THE EVOLUTIONARY DYNAMICS OF GENETIC DETERMINANTS OF *Plasmodium falciparum* RESISTANCE TO SULFADOXINE/PYRIMETHAMINE (SP) IN SOUTH EASTERN TANZANIA

BY

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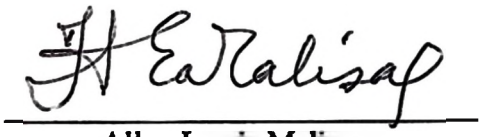
ABSTRACT

This study reports a systematic follow up of genetic changes in the genes encoding the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes of *Plasmodium falciparum*, in isolates from three rural districts of South-eastern Tanzania. The enzymes are the target of antimalarial drug, sulphadoxine-pyrimethamine (SP). The population-wide analysis of resistance mutations in the *dhfr* and *dhps* genes was applied to examine the influence of different drug use policies and their potential for the selection for SP resistance. A total of 47 244 bloodspot filter paper samples were collected from all individuals of all ages in randomly selected households in a series of annual surveys conducted between 2000 - 2006. Twenty percent (9 662) of all samples were found positive for *P. falciparum* infection on microscopy and hence were used for the genetic studies. DNA was extracted from *P. falciparum*-positive samples and *dhfr* and *dhps* genes were amplified by a nested polymerase chain reaction (PCR) and resistance conferring point mutations determined. Size polymorphisms at three sets of microsatellite loci linked to *dhfr* and three other sets of unlinked microsatellite loci were analysed by PCR amplification and electrophoresis on an automated sequencer. The influence of National treatment policy on the parasite reservoir was profound. The change of first line therapy from CQ to SP brought about highly significant increase of the frequencies of *dhfr* triple and *dhps* double mutants. Artemisinin-based combination therapy (ACT-SP+Artesunate) in Rufiji had a small and non-significant impact on the frequency of *dhps* double and *dhfr* triple mutant alleles, but significantly disrupted their

association. *Dhfr*-linked microsatellites revealed high diversity around the *dhfr* sensitive alleles and significantly reduced diversity around mutant *dhfr* alleles. The majority of triple mutant alleles had one flanking microsatellite haplotype which has previously been shown to be derived from Southeast Asia, while the double mutant alleles had multiple haplotypes which were independently derived. Distribution of major lineages indicates that there is extensive genetic exchange among the geographic regions. Unlinked microsatellites confirmed the extent of allele sharing among the regions and revealed a major trend for reduced transmission intensity, which was apparently independent of the ACT intervention.

DECLARATION

I, Allen Lewis Malisa, do hereby declare to the Senate of the Sokoine University of Agriculture that, this thesis is my own original work and has neither been submitted nor currently being submitted for a degree award in any other institution.



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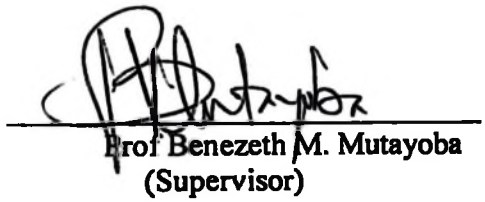

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DEDICATION

To my lovely wife Emiliana and my sons Allenbright, Emily and Emmanuel for their love, patience, support and understanding.

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ABBREVIATIONS AND SYMBOLS

Σ	Summation
χ^2	Chi square
^0C	Degrees centigrade
ACT	Artemisinin-based combination therapy
ALU	Artemether + Lumefantrine
ART	Artesunate
Bp	Base pair
CI	Confidence interval
CQ	Chloroquine
COARTEM®	Trade name for Artemether Lumefantrine
CSPD	Chemiluminescent substrate
CT	Combination therapy
DDT	Dichloro-diphenyl-trichloroethane
DHFR	Dihydrofolate reductase (*dhfr* the genomic coding for DHFR)
DHFR-TS	Dihydrofolate reductase-Thymidylate synthase
DHPS	Dihydropteroate synthase (*dhps* the genomic coding for DHPS)
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleotide triphosphates
DRC	Democratic Republic of Congo
ECF	Enhanced chemi-fluorescence

EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immuno-sorbent assay
EM	Electron microscope
g	specific gravity
gm	Gramme
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
He	Expected heterozygosity
HIV	Human Immunodeficiency Virus
ID	Identification
IMPACT	Interdisciplinary monitoring program for antimalarial combination Therapy
IPTi	Intermittent preventive treatment of infants
IPTp	Intermittent preventive treatment in pregnancy
ITN	Insecticide treated net
Kb	Kilo base pair
Kg	Kilogram
LD	Linkage Disequilibrium
MARA	Mapping Malaria Risk in Africa
$MgCl_2$	Magnesium chloride
Ml	Milliliter (s)
mM	Millimolar

Mmol/L	Milli mole per litre
MOI	Multiplicity of infection
Ng	Nanogrammes
Nm	nanometre
OD	Optical density
PABA	Paraamino benzoic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Measure of acidity or alkalinity of a solution
pmol/L	Pico mole per litre
PPPK-DHPS	Pyrophosphokinase-dihydropteroate synthase
RDT	Rapid diagnostic test
SNPs	Single nucleotide polymorphisms
SP	Sulfadoxine and Pyrimethamine
SSOP	Sequence Specific Oligonucleotide Probing
SUA	Sokoine University of Agriculture
Taq	*Thermus aquaticus*
TB	Tuberculosis
TBE	Tris, borate and EDTA
U	Units
µg/ml	Microgramme per milliliter
ug/µl	Microgramme per microliter

µl	Microlitre
µM	Micromole
USD	United states of America dollar
UTL	Useful therapeutic life
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Effective treatment of malaria is fundamental to the global strategy to control malaria. However, resistance to commonly used antimalarial drugs is a major cause of mortality and a significant drawback for controlling malaria in endemic countries. Notwithstanding, in recent years, the pace at which the parasite has developed resistance to antimalarial drugs by far surpasses the pace at which new antimalarial drugs are being developed, posing one of the huge and formidable challenge in the treatment and containment of the disease. The search for an effective vaccine against the disease continues but a successful outcome in the near future remains uncertain. It is therefore a priority to investigate ways to prolong the useful therapeutic life (UTL) of currently available antimalarial drugs, without unduly restricting the access to antimalarial drugs. The latter is key to addressing the 300 to 500 million new illnesses and nearly 3 million deaths that occur because of the disease each year, 90% of which occurring in Sub-saharan Africa (WHO, 1996).

In East and Southern Africa, spread of chloroquine (CQ) resistance begun in 1980s reaching critical levels in some countries by early 1990s (Bloland *et al.*, 1993). Malawi was the first country in Africa to replace CQ with Sulfadoxine/Pyrimethamine (SP) as first line treatment of uncomplicated malaria in 1993, followed by Kenya, Botswana and South Africa (Kwa Zulu Natal) in 1997 (Bloland *et al.*, 1993; Bloland *et al.*, 1998).

Although Tanzania did not replace CQ with SP until late 2001, as early as 1984, SP had been the policy of Muheza district designated hospital following CQ critical levels of resistance in the district (Mutabingwa *et al.*, 2001). SP resistance became widespread shortly after its adoption as first line partly because its resistance had already been established in many countries (Omar *et al.*, 2001; Kublin *et al.*, 2002; Pearce *et al.*, 2003; Roper *et al.*, 2003), following its extensive use as second line therapy and also due to its long half-life (Nzila *et al.*, 2000b). The molecular basis of *P. falciparum* resistance to SP is point mutations in the genes encoding folate biosynthetic enzymes, the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), leading to reduced binding affinity of antifolate drugs to the enzymes (Zolg *et al.*, 1989; Wu *et al.*, 1996; Triglia *et al.*, 1997). Artemisinin based combination cherapy (ACT) is the World Health Organisation (WHO) recommended replacement therapy in areas where SP failure rate prompts replacement. Already most of east and south African countries have revised their antimalarial treatment policies by adopting ACTs. Tanzania replaced SP with Artemether-Lumefantrine (COARTEM®) as first line drug for the treatment of non severe malaria in November 2006.

Policy change is very expensive and a difficult decision process given the struggling nature of the economy of most malaria endemic countries. One of the greatest challenges to this decision making process is the justifiable fear that resistance will develop rapidly to the replacement drug, initiating an endless cycle of drug replacement and escalating costs. Therefore, cost-effective strategies to delay development of resistance while

continuing to provide effective treatment to those who are ill become exceedingly important. Although use of ACT is one such strategy (White, 1999), monitoring of proper use including compliance and deterrent use of component drugs of the combination in a monotherapy form is key to the fulfilment of this goal. Similar to the strategy used extensively for treatment of tuberculosis (TB) and human immunodeficiency virus (HIV) infections, ACT aims to provide highly efficacious treatment for malaria in a way that limits the ability of resistant parasites to survive and be transmitted. There is scientific evidence suggesting that, ACT reduces gametocyte carriage by approximately 90%, potentially reducing the likelihood that resistant parasites spread within the community as well as potentially reducing overall malaria transmission rates (Price *et al.*, 1996; White *et al.*, 1998).

In order to prevent the selection of resistance to new antimalarial drugs and the spread of resistance to those in current use, it is important to understand how resistance develops and spread. In particular, it must be determined whether resistance emerges once and then spreads over a large geographic area, or whether resistance emerges many times, on many genetic backgrounds. This question can be investigated by studying the population genetics of *P. falciparum*. Short sequence repeats, known as microsatellites, have been identified throughout the *P. falciparum* genome, and over 900 microsatellite markers have now been mapped in *P. falciparum*, with one occurring approximately every 2–3 kb (Su and Wellems, 1996; Su *et al.*, 1999). Microsatellites are repeats of 2–6 bp simple sequence units found in many eukaryotic genomes and are usually found in non-coding

regions of DNA; therefore they are assumed to be selectively neutral (Goldstein and Schlotterer, 1999). They seem to be inherited according to standard Mendelian genetic principles but show high genetic variability due to their variable number of repeats. The mutation rate in areas of microsatellite DNA appears to be higher than the rest of the DNA (reviewed in Ellegren, 2000). Microsatellites are believed to evolve by replication slippage, whose rate is sufficiently high (mutation rate of 10^{3}–10^{4} per locus per generation) to drive short repeat stretches, embedded within unique DNA, into arrays of appreciable length (Levinson and Gutman, 1987). By constructing haplotypes from linked microsatellite markers, researchers can infer the evolutionary history of a resistance allele. Early studies have indicated that resistance to both chloroquine and sulfadoxine–pyrimethamine has emerged in Southeast Asia and subsequently spread to Africa (Wootton *et al.*, 2002; Nair *et al.*, 2003; Roper *et al.*, 2003; Roper *et al.*, 2004). Recently, a study conducted in western Kenya, an area of intense malaria transmission, found evidence of multiple origins of the highly resistant triple mutant *dhfr* genotype of *P. falciparum* (McCollum *et al.*, 2006).

