance of correct treatment of confirmed malaria cases in slowing the development of parasite resistance to antimalarial drugs in Kisii and in particular when using the ACTs. Moreover sulfadoxine-pyrimethamine being a failing drug is poor candidate for these.
Allele-specific oligonucleotide hybridization methods have been widely used for the examination of single-base polymorphisms in genomic DNA, and for identification of mutations associated with drug resistance in some microorganisms and some genetic diseases (Victor et al., 1999; 2001; Haliassos et al., 1989; Beecham et al., 1991; Conway et al., 1999; Alloueche et al., 2000; Abdel-Muhsin et al., 2002). In this study, this technique was adopted and optimized to detect point mutations in codons 51, 59 and 108 of the \textit{dhfr} gene associated with high levels of resistance to pyrimethamine in the lab settings. The optimal conditions did deviate from the published ones (Abdel-Muhsin et al., 2002) but these were within the acceptable ranges. The method is specific, sensitive, and very robust. It is able to detect \textit{dhfr} alleles in samples with parasitemia less than 100 parasites/ul of blood and distinguishes minority genotypes when they coexist at very low proportions (1\%) in mixture with other clones (Abdel-Muhsin et al., 2002; Ranford-Cartwright et al., 2002). The method could be applied in the detection of mutations in genes associated with resistance to other antimalarials such as chloroquine, in which resistance has been attributed to a single point mutation in the \textit{Pfcrt} (Djimde et al., 2001; Fidock et al., 2000) and in \textit{Pfmdr1} genes (Reed et al., 2000). Also possibly applicable in artemisinins, which are now known to inhibit pfATP6, the sarcoplasmic reticulum Ca++ ATPase (SERCA) ortholog of \textit{P. falciparum}. A single amino acid alteration in the segment 3 of SERCAs can determine susceptibility to artemisinins, as have been seen in L263E replacement of a malarial amino acid residue by a mammalian one (Eckstein-Ludwig et al., 2003; Uhleman et al., 2005).
The combination of this technique and a 96-well plate PCR could allow extensive epidemiologic surveys of antimalarial genes to be carried out. This may allow analysis of evolutionary patterns of drug resistance and the subsequent identification of targets that may control the spread of the same more so in our country in face of new treatment policies. The use of a non-radioactive labeling system could be an additional advantage in this technique, eliminating the possible safety risk associated with radioactive labeling. However, the non-radioactive systems may be less sensitive in revealing low-level parasites, especially when they occur as mixtures with different genotypes. The method described here is able to detect minority parasite populations at a proportion of 1% relative to the majority clone (Abdel-Muhsin et al., 2002; Ranford-Cartwright et al., 2002). This method has a potential diagnostic role since it is sensitive enough to detect low-level resistant clones in a mixture, which may eventually increase in density through selection following treatment (Gatton et al., 2004). This molecular method and its application in surveillance programmes to detect and monitor drug-resistant strains may offer an additional tool in epidemiological surveys of drug efficacy and resistance monitoring following changes in drug deployment policy in malaria-prone countries.
4.2 Conclusions

There is an indication of a declining trend of SP drugs efficacy in Kisii, the study site. These drugs are of little use in monotherapy and are poor candidates for combination therapy in artemisinin-based combination therapy (ACT). Being failing drugs, their use will only lead to increased drug pressure thus more selection of the resistant strains. Proper and accurate diagnosis plays a critical role in the control of malaria by proper treatment of the already infected cases. Though microscopy has been one of the most affordable and readily available method, it is still lacking specificity, sensitivity and reproducability. It could be contributing heavily and negatively to the success of malaria-control strategies.

This study has demonstrated that the allele-specific hybridization method is specific and very sensitive in detecting point mutations in the *P. falciparum dhfr* gene. The method has a clear value in surveying frequencies of genes conferring drug resistance in field populations and is cost effective. The technique was found to be superior to PCR-RFLP in malaria diagnosis and resistance monitoring, especially for large-scale screening of single point mutations and/or other sequence polymorphisms in many other disease gene markers.
4.3 Recommendations

- The possibility of having molecular diagnosis in all health facilities should be explored to minimize the problem of misdiagnosis. This will allow for efficient usage of drugs, reduce drug pressure hence the spread of resistance. Meanwhile the microscopists in these facilities especially those from epidemic areas, need more training to alleviate this problem.

- It is recommended that dot blot radionuclide-based technique be adopted for the early detection of development of multidrug-resistance in the artemesinin-based combination therapies, as constant surveillance is needed. This will assist in rapid detection of new emerging multidrug-resistant foci in both endemic and epidemic areas. The technique could also be extended to other infectious diseases in the African continent.

- In established multidrug-resistant areas, for example, the lowlands neighbouring the epidemic areas like Kisii district, efforts are needed to limit geographical spread, which is a great threat to the less immune neighbours. New drug candidates are urgently needed and the malaria vaccine development enhanced.

- In as much as reliance should not be placed on drugs alone to control malaria in epidemic and endemic areas, it is vital that all measures possible should be taken to protect such new compounds by judiciously selecting combinations with other antimalarials and ensuring that essential procedure is followed in the dispensation. Rational drug use must be reinforced and proper diagnosis emphasized to limit drug pressure.
• Molecular epidemiological studies on drug resistance ought to be carried out in more areas where malaria is endemic or epidemic, to map out the extent of mutation associated drug-resistance. The possibility of using short acting antimalarial as opposed to long-acting ones need to be exploited because the long-acting drugs give the parasite a chance to select for resistance. There is urgent need for accelerated research on reversing the existing resistance by use of resistance-reversers, drug-enhancers and novel drug combinations. Studies should be done to establish whether withdrawing a drug from use would result in the disappearance of parasites resistant to that drug. This may show whether the possibility of rotating the limited number of safe, effective and affordable antimalarial drugs could be considered.