In the present study, a high throughput polymerase chain reaction and sequence specific oligonucleotide probe based aproach (PCR-SSOP, described previously by Pearce *et al.* (2003) and used elsewhere in ELISA form (Alifrangis *et al.*, 2005), was employed to track population genetic changes occurring under two contrasting antimalarial drug policies of SP monotherapy in Kilombero and Ulanga districts and SP+artesunate (ART) combination therapy in Rufiji district using samples collected under the interdisciplinary

Monitoring Project for Antimalarial Combination Therapy in Tanzania (IMPACT-TZ). By using pairs of *dhfr*-linked and unlinked microsatellite markers this study investigated the evolutionary origin and spread of the resistant forms of the *dhfr* genotypes of the *P. falciparum*. IMPACT-Tz is a multiyear implementation research evaluation project that rests on a collaborative platform comprising the United States Centers for Disease Control and Prevention, the Ifakara Health Research and Development Centre, the National Institute for Medical Research, Muhimbili University College of Health Sciences, the London School of Hygiene and Tropical Medicine, and the Tanzanian Ministry of Health, including its National Malaria Control Programme, the Tanzania Essential Health Interventions Project, the Audit Morbidity and Mortality Project, and the Council Health Management Teams of Rufiji, Kilombero and Ulanga districts.

1.2 Objectives

1.2.1 Broad objective

To carry out a population based genetic study of *P. falciparum* genes coding for SP target enzymes dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) with the aim of analysing the genetic changes which underlie the emergence and intensification of SP resistance in the context of different treatment policies in Rufiji and Kilombero/Ulanga districts.

1.2.2 Specific objectives

(i) To establish the common *dhfr* and *dhps* point mutation haplotypes in the study communities and to measure and compare their frequencies.

(ii) To compare the frequencies of point mutation haplotypes in Kilombero/Ulanga and Rufiji districts in a sequence of cross sectional surveys carried out during the study period 2000-2006.

(iii) To determine the impact of national policy change in recommended firstline treatment for malaria from CQ to SP during 2001 by contrasting *dhfr* and *dhps* frequency changes during one year of firstline CQ (2000-2001) with changes during one year of firstline SP (2001- 2002).

(iv) To examine whether there was an impact of ACT on drug pressure by comparing *dhfr* and *dhps* point mutation haplotype changes in the Rufiji population with those in Kilombero/Ulanga at cross sectional surveys in 2002, 2004, 2005 and 2006.

(v) To determine the spatial distribution of point mutation haplotypes in the study communities by summarising the survey data for individual wards within each of the districts.

(vi) To study the polymorphic microsatellite repeats flanking the *dhfr* gene and examine the evolutionary relationships between the allelic haplotypes found at this locus.

(vii) To compare microsatellite haplotypes flanking *dhfr* and examine the extent of allele sharing (gene flow) between Rufiji and Kilombero/Ulanga sub populations.

(viii) To analyse unlinked microsatellite markers in the Rufiji and Kilombero/Ulanga populations at two time points to examine the multiplicity of infection.

1.3 Hypotheses

(i) Increased drug pressure selects for SP resistant *P. falciparum* genotypes.

(ii) Antimalarial combination therapy of SP + ART will reduce drug pressure on SP resistance mutations (measurable by frequency changes of resistance mutations at the *dhfr* and *dhps* loci).

(iii) ACT will reduce transmission (measurable by a reduction in prevalence of *P. falciparum* infection and/or by a reduction in the multiplicity of infections (MOI), which is a measure of the number of co-infecting genotypes within an infected individual).

(iv) Mutations accumulate within resistance lineages, i.e. double mutants become triple mutants and this is the molecular basis of resistance intensification in the parasite population.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria: The disease

Malaria occurs in over 100 countries and more than 40% of the people in the world are at risk. Africa, Southeast Asia, the Middle East, the Indian subcontinent, large areas of Central and South America, Hispaniola (Haiti and the Dominican Republic), and Oceania are considered malaria-risk areas. Malaria is one of the world's most important parasitic diseases, with an estimated 200-500 million cases, killing between 1.7 - 3 million people per year (Breman, 2001), 90% of which occurring in Sub Saharan Africa. Mortality due to malaria occurs mostly in young children under the age of five. Young children, pregnant women and non-immune visitors to endemic areas hold the highest risk of getting severe and potentially fatal malaria.

The economic impact of malaria

Malaria targets the poor more than any other disease and malaria endemic countries represents the worlds poorest. The costs of malaria in terms of burdens on the health systems and economic activity lost are enormous. Malaria in Africa reaches its peak at the end of the rainy season or harvest time and people infected with a single episode of the disease are laid low for an estimated equivalent of 10 working days. Experience shows that, the lost days do not apply to the sick patient alone; rather it also applies to the time lost during caring for a sick relative. According to 1997 estimates from the WHO, the annual direct and indirect costs of malaria in Sub - Saharan Africa exceeds

USD2 billion. Many nations in Sub Saharan Africa have faced socio-economic instability and a dismantling of government sector malaria control programs (World Bank, 1993). Indeed, increased cost of insecticides, the vector's resistance to insecticides and the lack of an effective vaccine has resulted in reliance upon case management and effective curative chemotherapy as the primary approach to malaria control. Malaria parasite resistance to treatment with CQ and SP has already complicated malaria management and has been associated with increased malaria morbidity and mortality (Greenberg *et al.*, 1989; Trape *et al.*, 1998; Marsh, 1998). Indeed, recent rapid increase in resistance to SP, shortly after CQ widespread failure, has rendered both CQ and SP drugs redundant in most of East and Central part of African continent. Resistance of the malaria parasite, especially to CQ and to the antifolates, the two most cheap and safe antimalarial groups, present a real test to the progress on rolling back malaria, and a formidable challenge for the foreseeable future. Currently, the antimalarial combination therapy (CT) containing artemisinin derivative is WHO recommended therapy aimed at slowing down rapid development of resistance. Already most part of East and Southern Africa has replaced SP monotherapy with artemisinin based combination therapy.

2.2 The malaria parasite

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Four Plasmodia species cause disease in humans: *P. vivax, P. ovale, P. malariae* and the cause of most severe malaria disease and deaths, *P. falciparum*. Numerous other Plasmodia species cause disease in other mammals, birds, and reptiles. *P. falciparum* is the most

widespread and dangerous of the four human *Plasmodia* and if left untreated can be fatal. *P. falciparum* malaria is very serious infection and the most common form, the uncomplicated malaria, may sometimes advance to complications that include cerebral and placental malaria. The other three species are debilitating and are characterised by chills, fever and weakness, and therefore, malaria caused by them is rarely fatal and mostly self-limiting in nature. The only problem is that, with *P. malariae* and *P. vivax*, there can be relapses if the disease is not properly treated.

Life cycle

The four species of *Plasmodium* mentioned above require both a mosquito vector and a human host in order to survive. Their life cycles are very complex allowing them to change their biochemical activities in order to survive in the two hosts they require. The parasites are transmitted from person to person by a female mosquito of the genus *Anopheles*. Only the females of this genus feed on blood. The males are not disease carriers as they feed only on plant juices and nectar. There are almost 380 species of the anopheline mosquito with about sixty of them being able to transmit malaria. The *A. gambiae* is one of the most known and effective vectors of the parasite. In Tanzania, until recently, *A. gambiae, A. arabinsis* and *A. funestus* were the three known species of a mosquito vector transmitting the disease, with each species dominating the others at different geographical locations of the country. However, recent discovery of yet another species, *Anopheles merus* in coastal areas of the country (Emmanuel Kigadye, 2007,

personal communication) which is also known to transmit the disease further complicates the problem.

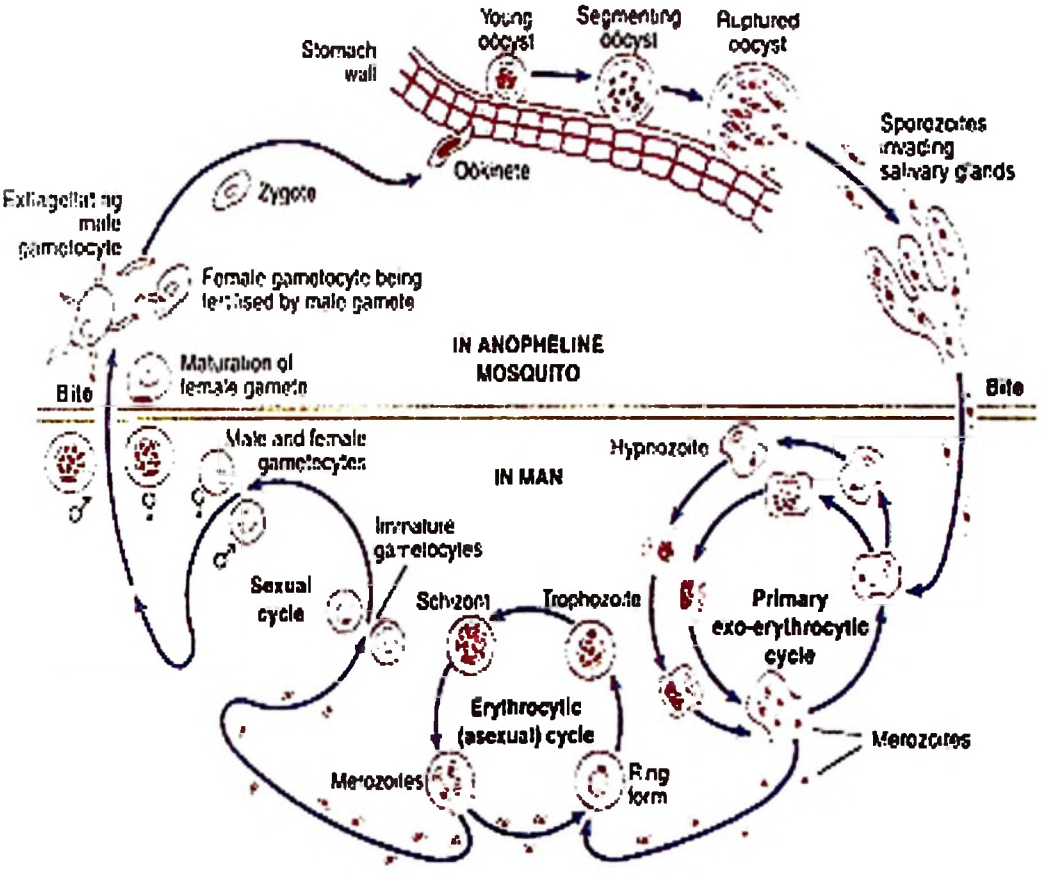

Figure 1. *Plasmodium* life cycle. Source: Kakkilya (2006)

Plasmodium develops in the mid-gut of the mosquito through fertilisation by fusion of the male microgamete and female macrogamete which are ingested in a blood meal stolen by a mosquito from human host (Figure 1). This diploid zygote will then mature and become a motile ookinete, which will penetrate and pass the mid- gut wall of the stomach and settle below the mid-gut lamina on the external side of the mid-gut. The

ookinete will develop into the oocyst, after meiotic reduction, in which asexual mitotic replication takes place, leading to the formation of thousands of sporozoites which travel to the salivary glands of the mosquito. These sporozoites are passed on every time the infected insect bites a person to take a blood meal. These parasites are then carried to the liver of the human host where they invade the hepatocytes and multiply. After almost 9 – 16 days, depending on which of the species of the malaria parasite, they return to the blood and penetrate the red blood cells where they multiply again progressively breaking down red blood cells.

This is what induces the clinical symptoms of malaria. Clinical symptoms of malaria include fever, shivering, sweats, anorexia, nausea, vomiting, headache, muscle and joint pains, general body malaise and many more which quickly disappear once the parasite is killed. Malaria spreads when the mosquito feeds on an infected person and then bites an uninfected one. The spreading is dependent on the survival of the parasite and this in turn, depends on the favourable conditions for the survival of the anopheles mosquito. High temperatures between 21 and 32^0C and relative humidities of at least 60% are the environmental conditions most favourable to its survival. The development of the mosquito vector ceases at temperatures below 15^0C, which is why it is mostly prevalent in tropical areas, 90% of which occurs in Sub Saharan Africa.

2.3 Malaria diagnosis

Several approaches to the diagnosis of malaria are currently available. Each approach presents characteristics such as cost, ease of performance and accuracy, which will determine its applicability to different settings.

2.3.1 Clinical diagnosis

Clinical diagnosis is the most widely used approach in poor endemic settings. It has been the only feasible one in many situations, particularly in rural areas and at the periphery of the health care system where laboratory support to clinical diagnosis does not exist (WHO, 2000). Among the many clinical signs and symptoms associated with malaria, the most prominent is fever, which is often accompanied by chills, sweats, anorexia, headaches, muscle pains, nausea, vomiting and malaise. Residents of endemic areas like Tanzania, are often familiar with this combination of symptoms, and frequently self-diagnose malaria based on symptoms alone. In addition to these symptoms of uncomplicated malaria, other manifestations may develop signalling severe malaria, which is almost always due to *P. falciparum*. These include confusion or drowsiness with prostration and may advance to cerebral form of malaria manifesting with neurological focal signs such as, severe anaemia, coma, respiratory difficulties and others. Clinical diagnosis is inexpensive to perform, and requires no special equipment or supplies. However, these symptoms of malaria are very non-specific and overlap with those of other febrile illnesses (WHO, 2000). A diagnosis of malaria based on clinical grounds alone is therefore unreliable, and when possible should be confirmed by

laboratory tests. In spite of this lack of specificity, in some settings disease management based on clinical diagnosis alone is justifiable.

2.3.2 Microscopic diagnosis

Conventional light microscopy is the established method for the laboratory confirmation of malaria. The careful examination by an expert microscopist of a well prepared and well stained blood film remains currently the "gold standard" for detecting and identifying malaria parasites. In most settings, the procedure consists of: collecting a finger-prick blood sample; preparing a thick blood smear (in some settings a thin smear is also prepared); staining the smear (most frequently with Giemsa); and examining the smear through a light microscope (preferably with a 100X oil immersion objective) for the presence of malaria parasites (Payne, 1988). Light microscopy offers many advantages; when used by skilled and careful microscopists, it is a sensitive method that can detect densities as low as 5–10 parasites per µl of blood (WHO, 1990). Under general field conditions, however, the detection capabilities of a typical microscopist might be more realistically placed at 100 parasites per µl of blood (Anonymous, 1988). When parasites are found, they can be characterized in terms of their species (*P. falciparum*, *P. vivax*, *P. ovale*, or *P. malariae*) and the circulating stage (e.g. trophozoites, schizonts, gametocytes).

Occasionally, expert microscopists can detect morphological alterations induced by recent drug treatment. In addition, the parasite densities can be quantified (from ratio of

parasites per number of leukocytes or erythrocytes). Such quantifications are needed to demonstrate hyperparasitaemia (which may be associated with severe malaria) or to assess parasitological response to chemotherapy. The method is relatively inexpensive, with cost estimates for endemic countries ranging from about US$ 0.40 to US$ 0.80 per slide examined. Such figures, however, do not reflect the true cost to the health system or to the patient, which may be substantially higher. In addition, the cost per test will increase if utilization is low, or if microscopy in the health facility is used only for malaria diagnosis. It is a general diagnostic technique that can be shared with other disease control programmes, such as those against tuberculosis or sexually transmitted diseases. The method can provide a permanent record (the smears) of the diagnostic findings and be subject to quality control. Microscopy suffers from three main disadvantages. First, it is labour-intensive and time-consuming, normally requiring at least 60 minutes from specimen collection to result. Second, its accuracy depends absolutely on good techniques, reagents, microscopes and, most importantly, well trained and well supervised technicians. Unfortunately these conditions are often not met, particularly at the more peripheral levels of the health care system. In these circumstances, microscopic diagnosis risks becoming an unreliable tool that uses up scarce resources for doubtful results. Third, there are often long delays in providing the microscopy results to the clinician, so that decisions on treatment are often taken without the benefit of the results.

2.3.3 Presumptive treatment

In highly endemic areas, particularly in Africa, the great prevalence of asymptomatic infections and lack of resources (such as microscopes and trained microscopists) have led peripheral health facilities to use "presumptive treatment". Patients who suffer from a fever that does not have any obvious cause are presumed to have malaria and are treated for that disease, based only on clinical suspicion, and without the benefit of laboratory confirmation. This practice is dictated by practical considerations and allows the treatment of a potentially fatal disease. But it also leads frequently to incorrect diagnoses and unnecessary use of antimalarial drugs. This results in additional expenses and increases the risk of selecting for drug-resistant parasites.

2.3.4 Rapid diagnostic tests (RDTs)

Also known as antigen detection, these tests are based on the identification of antigens derived from malaria parasites in lysed blood, using immunochromatographic methods. Most frequently they employ a dipstick, card, pad, well, cassette or test strip bearing monoclonal antibodies directed against the target parasite antigens. The tests can be performed by individuals with minimal training and with the different tests that are commercially currently available; the procedure may involve 2 to 6 steps and take 5 to 30 minutes. The cost of the RDT also varies from test to test and from country to country, ranging from US $1.50 to $15.0 per test. The field is evolving rapidly, and technical improvements are continually being announced that will undoubtedly enhance the capabilities of RDTs for malaria diagnosis. Although RTDs have been reportedly

easy tools to use, their results need to be interpreted with caution because of some serious weaknesses. With these tests, the circulating antigen will be detectable for many days even after the elimination of viable *P. falciparum* from the blood stream. A positive test therefore may not always indicate an active infection. As both the asexual as well as the sexual forms of the parasites expresses antigens targeted by RDTs and much as the schizonticidal drugs are having no effects on the gametocytes of *P. falciparum* (except for the artemisinin compounds), RDTs may not be reliable tools to predict the therapeutic response.

2.3.5 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). Practically, molecular methods employing the use of PCR and/ or gene sequencing have been used extensively to study drug resistance in malaria. This has allowed the prediction of resistance to some drugs; although, the predictive values of these molecular tests are still being evaluated. It has been shown for instance that, occurrence of three mutations in the *dhfr* gene simultaneously with two mutations in the *dhps* gene of the same parasite is sufficient to predict SP failure in clearing such genotypic parasites (Kublin *et al.*, 2002)

2.4 Control of malaria

Malaria control is too complex to be addressed by a single approach, and any attempt to do so may prove unfruitful. It is important to direct the strategy to the prevailing ecological and epidemiological conditions (Mouchet and Carnavale, 1998). To illustrate this, definitions of the four main patterns of epidemiology (hypoendemic, mesoendemic, hyperendemic and holoendemic) are based on indicators which can be measured in the community. Of significance, the immune status of the population and the patterns of malaria seen will be different in these four situations and will also affect the strategy for control. Therefore, these will be dictated by the prevailing transmission patterns and will be orientated to the following outcomes: mortality control, transmission control, and eradication.

2.4.1 Mortality control

The major impact of malaria in any community is that of the death of individuals. To prevent a person dying from the disease, prompt and appropriate treatment is necessary. The strategy of mortality control involves detecting presumptive cases, determining which cases are parasites positive, and administering effective treatment. Mortality control is the main focus of the current "Global Malaria Control Strategy" (WHO, 1993) and will be discussed further under the section of treatment of malaria in this thesis.

2.4.2 Transmission control

The transmission control strategy recognizes that malaria is an important cause of morbidity as well as mortality. The disease is extremely debilitating and extracts a high price from the communities affected. Children are anaemic and unable to concentrate at school (Premji *et al.*, 1995; Shiff *et al.*, 1996), and society as a whole is debilitated. Whilst appropriate treatment is one aspect of the transmission control strategy, vector control is also a major player, and, when properly applied, these aspects together have an impact on both the mortality and morbidity of malaria.

This approach is effective in most epidemiological conditions and is an effective control strategy for a sustained attack on the malaria problem. It is adaptable to the use of insecticide-treated mosquito nets as well as indoor spraying of insecticide (Kouznetsov, 1977; Curtis, 1996; Curtis and Mnzava, 2000). It can be implemented in specific circumstances where malaria is a local priority or on a wide scale as part of a major program of intervention. Transmission control requires coordination and the development of strategic plans to intervene against malaria (Kouznetsov, 1977). A high level of expertise is needed with personnel trained in epidemiology and vector control as well as in planning, mapping, and communications to coordinate and supervise the operations. However, these would be the prerequisites in countries which have made commitments to controlling malaria.

Concerns have been raised by some health authorities (Marsh, 1992; Coleman *et al.*, 1999) that transmission control will eventually reduce local immunity acquired from

longstanding infection in the population. This is true. Effective transmission control will reduce the incidence of infection and reinfection in the community, and eventually people will lose their acquired immunity. Therefore, such interventions should be planned in a sustainable manner. It is important for the local government to recognize this when making commitments to malaria control.

Dichloro-diphenyl-trichloroethane (DDT) is a proven highly effective insecticide for the control of mosquito, although its use continues to be debatable due to its environmental and health effects (Curtis, 1994). Probably, the most remarkable demonstration of the effectiveness of DDT to kill maria-carrying mosquitoes, was its use in Zanzibar during late 1950s and early 1960s, resulting in the reduction of the percentage of malaria cases from 70% in 1958 to <5% in 1964 (Bast *et al.*, 1994). Following suspension of the DDT spraying program in 1964, the malaria rate was back up to 50 to 60% by 1984. Despite the on going hot debate on the use of DDT, several countries in Africa have deployed DDT-spraying programs in recent years that have yielded impressive results (Sanders, 2006). South Africa, which reintroduced DDT in 2003 after a seven-year ban, saw its malaria rates decreasing by 80%. Other nations experimenting with DDT include Eritrea, Zambia Tanzania, Kenya and Madagascar (Sanders, 2006).