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APPENDICES

APPENDIX I

PRIMERS

DOT BLOTTING dhfr and dhps
Primer AMP1: TTTATATTTTCTCTTTTTTA
Primer AMP2: CATTTTTATATTCCGTTTTCT
Primer SP1: ATGATGGAACAGTCTGCGAC
Primer SP2: ACATTTTATTACGTTTTTC
Primer 186: GTTTAAATCACATGTTTGCACTTTCC
Primer M3717: CCATTCCTCAGTGATACACAC
Primer 185: TGATAACCCGAATATAAGCATATAG
Primer 218: ATAAATAGCTGTAGGAAGCAATTG

GENOTYPING-MSP-I and MSP-2
Primer O1: CACATGAAAGTTATCAAGAACCTTGTC
Primer O2: GTACGTCTAAATTCATTGCACG
Primer N1: GCAGTATGACAGGTTATGG
Primer N2: GATTGAAAGGTATTGAC
Primer S3: GAAGGTAATTGAAACATTTGTC
Primer S2: GAGGGATGTTGCTGCTCCACAG
Primer S1: GAGTATAAAGGGAAGATATG
Primer S4: CTAGAAACCATGCAATAGTTCC

PCR - RFLP

NESTII

dhfr
M3 5’TTTATGATGGAACAGTCTGCGACGTT3’
F/ 5’AAATTCCTTTGATAAACACGGGAACTTTTAT3’
F 5’GAAATGTAATTCCCTAGATATGgAATATT3’
M4 5’TTAATTTCACAAGTAAACTATTAGGCTTTCC’

dhps
K/ 5’TGGCTAGTGTTTATAGATATAGGatGAGcATC3’
K/ 5’CTATAACGAGGTATTgCATTTAATgCAAGAA3’
J 5’TGGCTAGTGTTTATAGATATAGGTGGAGAAgC3’
L 5’ATAGGATAACTATTTGATATGAGGAccAGGATTCg3’
L/ 5’TATTACCAACATTTTGATCATTgGCAAccGG3’

NESTI

dhfr
M1 5’TTTATGATGGAACAGTCTGC3’
M5 5’AGTATATACATGCTAAGA3’

dhps
R2 5’AACCTAAACGCTGTCGTTCAA3’
R/ 5’AATTGTGATTTTGCCACAA3’
APPENDIX II

RADIOLABELLING PROBES for *dhfr* and *dhps*

dhfr108Ser: AACAAGCTCGAAGACATTCCAA

dhfr108Asn: AACAAACTGGGAAACATTCCAA

dhfr108Thr: AACAACCTCGAAGACATTCCAA

dhfr51Ile: CCATGGAAATGTATTTCGCTAG

dhfr51Asn: CCATGGAAATGTATTTCGCTAG

dhfr59Arg: GAAATATTTTCGTGCAGTTAC

dhfr59Cys: GAAATATTTTTGTGCAGTTAC

dhps436/437Ser/Gly: GAATCTTCTGTCCTTTT

dhps436/437Phe/Gly: GAATCCTTTTGTCCTTTT

dhps436/437Ala/Gly: GAATCCGCTGTCCTTTT

dhps436/437Ser/Ala: GAATCCTCTGTCCTTTT

dhps436/437Ala/Ala: GAATCCGCTGTCCTTTT

dhps436/437Phe/Ala: GAATCCTTTGTCCTTTT

dhps540Lys: CAATGGATAAACTAACAA

dhps540Glu: CAATGGATGAACTAACAA
## APPENDIX III

### RESTRICTION ENZYMES for dhfr

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Incubation Temp</th>
<th>Point mutation</th>
<th>Retriction site</th>
<th>Pattern(bp)</th>
</tr>
</thead>
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<tr>
<td>AluI</td>
<td>37</td>
<td>$^{AGC}_{108}^{AAC}$</td>
<td>5'...$^{AG}$C$^{CT}$...3'</td>
<td>326, 196</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>3'...TC$^{GA}$...5'</td>
<td></td>
</tr>
<tr>
<td>BsrI</td>
<td>65</td>
<td>$^{AGC}_{108}^{AAC}$</td>
<td>5'...ACTGGN...5'</td>
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<tr>
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<td></td>
<td></td>
<td>3'...TGAC$^{CN}$...5'</td>
<td></td>
</tr>
<tr>
<td>Tsp509I</td>
<td>65</td>
<td>$^{AAT}_{51}^{ATT}$</td>
<td>5'...$^{A}$ATT...3'</td>
<td>163, 121</td>
</tr>
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<td>60</td>
<td>$^{AGC}_{108}^{ACC}$</td>
<td>A</td>
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<td></td>
<td></td>
<td></td>
<td>5'...CC$^{*}$GG...3'</td>
<td>181, 141</td>
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<tr>
<td>XmnI</td>
<td>37</td>
<td>$^{TGT}_{59}^{CGT}$</td>
<td>'5...GAANN$^{*}$NNTTC...3'</td>
<td>163, 137</td>
</tr>
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<td></td>
<td></td>
<td>'3...CTTNNNNAA$^{*}$G...5'</td>
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