2.4.3 Eradication of malaria

Eradication can be considered only in certain areas, e.g., in places where malaria has been eradicated and where it has been reintroduced and in areas of hypoendemic malaria where there are sufficient resources to undertake the process and where there is little

likelihood of future introduction. The advantage of an eradication program is that it is time limited and, once it has achieved its objective, can be terminated with little further oversight (WHO, 1957). The relative successes of the various malaria control activities commenced in the mid-20th century. Clearly, eradication programs were extremely successful, but eradication could not be achieved in many places and the technique must be considered not appropriate in most areas of endemic infection.

2.5 Malaria treatment

Antimalarial drugs can normally cure malaria if they are taken promptly and appropriately. The numbers of drugs that are being used to treat and prevent malaria are severely limited. Most drugs used in treatment are active against the parasite forms in the blood (the form that causes disease) and most common include: CQ, SP (Fansidar®), amodiaquine, mefloquine (Lariam®), halofantrine (Halfan®), atovaquone-proguanil (Malarone®), quinine, doxycycline, artemisin derivatives and most recently artemisinin based combination therapies (ACTs) (Appendix xvii). The choice of these drugs to treat malaria depends on: the area where the infection was acquired and its drug-resistance status, the clinical status of the patient, pregnancy, drug allergies and any accompanying illness or condition.

Chloroquine (CQ), which is one of the aminoquinoline family, was the mainstay of malaria treatment and control for almost four decades in most endemic areas, due to its effectiveness, safety and affordability. Parasite resistance to this drug was first observed

in Thailand in 1957 and then on the border of Colombia and Venezuela in 1959. By the late 1970s resistance had spread to East Africa and by the mid-1980s had become a major problem in several areas of the continent (Wernsdofer and Payne, 1991). This led to most of African countries dependence on other alternatives such as antifolate combination drugs. These antifolates, which include combinations such as sulfadoxine and pyrimethamine, target and inhibit the DHFR and DHPS enzymes of the parasite. Inhibition of the DHFR and DHPS enzymes leads to the disruption of DNA synthesis in the parasite. As a formidable challenge to malaria control, resistance to antifolates has reached unacceptable levels in most of Eastern and Southern African countries forcing an increasing number of countries to adopt ACTs. ACTs produce rapid clinical and parasitological cure, parasite resistance is yet to be documented, reduce gametocyte carriage rate, and are generally well tolerated (Cravo *et al.*, 2006; WHO, 2006). However, the cost of treatment could be higher by a factor of up to 10-fold and this may be a hindrance in poor communities, Tanzania inclusive.

2.6 Antimalarial usage and development of resistance: a historical perspective

The combination of such factors as the increased cost of insecticides, the mosquito's resistance to insecticides and lack of effective vaccine has resulted in reliance upon prompt case diagnosis and effective curative chemotherapy as the main strategy for malaria control (Gregson and Plowe, 2005). The history of the usage of antimalarials by humans' dates back to centuries, where the earliest use recorded extracts from plants used by the Chinese. Chinese started extracting artemisinin from the Chinese herb

known as Quinghao (*Artemisia annua*) some 1500 years ago. Later on, Quinine was discovered from the bark of Peruvian "Fever tree" (*Cinchona spp*) in the 19th century (Cowman, 1997).

The use of quinine as chemotherapeutic agent began shortly after World War I and has been of major clinical importance in combating malaria, and it remains the cornerstone for the treatment of both drug resistant and severe infections. Later, the discovery of 4-aminoquinolones briefly before World War II, as quinine analogues revolutionised the field of malaria case management. Chloroquine and amodiaquine were the most useful of the four synthetic quinine analogues discovered, because of the least contraindications. Amodiaquine is still not recommended for prophylactic use because of adverse reactions (Cowman, 1997).

Chloroquine being highly schizontocidal compound was easily accepted and readily adopted due to its cheapness, tolerability and minimal side effects, and by mid 1940s it was a widespread drug of choice for malaria treatment. The other important benefit of CQ was its antipyretic activity. This is associated with prompt fever clearance creating a perception of a curative effect and as such, this perception had so much to play in the development and spread of choloroquine resistant parasites, because full dosage regimen was often ignored given the perceived cure that followed clearance of fever (Gregson and Plowe, 2005). Earliest reports of CQ resistance were in 1957 in Southeast Asia, followed shortly in 1959 by South America and by 1978 report of resistance in Africa

already existed (Figure 2). According to Payne (1989), the spread was in the form of dispersal from Southeast Asia through to India and eventually East Africa. From East Africa, resistance radiated outwards to the South and Northwest. This idea of spread rather than repeated *de novo* emergence of resistance were later confirmed by molecular studies of CQ resistance (Wootton *et al.*, 2002), which showed that the Asian form of resistant CQ (*P. falciparum* CQ resistance transorter, pfcrt gene carrying parasites) had spread across Africa.

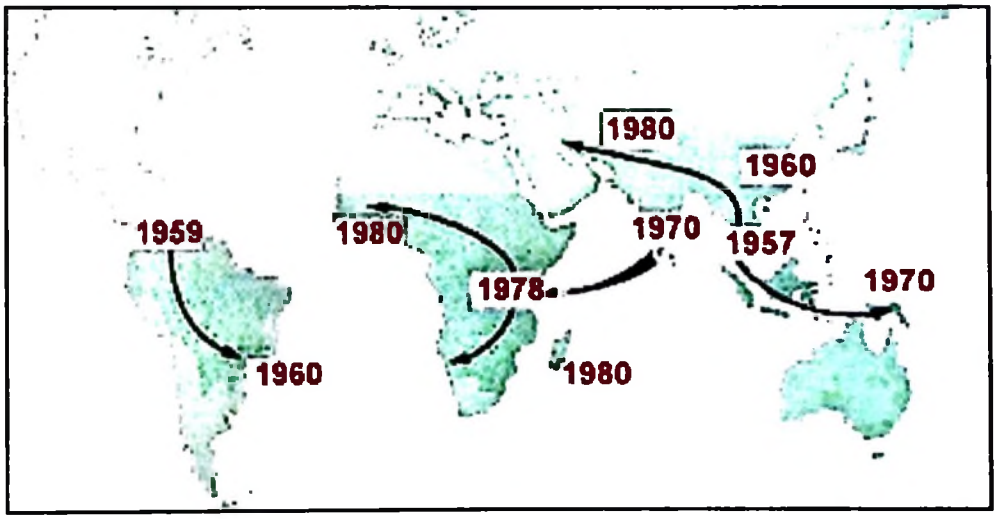

Figure 2. The spread of chloroquine resistant *P. falciparum*. Source: Pyne (1989)

As CQ came into widespread usage in the 1940s, simultaneous drug development was underway, focusing on derivatives of pyrimidine, based upon its presence in nucleic acids and its metabolism by protein systems that were effectively blocked by antimalarial sulfonamides. Research efforts on the area proved successful following the discovery of antifolate biguanides, proguanil and chlorproguanil in 1945. Initially identified as a drug with low toxicity and high activity against avian malaria. proguanil,

early reports on its use (as Paludrine) for both prophylaxis and treatment were very encouraging (Maegraith *et al.*, 1945, 1946; Jones *et al.*, 1948; Seaton and Lourie, 1949), despite lower schizontocidal action compared with quinine or mepacrine (Covell *et al.*, 1949). Later in late 1940s and early 1950s, proguanil use as prophylaxis for plantation workers in Southeast Asia and elsewhere, led to widespread drug selection pressure on the parasite with an eventual development of resistance to the drug.

Shortly after discovery of proguanil, pyrimethamine discovery followed in early 1950s. As the DHFR enzyme inhibitor, pyrimethamine (Daraprim) was a remarkably effective casual prophylactic and therapeutic agent (Archibald, 1951; Goodwin, 1952b; Vincke and Lips, 1952; Delannoy and Hugon, 1954; Miller, 1957), even against CQ-resistant parasites (Powell *et al.*, 1964). Yet, a concern about the rapid development of parasite resistance to pyrimethamine and its slow schizontocidal activity was raised shortly after its introduction (Coatney *et al.*, 1952; Goodwin, 1952a; Petersen, 1987) and (Figure 3). Resistance was hugely exacerbated by the continued use of pyrimethamine as prophylactic (Clyde and Shute, 1954; Jones, 1954). The occurrence of *P. falciparum* resistance to pyrimethamine was first reported in Muheza, Northeastern Tanzania in 1954 (Clyde and Shute, 1954). As such, when pyrimethamine was given as a weekly prophylaxis to children for 1 year, pyrimethamine resistance increased throughout the course of the year, reaching 60% resistance at the end of the year (Clyde and Shute, 1957). Reports of the spread of pyrimethamine continued to mount.

In East Africa in particular, there appeared to be a spreading pyrimethamine phenotype from Sudan and Kenya to Tanzania. Perhaps similar to the spread of CQ resistance from Southeast Asia, at many points, there was clearly a *de novo* emergence independently in response to the use of pyrimethamine, particularly in the disjointed distribution of pyrimethamine resistance in West Africa. The increased exposure of nonimmune persons to these resistant strains opened interest in the sulfonamides and sulfones as antimalarials. Protonsil containing sulphonamide as active ingredient was developed in 1932. A trial conducted in 1937 using protonsil for treatment of falciparum malaria proved 100% cure rate in 93 individuals following four, 12 hourly injection (Hill and Godwin, 1937; Niven, 1938).

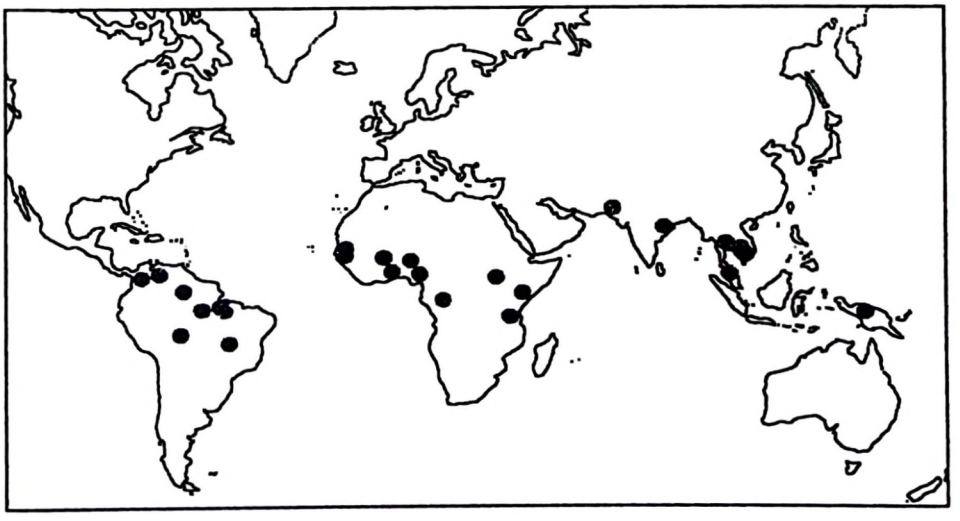

Figure 3. Reported pyrimethamine resistance in Africa. Source: Peters (1970)

Interest in sulfonamides then waned, partly because of the introduction of synthetic antimalarials and the continued effectiveness of quinine, until sulfa drugs with improved

toxicity and longer half-lives were developed in late 1950s and 1960s (Hill, 1963). Sulfadoxine in particular, demonstrated considerable promise as a prophylactic and curative drug against *P. falciparum* in Tanzania (Laing, 1965). Several early trials confirmed that a single dose of sulfadoxine was an effective, although slow schizontocide. In order to extend the useful therapeutic life of pyrimethamine, it was combined with sulfonamide, following a 1959 study which found that, sulfadoxine potentiated pyrimethamine in human falciparum infections, was more effective than either drug alone (Greeberg and Richeson, 1950; Hurly, 1959).

Sulfadoxine and Pyrimethamine are competitive inhibitors of enzyme components of the folate biosynthesis pathway (Bzik *et al.*, 1987; Brooks *et al.*, 1994; Triglia and Cowman, 1994). Sulfadoxine target dihydropteroate synthase (bifunctionally combined with hydroxymethylpterin pyrophosphokinase, PPPK-DHPS, Brooks *et al.*, 1994; Triglia and Cowman, 1994) while pyrimethamine targets dihydrofolate reductase (bifunctionally combined with thymidilate synthetase, DHFR-TS, Bzik *et al.*, 1987). The two inhibitors work synergistically in disrupting folate synthesis and the parasite life cycle. The synergy is interfered following development of parasite resistance to one of the two component drugs. In 1982, SP became the second line drug for the treatment of malaria in cases of CQ failure in Tanzania.

According to Mutabingwa *et al*, (2001), SP was the first line treatment policy of uncomplicated malaria in Muheza district hospital by 1984, following the alarming rates

of CQ resistance in the district. However, nationally, SP became the first line antimalarial treatment policy of Tanzania in 2001. The first reported national use of SP as first line antimalarial treatment in Africa was in Malawi in 1993. Kenya, Botswana and South Africa (except Kwa Zulu Natal which adopted in 1988) followed in 1997 (Bloland *et al.*, 1993; Bloland *et al.*, 1998). Warries that the combination (SP) might prove to be ineffective long term solution started to build shortly after its inception, following increasing reports of clinical failure from Southeast Asia and South America in semi immune persons (Bunnag *et al.*, 1980). An early paper on efficacy of SP wisely cautioned that the combination of sulfadoxine and pyrimethamine, whose dose activity regression lines are nearly flat, might lead to rapid development of parasite drug resistance (Jacobs *et al.*, 1963; Harinasuta *et al.*, 1967). Generally, resistance to SP was relatively quick to develop, although slower than pyrimethamine alone. Although *in vivo* efficacy assesment of SP begun in early 1980s (Kilimali and Mkufya, 1985), earliest reports of emerging SP resistance in Africa came from Muheza district in Tanzania between 1994 and 1995 (Ronn *et al.*, 1996; Trigg *et al.*, 1997)

Mechanisms of drug resistance

A number of organisms have found ways to develop resistance against chemical compounds. Some of these include parasites resistant against anti-parasitic drugs, mosquitoes against insecticides, bacteria against antibiotics and even cancer against cancer drugs (Fidock *et al.*, 2000; Djimde *et al.*, 2001; Warhurst, 2001). There are many different ways by which microorganisms might exhibit resistance to drugs. The

following are fairly well supported (Jawetz *et al.*, 1991): (1) Microorganism may produce enzyme that destroy the active drug (drug in activation), example: Staphylococci resistant to penicillin G produce a β-lactamase that destroy the drug. (2) Microorganism may change their permeability to the drug, examples: Tetracyclines accumulate in susceptible bacteria but not in resistant bacteria. Also, CQ resistant parasites have ability to pump out CQ (efflux) but not the sensitive strains (Krogstad *et al.*, 1987). (3) Micro organisms develop an altered structural target for the drug, examples: Chromosomal resistance to aminoglycosides is associated with the loss or alteration of a specific protein in the 30S subunit of the bacterial ribosome that serves as a binding site in susceptible organisms.

Also, *P. falciparum* parasites bearing mutated dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes show decreased binding affinity to both pyrimethamine and sulfadoxine (Hyde, 1990; Wu *et al.*, 1996). (4) Microorganisms may develop an altered metabolic pathway that by-passes the reaction inhibited by the drug. Example: Some sulphonamide-resistant bacteria and protozoa do not require extracellular paraamino benzoic acid (PABA) but, like mammalian cells, can utilise pre-formed folic acid (folate salvage pathway). (5) Microorganisms may develop an altered enzyme that can still perform its metabolic function but is much less affected by the drug than the enzyme in the susceptible organisms. Example: In trimethoprim-resistant bacteria, the dihydrofolic acid reductase is inhibited far less efficiently than in trimethoprim-susceptible bacteria (Jawetz *et al.*, 1991). Once a pathogen has chanced

upon an effective strategy, the newly acquired or mutated genes that confer that resistance are frequently spread throughout the pathogen population and may even be transferred to pathogens of different species that are treated with the same drug (Jawetz *et al.*, 1991).

2.7 Drug resistance in malaria

Drug resistance is an important problem facing malaria control that continues to grow with time. Drug resistance in malaria has been defined as the ability of the parasite to survive and or multiply despite being treated with required concentration of the drug or higher than the dose recommended as long as it is tolerable by the individual (Bloland, 2001). This resistance is believed to have come about due to selective pressure of the drugs by the parasite. This could be due to massive deployment of the drug to the population or the subject taking an incomplete treatment of the drug, and it has recently been discovered that the parasites, through point mutations in certain codons of their CQ transporter (*pfcrt*) or *dhfr* and *dhps* genes can avoid inhibition by CQ or antifolates.

The first antimalarial drug resistance to be recorded was for *P. falciparum* as early as in 1961 in Thailand and the first confirmed cases of CQ resistance in Africa were recorded in 1978. With the advent of CQ resistance, scientists had to come up with replacement drugs that were both affordable and non-toxic. These new drugs are the antifolate combination drugs, which are composed of pyrimethamine as DHFR inhibitors and sulfa drugs, as DHPS inhibitors. The loss of UTL of CQ and most recently of SP as well

means that what is available at the moment for malaria treatments are drugs that are both expensive and could be toxic.

2.8 Biochemical mechanisms of drug resistance by the parasite

2.8.1 Chloroquine resistance

Chloroquine works by inhibiting heme polymerisation, which occurs when the parasite is producing large amounts of toxic by-products that are formed when the malaria parasite is digesting hemoglobin. This by-product is polymerised in its food vacuole to produce a non-toxic hemazoin, which is the malaria pigment. Chloroquine prevents the neutralisation of this toxic by-product, ferreprotoporphyrin IX by raising the pH in the food vacuole. Resistance occurs due to the parasite being able to increase its rate of metabolising CQ by pumping it outside the food vacuole (CQ efflux) and thereby preventing its accumulation to reach the concentration prohibitive to the heme polymerisation process. Krogstad *et al.*, (1987) demonstrated that susceptible and resistant parasites initially accumulate CQ at the same rate (28 to 29 fmol/106 parasitized erythrocytes per min) but later it became clear that the sensitive *P. falciparum* parasites retains significantly higher levels of CQ than the resistant ones (Krogstad *et al.*, 1987).

Molecular studies identified point mutation in the transmembraneous protein (*P. falciparum* CQ transporter protein, *Pfcrt*) as the main cause of efflux phenomenon in resistant parasites (Fidock *et al.*, 2000; Djimde *et al.*, 2001; Warhurst, 2001). Following

the discovery of *Pfcrt* K76T mutation as the main cause of CQ resistance, it has since being widely used for the surveillance of CQ resistance in endemic settings (Sidhu *et al.*, 2002; Warhurst, 2003; Cooper *et al.*, 2005; Khalil *et al.*, 2005; Alifrangis *et al.*, 2006).

2.8.2 *P. falciparum* folate biosynthesis and mechanism of resistance to sulfadoxine and pyrimethamine

Folate metabolism is critically important in the survival of malaria parasite. For over half a century, the pathway has been targeted in both treatment and prophylaxis of the disease, following the discovery of antifolates for the treatment of malaria in early 1950s (Archibald, 1951; Goodwin, 1952b). The most widely used antimalarial drugs of this type include proguanil, pyrimethamine, sulfadoxine and more importantly, the SP, which until recently, have long provided chemotherapy at a price affordable by poorer nations and have been especially important since the principal antimalarial drug, CQ became ineffective in most endemic areas.

The biosynthetic pathway involves conversion of GTP to polyglutamated derivatives of tetrahydrofolate, the essential cofactors for DNA synthesis (Cowman *et al.*, 1988; Wang *et al.*, 2004). The process is mediated by five enzymes (Figure 4), two of which, dihydropteroate synthase, DHPS (bifunctionally combined with hydroxymethylpterin pyrophosphokinase, PPPK-DHPS) and dihydrofolate reductase, DHFR (bifunctionally combined with thymidilate synthetase, DHFR-TS) are the target of sulfadoxine and pyrimethamine drugs, respectively. The two drugs are competitive inhibitors of their

target enzymes, and they work synergistically in disrupting folate biosynthetic pathway and the parasite life cycle. The mechanism of *P. falciparum* resistance to sulfadoxine and pyrimethamine was identified through laboratory based *in vitro* sensitivity and transfection experiments on *dhfr* (Zolg *et al.*, 1989; Wu *et al.*, 1996) and *dhps* (Triglia *et al.*, 1997; Wang *et al.*, 1997b; Triglia *et al.*, 1998). A single mutation at codon 108 of *dhfr* (S108N substitution), decreases sensitivity to pyrimethamine by 100 fold (Wu *et al.*, 1996). Additional mutation(s) at codon 51 (N51I) and/or codon 59 (C59R) progressively increase levels of resistance to pyrimethamine (Hyde, 1990; Wu *et al.*, 1996). An addition of non synonymous mutation at codon 50 (C50R) further confers an increased levels of resistance to pyrimethamine. However, this mutation has not been described at sites other than South America (Cortese and Plowe, 1998). Occurrence of a fourth mutation at codon 164 (I164L) confers by far highest levels of resistance to SP (Plowe *et al.*, 1998). So far, the quadruple mutant genotype (N51I +C59R +S108N+ I164L) has been found in multiple sites in Southeast Asia and South America. However, the presence of the I164L mutation in Africa has been elusive and has only recently been observed (Hastings *et al.*, 2002; Staedke *et al.*, 2004; Alker *et al.*, 2005; McCollum *et al.*, 2006).

At *dhps*, 14 substitutions at five sites have been observed worldwide (Table 3), of which, six have been reported in Africa. The A437G and K540E mutations are the most prevalent hence most frequently reported in Africa (Wang *et al.*, 1997a; Wang *et al.*, 1997b; Pearce *et al.*, 2003; Roper *et al.*, 2003; Mugittu *et al.*, 2004). The role of point

mutations at each locus in conferring resistance to SP *in vivo* has been inferred from studies showing predictive association of particular mutations with treatment failure (Omar *et al.*, 2001; Kublin *et al.*, 2002) and from lack of predictive association (Edoh *et al.*, 1997; Jelinek *et al.*, 1997; Khan *et al.*, 1997; Basco *et al.*, 1998; Cortese and Plowe, 1998; Rallon *et al.*, 1999; Basco *et al.*, 2000; Doumbo *et al.*, 2000; Nzila *et al.*, 2000a; Alifrangis *et al.*, 2003a; Mugittu *et al.*, 2004)

2.8.3 Folate salvage and biosynthesis pathways

Most microorganisms are able to synthesise the folates they need from the simple precursors GTP, *p*-aminobenzoic acid (PABA) and L-glutamate (Figure 4). Higher organisms such as humans are unable to do this, so they depend on dietary intake of pre-formed folate as an essential nutrient. *P. falciparum* has the ability to exploit both of these routes (Milhous *et al.*, 1985; Krungkrai *et al.*, 1989; Wang *et al.*, 1997b, 1999) by utilising folate either provided in culture medium *in vitro* or salvaged from the host plasma *in vivo*, or converting the above precursors *de novo* into folate derivatives. A key question with important implications for antifolate therapy concerns the relative importance of the biosynthetic pathway compared to folate salvage from human host *in vivo*. Consequently, the importance of sulfadoxine in killing parasites *in vivo* has been a subject of doubt, criticism and speculation owing to the ability of parasites to source folate externally (Sibley *et al.*, 2001). It has been shown that, different parasite lines display different abilities to use exogenous folate and some grow almost normally in very high concentrations of sulfadoxine *in vitro*, indicating that *dhps* is non essential in

the biosynthesis of folate (Wang *et al.*, 1997b; Wang *et al.*, 1999). Consequently, the ability of the parasite to salvage folate from the host has been postulated to be the explanation of why in the majority of populations where SP resistance occurs, resistant alleles at *dhfr* are found in higher frequencies than at *dhps*. Inhibition studies of the *dhps* activity with high levels of sulfadoxine indicates that, at least in certain strains grown *in vitro*, blockage of folate biosynthesis can apparently be bypassed *via* the salvage route, suggesting that biosynthesis may be dispensable (Wang *et al.*, 1997b).

In the field, however, a strong correlation has been found between sulfadoxine -resistant forms of the parasite carrying mutations in the *dhps* gene and the usage of Fansidar, the clinical formulation of sulfadoxine and pyrimethamine (Plowe *et al.*, 1997; Wang *et al.*, 1997a; Plowe *et al.*, 1998; Nzila *et al.*, 2000a; Sibley *et al.*, 2001), suggesting that in normal infections of the human host, folate salvage cannot completely satisfy the parasite's requirements. It has been proposed that the observed synergy between sulfadoxine and pyrimethamine may result from the ability of pyrimethamine to interfere with the efficient utilization of such salvaged folate (Sims *et al.*, 1999; Wang *et al.*, 1999).

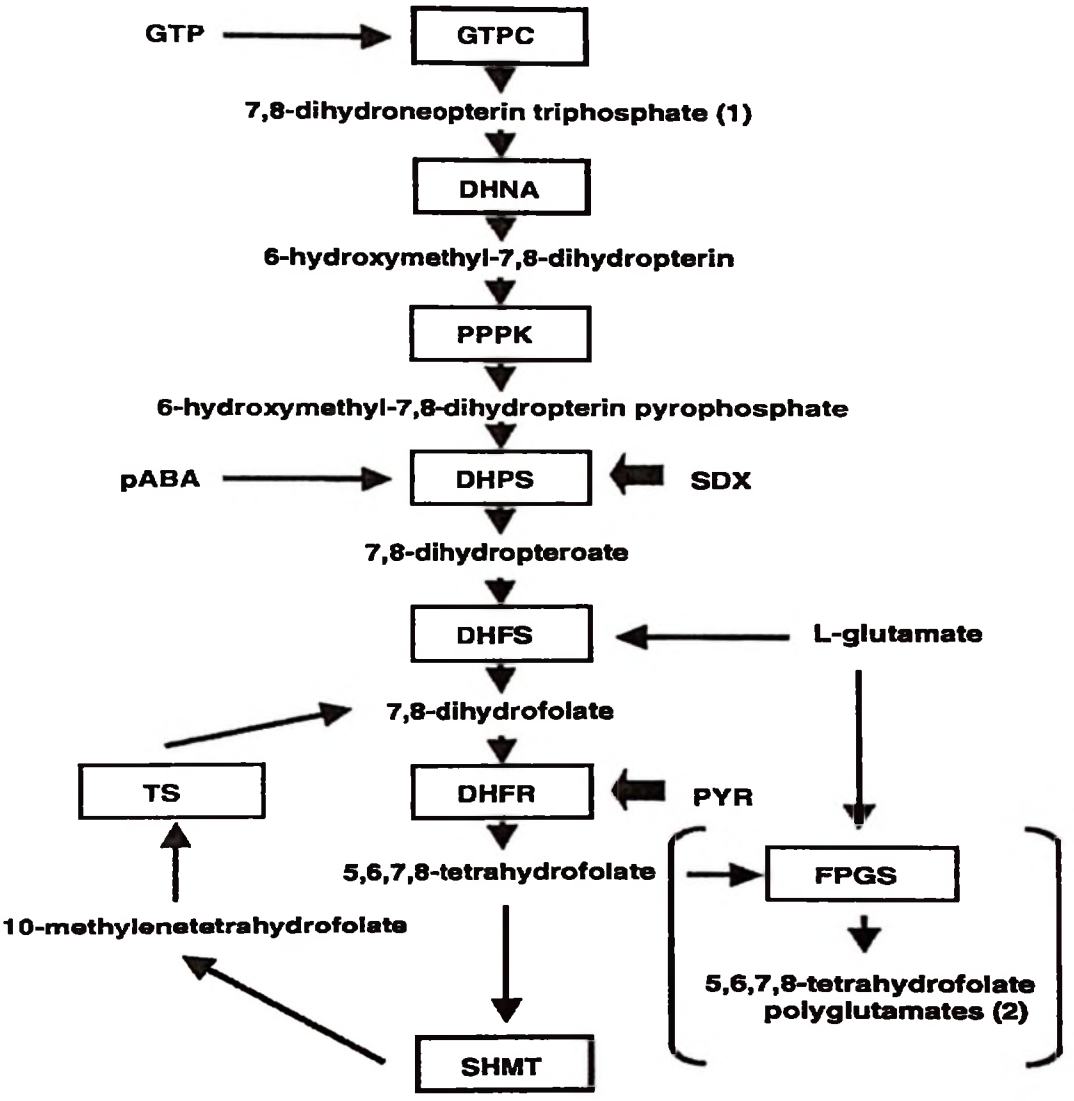

Figure 4. Schematic diagram of folate biosynthetic pathway showing Sulfadoxine (SDX) and Pyrimethamine (PYR) action points, the tetrahydofolate product of the cycle and its utilization in the thymidylate cycle. GTP, guanosine triphosphate; GTPC, guanosine triphosphate cyclohydrolase; DHNA, dihydroneopterin aldolase; PPPK, hydroxymethyldihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthase; SHMT, serine hydroxymethyltransferase; TS, thymidylate synthase.

Transfection studies have shown that, parasites with truncated form of *dhps* are not viable (Wang *et al.*, 2004) indicating that *dhps* activity above a low yet critical level is essential for viable parasites, regardless of the availability of salvageable folate. Earlier studies had assumed that, high concentrations of sulfadoxine were completely inhibiting all DHPS activity with negligible effect on parasite viability because of folate salvage activity. Analysis of *dhps* mutant alleles in field populations of *P. falciparum* have shown that, positive selection is operating on *dhps* A437G+K540E double mutant resistant allele during a period of SP first line use in South African populations (Roper *et al.*, 2003).

CHAPTER THREE

3 0 MATERIALS AND METHODS

3.1 Study area, subjects and samples

Annual community surveys were conducted during 2000, 2001 and 2002, 2004, 2005 and 2006 in three rural districts of Southeastern Tanzania (Figure 5), Rufiji (Population ≈ 170,000), Kilombero (Population ≈ 220,000) and Ulanga (Population ≈ 160,000). The surveys were part of large combination therapy pilot implementation programme in Tanzania, known as the Interdisciplinary Monitoring Programme for Antimalarial Combination Therapy (IMPACT-TZ). For the purpose of IMPACT study, Kilombero and Ulanga districts were treated as a unit district because human movement between these two districts is high and the study population spans the border region. Although geographically contiguous, these two districts (Rufiji and Kilombero/Ulanga) are essentially isolated from each other as movement between these communities is unlikely due to the long distance required to get around the vast Selous game reserve.

The two areas are well matched in terms of predicted intensity and duration of malaria transmission and risk (MARA), relative access and overall utilisation of health services (based on surveys), fairly usage of insecticide treated nets (ITNs) and relative proportion of urban peri-urban, rural population. *P. falciparum* malaria transmission in these districts is intense and perennial with some seasonal fluctuation. Each survey year, an estimated total of between 6,000 – 11,000 adults and children belonging to randomly selected households participated in the study. A finger-prick blood sample for blood

slide and filter paper bloodspot were collected from each individual in the household. The filter paper bloodspots were air-dried and stored at room temperature in self-sealing plastic bags with desiccant and stored on dry area at room temperature. All blood slide samples were screened by microscopy for *P. falciparum* positivity. Bloodspots from microscopically positive subjects were selected and preserved at room temperature for molecular genotyping.

3.2 Ethics

Scientific and ethical clearance was granted from the Medical Research Council of the National Institute for Medical Research in Tanzania, the Centre for Disease Control and Prevention, USA, and the London School of Hygiene and Tropical Medicine. Consent was obtained from all individuals or their guardians before collection of samples.

3.3 DNA extraction

DNA was extracted from bloodspots dried on filter papers in a 96-well plate format. A sector of the dried blood spot filter paper was excised using a sterile blade or scissors, and soaked in a 1 ml of 0.5% saponin in 1x phosphate buffered saline (PBS) overnight. The sample was then washed twice in 1 ml of 1x PBS. The segment was finally boiled for 8 min in 100 µl PCR quality water with 50 µl 20% chelex suspension (pH 9.5).

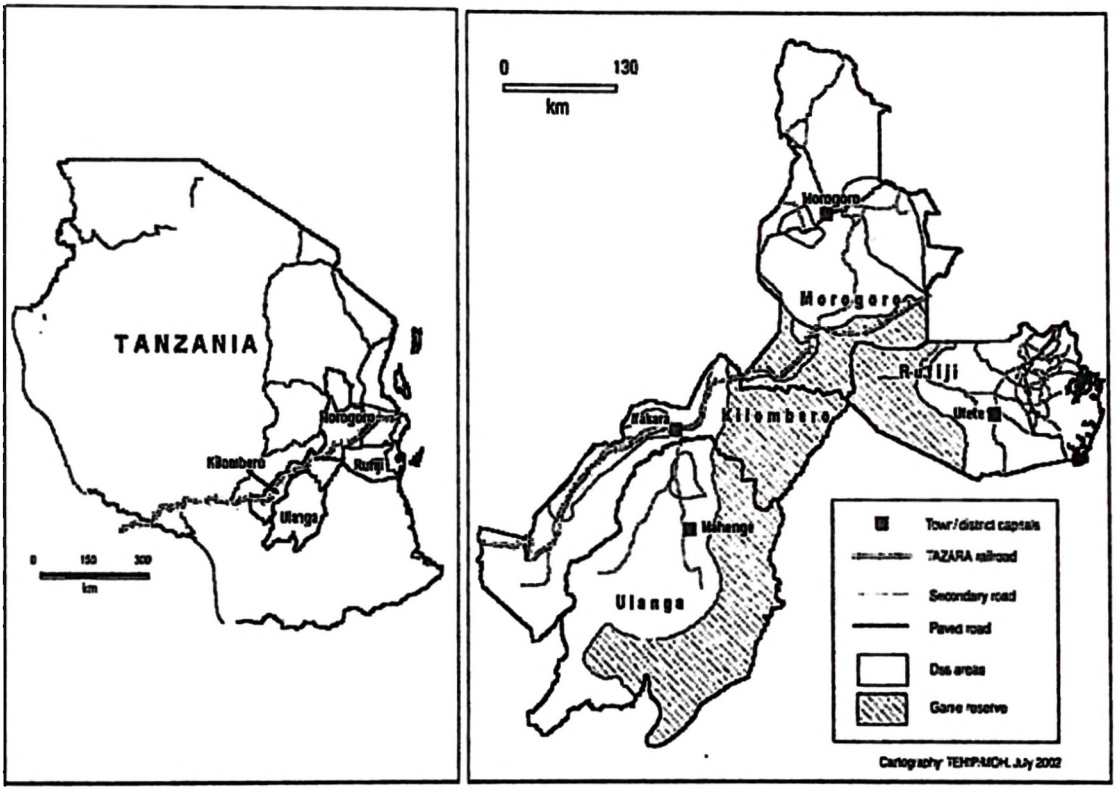

Figure 5. Map of Tanzania showing the study sites (insert).

3.4 PCR amplification of *dhfr* and *dhps* genes

Nested PCR was used to amplify a 594 bp fragment of *dhfr* and a 711 bp fragment of *dhps* each containing the coding sequences where known sites of sequence polymorphisms are found. PCR amplifications were each performed in a 96-well plate format. Primer sequences and reaction conditions were as described by Pearce *et al.* (2003) and detailed in Table 1. The 25-µl PCR reaction volumes contained oligonucleotide primers at 0.25 µM final concentrations, 2mM $MgCl_2$, 250 µM each deoxyribonucleotide triphosphate (dNTPs), and 1x Taq polymerase. A 1µl DNA

template was added to the outer (primary) PCR reaction mixtures. One µl of the outer *dhps* PCR products was added into the inner (secondary) PCR reaction mixtures while the outer *dhfr* PCR products were diluted three fold before a 1 µl was introduced into the inner PCR reaction mixtures.

Table 1. PCR primer sequences and reaction conditions for the nested amplification of *dhfr* and *dhps*

Gene and Primer	Primer sequence	PCR conditions
dhfr Outer, 650 bp		94°C x 3 min 94°C x 1 min, 52°C x 2 min, 72°C x 1 min, 40X; 72°C x 10 min
M1	5'-TTTATGATGGAACAAGTCTGC- 3'	
M7	5'- CTAGTATATACATCGCTAACA-3'	
Inner, 594 bp		94°C x 3 min 94°C x 1 min, 44°C x 2 min, 72°C x 1 min, 4X; 94°C x 1 min, 44°C x 1 min, 72°C x 1 min, 34X; 72°C x 10 min
M3b	5'-TGATGGAACAAGTCTGCGACGTT-3'	
M9	5'-CTGGAAAAAATACATCACATTCATATG-3'	
Dhps, Outer, 770 bp		94°C x 3 min 94°C x 1 min, 51°C x 2 min, 72°C x 1 min, 40X; 72°C x 10 min
N1	5'-GATTCTTTTTCAGATGGAGG-3'	
N2	5'-TTCCTCATGTAATTCATCTGA-3'	
Inner, 711 bp		
R2	5'-AACCTAAACGTGCTGTTCAA-3'	As described above for *dhps* outer primer sequence
R/	5'-AATTGTGTGATTTGTCCACAA-3'	

3.5 *Dhfr* and *dhps* point mutation detection

PCR and sequence specific oligonucleotide probe based method (PCR-SSOP or dot blot technique) was employed for the detection of point mutations which were further used to construct haplotypes. This high throughput method was previously described by Pearce *et al.* (2003). Haplotypes are combinations of point mutations or single nucleotide polymorphisms (SNP) that are in the same gene of the same parasite, as distinct from

association of point mutations that occur because there is a mixture of parasites of different genotypes within a single infection (Pearce *et al.*, 2003).

Haplotypes are biologically meaningful, since they determine the resistance properties of the parasites that are exposed to drugs at the time of treatment. For example, a triple mutant *dhfr* haplotype (N51I + C59R + S108N) has 3-fold higher pyrimethamine resistance *in vitro* than either of the double *dhfr* mutant haplotypes (N51I + S108N or C59R + S108) (Sirawaraporn *et al.*, 1997). The parasite DNA was screened for known *dhfr* and *dhps* sequence variants (point mutation) that encode amino acid substitutions at codons 50, 51, 59, 108, 140 and 164 of the *dhfr* gene and 436, 437, 540, 581 and 613 of the *dhps* gene as summarised in Table 3, using probe sequences listed in Table 2. PCR products were spotted in a 12 by 8-grid and cross linked onto nylon membranes and probed for sequence polymorphisms by hybridisation to specific oligonucleotide probes described previously in Pearce *et al.*, (2003) (Table 2). For analysis of samples collected in 2000, the visualization of hybridised digoxygenin labelled probes on membranes was performed by the alkaline phosphatase-catalysed breakdown of the chemoluminescent substrate, CSPD (Roche Boehringer Mannheim, Mannheim, Germany) and visualised by exposure on Hyperfilm-ECL (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom), according to Boehringer Mannheim recommendations and previously described by Conway *et al.* (1999).

Table 2. Summary of each of the sites at which SNP occurs which is known to be associated with SP resistance and the oligonucleotide probe designed to detect it

a) *Dhfr*

Probe	Amino acid	Probe sequence[a]
Codon 50 and 51		
CN*	Cys Asn	TGG AAA TGT AAT TCC CTA
CN2*	Cys Asn	TGG AAA TGT AAC TCC CTA
RN	Arg Asn	TGG AAA CGT AAT TCC CTA
RN2	Arg Asn	TGG AAA CGT AAC TCC CTA
RI	Arg Ile	TGG AAA CGT ATT TCC CTA
CI	Cyst Ile	TGG AAA TGT ATT TCC CTA
Codon 59		
C*	Cys	AA TAT TTT TGT GCA GTT A
R	Arg	AA TAT TTT CGT GCA GTT A
Codon 108		
N	Asn	A AGA ACA AAC TGG GAA AG
S*	Ser	A AGA ACA AGC TGG GAA AG
T	Thr	A AGA ACA ACC TGG GAA AG
Codon 140		
V*	Val	AT GAA GAT GTT TAT ATC A
L	Leu	AT GAA GAT CTT TAT ATC A
Codon 164		
I*	Ile	GT TTT ATT ATA GGA GGT T
L	Le	GT TTT ATT TTA GGA GGT T
b) *Dhps*		
Codon 436 and 437		
SA*	Ser Ala	GAA TCC TCT GCT CCT TTT
SG	Ser Gly	GAA TCC TCT GGT CCT TTT
FA	Phe Ala	GAA TCC TTT GCT CCT TTT
FG	Phe Gly	GAA TCC TTT GGT CCT TTT
AA	Ala Ala	GAA TCC GCT GCT CCT TTT
AG	Ala Gly	GAA TCC TCT GGT CCT TTT
CA	Cys Ala	GAA TCC TGT GCT CCT TTT
Codon 540		
K*	Lys	ACA ATG GAT AAA CTA ACA
E	Glu	ACA ATG GAT GAA CTA ACA
Codon 581		
A*	Ala	A GGA TTT GCG AAG AA A CA
G	Gly	A GGA TTT GGG AAG AAA CA
Codon 613		
A*	Ala	GA TTT ATT GCC CAT TGC
T	Thr	GA TTT ATT ACC CAT TGC
S	Ser	GA TTT ATT TCC CAT TGC

[a] Entries in column 3 indicate the amino acid changes that occur at the relevant codons following the point mutation. The sequence in the bold represent the codon within which the point mutation occurs. * Wild-type or sensitive codon

For analysis of samples collected between 2001 and 2006 the probed blots were visualised using enhanced chemifluorescent (ECF) substrate and detection using a phosphoimager scanner (STORM). Inspection of autoradiographic films was carried out by light box illumination, while the phophoimager output was recorded through viewing of digitally captured images of chemiflourescent signal. The change in the method by which probe hybridisation signal was visualised did not affect the results in any way since the probes and hybridisation conditions were unchanged.

The stringency and specificity of the hybridisation process was confirmed by inspection of a series of 4 controls with a known single genotype variant sequence. All blots with non-specifically bound probes were stripped and re-probed. A SNP was considered to be present in the PCR product when the intensity of signal was higher than that of the background. The blots were scored independently by two people.

The analysis was aimed to establish the relative abundance of different point mutation haplotypes at *dhfr* and *dhps*. Since bloodstage *P. falciparum* is haploid, this is very straightforward when an infection consists of a single genotype because only one form of sequence at every SNP locus is seen. When infections are composed of multiple genotypes a mixture of different sequence variants occur making the inference of point mutation haplotypes within that infection more difficult.

The presence, absence, and relative abundance of hybridisation signal for every probe were recorded at each locus. A sample was considered to have a single haplotype when only one sequence variant was found at each locus. Blood samples were categorised as having a single, a majority or a mixture of sequence at every SNP locus. Majority and mixed genotype infections were differentiated according to the relative intensity of signal. To determine the relative abundance of different point mutation haplotypes in the parasite population, one haplotype only was counted from each infection and those mixed infections where haplotypes could not be resolved were omitted from the calculation of haplotype frequencies. Hence, frequency data is based upon a subset of isolates which were unmixed or had a predominating majority haplotype. A breakdown of the proportions of isolates which successfully PCR amplified and were subsequently genotyped as single, majority or mixed haplotype infections are given in Table 5 and 6.

3.6 Microsatellite analysis

3.6.1 *Dhfr*-linked microsatellite loci

The evolutionary history of *dhfr* resistance alleles and the extent of gene flow between the study districts sub populations were inferred by constructing haplotypes of amplified microsatellite loci linked to the *dhfr* gene. In order to analyse microsatellite immediately flanking sequence polymorphism on chromosomes carrying specific allelic forms of *dhfr*, the genotype data obtained by PCR-SSOP method described above were used to identify and locate DNA sample bearing that particular allelic form.

Table 3. Summary of nucleotide and amino acid substitutions at A) *dhfr* and B) *dhps* genes

A) *Dhfr*

Codon	50	51	59	108	164
Wild type	Cys (C) TGT	Asn (N) AAT AAC	Cys (C) TGT	Ser (S) AGC	Ile (I) ATA
Mutant	**Arg (R) CGT**	**Ile (I) ATT**	**Arg (R) CGT**	**Asn (N) AAC Thr (T) ACC**	**Leu (L) TTA**

B) *Dhps*

Codon	436	437	540	581	613
Wild type	Ser (S) TCT	Ala (A) GCT	Lys (K) AAA	Ala (A) GCG	Ala (A) GCC
Mutant	**Phe (F) TTT Ala (A) GCT Cys (C) TGT**	**Gly (G) GGT**	**Glu (E) GAA**	**Gly (G) GGG**	**Ser (S) TCC Thr (T) ACC**

For the purpose of looking on these flanking sequence polymorphisms, this study deliberately selected a subset of samples that were unmixed at any polymorphic loci at *dhfr* (samples in which only single haplotype were obtained). Microsatellite sequence located 0.3 kb, 4.4 kb, and 5.3 kb upstream of codon 108 of the *dhfr* gene, which is on chromosome 4 was analysed. Primer sequences that were previously reported by Roper *et al.* (2003) and details described on Table 4 were used. The sample size comprised of 728 isolates (CNCS = 126, CNRN + CICN = 346, and CIRN = 256) of *P. falciparum*

carrying resistant or the sensitive *dhfr* chromosomes from Kilombero/Ulanga and Rufiji districts, respectively for both 2001/2002 and 2006. These isolates were all monoclonal based on the PCR-SSOP genotypic data.

3.6.2 Unlinked (selectively neutral) microsatellite loci

Unlike the analysis of microsatellite loci flanking the *dhfr* gene where samples were selected based on the specific allelic form previously identified by dot blot method, analysis of unlinked microsatellite loci were randomly selected irrespective of allelic forms identified in the sample. This study randomly selected 360 samples from the 2000 survey, half from each of Kilombero/Ulanga and Rufiji, and an equal number and distribution of samples from 2006 survey.

3.6.3 Microsatellite PCR amplification and analysis

Both linked and unlinked microsatellites were amplified in a semi-nested manner. The 1^0 reaction (11.0µl) composed of 1µ template DNA, 1x thermo buffer, and 3.0 mmol/L Mg^{2+}, 0.75-pmol/L of each primer and 1 unit of Taq Polymerase. The cycling conditions were: 2 min at 94^0C; and then 25 repeated cycles of 30 Sec at 94^0C; 30 Sec at 42^0C; 30 Sec at 40^0C; and 40 Sec at 65^0C; followed by 2 min at 65^0C. In the second round of PCR, a third fluorescent labelled primer (Applied Biosystems, Warrington, Cheshire UK) was incorporated and the final PCR reaction volume (11.0µl) consisted of 1x thermo buffer, 2.5 mmol/L Mg^{2+}, 2 pmol/L primer, 1 unit of Taq polymerase and 1 µl of the outer (1^0) PCR products. The cycling conditions were: 2 min at 94^0C; and then 25

repeated cycles of 20 Sec at 94^0C; 20 Sec at 45^0C; and 30 Sec at 65^0C; followed by 2 min at 65^0C.

Semi nested PCR products were diluted at 1:100 ratio and run with LIZ – 500 size standards on an Abi 3730 genetic analyser (Applied Biosystems, Warrington, and Cheshire, UK). Fragments were sized using the Gene Mapper software (Applied Biosystems, Warrington, and Cheshire, UK). For the selectively neutral microsatellites where the samples were random selected, multiply infected samples were a common encounter as revealed by multiple peaks, each representing clonally different parasite (haploid nature of blood stages of *P. falciparum*). In the case of *dhfr* linked microsatellite loci, as the samples were pre-selected, multiple allele in the same isolate was a rare occurrence. On the event of a two or more alleles being detected, the majority allele was used if the minority peak was $\leq$25% of the height of the majority and the sample was treated as monoclonal. Where peaks were of equivalent height or minority peak >25% of the majority, the data was recorded for other purposes but discounted for this analysis.

Table 4. Primer sequence and Chromosome target (all primers are listed 5` → 3`)

(a) *Dhfr* linked[1]

Name	Label	Sequence	Chromosome
DHFR.3kb.3R		GGC ATA AAT ATC GAA AAC	4
DHFR.3kb.F		AAT CCA ACA TTT TCA AGA	
DHFR.3kb.RVic	Green	TCC ATC ATA AAA AGG AGA	
MA 1.4kb.3F		GTT GTC AAT AAT TTC TGC ATC	4
MA 1.4kb.R		CGA TAT ATC TGA TGG GTG A	
MA 1.4kb.FFAM	Blue	TAC CAT AGC AGT CTT TGC A	
MA 2.3kb.3F		TAC ATA ATT CAT ATG AAC TTG	4
MA 2.3kb.R		CAC ATA TTA TAC AGG ACG A	
MA 2.3kb.FFAM	Blue	CCT GCA TTT GCA AGA AGT A	

[1] Roper *et al.* (2003)

(b) Unlinked[2]

Name	Label	Sequence	Chromosome
Poly α-3R		ATC AGA TAA TTG TTG GTA	4
Poly α-F		AAA ATA TAG ACG AAC AGA	
Poly α-RNED	Green	GAA ATT ATA ACT CTA CCA	
PfPk2-RNED	Yellow	CCT CAG ACT GAA ATG CAT	12
PfPk2-F		CTT TCA TCG ATA CTA CGA	
PfPk2-3R		AAA GAA GGA ACA AGC AGA	
TA109-FFAM	Blue	TAG GGA ACA TCA TAA GGA T	6
TA109-R		CCT ATA CCA AAC ATG CTA AA	
TA109-3F		GGT TAA ATC AGG ACA ACA T	

[2] Anderson *et al.* (1999)

3.7 Statistical analysis

Wrights fixation index (Fst) were calculated in Arlequin software (Schneider *et al.*, 2000). Allele frequencies of *dhfr* and *dhps* in Kilombero/Ulanga and Rufiji time series

were compared using Fisher's exact test in STATA version 9.0. To test the significance of the changes in frequence of one allele alone, χ^2 or Fisher's exact test was used. Gene diversity values were calculated as $He = [n/(n-1)][1- \Sigma Pi^2]$ where n is the number of samples and Pi is the frequency of the ith allele. The variance of the diversity was calculated using Nei and Roychoudhury's formula (Nei and Roychoudhury, 1974).

$$Var = 2/n\ (n-1)\ \{(n-1)\ [\Sigma Pi^3 - (\Sigma Pi^2)^2] + \Sigma Pi^2 - (\Sigma Pi^2)^2\}$$

CHAPTER FOUR

4. 0 RESULTS

This study has generated data in a population-wide analysis of genetic changes in the *P. falciparum dhfr* and *dhps* genes from Southeastern Tanzania. This chapter is divided into five sections; section 4.1 examines the influence of National policy change in 2001 from CQ to SP on the evolution of resistant *dhfr* and *dhps* alleles in Rufiji and Kilombero/Ulanga populations. Section 4.2 examines whether there was impact of SP+ART combination therapy on drug pressure by comparing *dhfr* and *dhps* point mutation haplotype changes in the Rufiji population with those in Kilombero/Ulanga at cross sectional surveys in 2002, 2004, 2005 and 2006. Section 4.3 assesses the spatial distribution of point mutation haplotypes in the study communities, using wards as subpopulations within each district. Section 4.4 studies the polymorphic microsatellite repeats flanking the *dhfr* gene and report the evolutionary relationships between the allelic haplotypes found at this locus. Section 4.5 analyses unlinked microsatellite markers in the Rufiji and Kilombero/Ulanga populations at two time points (2001/2002 and 2006) to examine the trend on multiplicity of infection.

4.1 Effects of policy change on resistant *dhfr* and *dhps* allele frequencies in Kilombero/Ulanga and rufiji districts

About 20 000 people were sampled in the 2000, 2001 and 2002 annual surveys and 4 949 asymptomatic infections identified. DNA was extracted from the 4 949 *P. falciparum* positive bloodspots and PCR of *dhfr* and *dhps* performed once, giving a

combined rate of PCR amplification success of 69% for both genes (Table 5). The amplified products were screened for all the variant sequences described in Table 3. Out of the 3 436 isolates which amplified for *dhfr*, 71% were single or majority genotype infections and the point mutation haplotypes could easily be resolved. Of the 3 412 samples which amplified for *dhps*, 81% were single or majority genotype with resolvable haplotypes. To test wherther the idea of fusing Kilombero and Ulanga districts was correct, frequencies of *dhfr* and *dhps* genes in a subset of samples in the 2000 survey were compared between the two districts. Results indicated that the two districts had remarkably similar *dhfr* and *dhps* haplotype frequencies, hence could not be differentiated (data not shown).

Table 5. Annual survey population, proportions that were malaria positive and their PCR amplification outcome for the period between 2000 – 2002 in Rufiji and Kilombero/Ulanga populations.

Year	Rufiji			Kilombero/Ulanga		
	2000	2001	2002	2000	2001	2002
Survey population	2 844	3 285	3 349	3 289	3 197	4 098
P. falciparum positive	778 (27.4%)	908 (27.6%)	854 (25.5%)	955 (29.0%)	580 (18.1%)	875 (21.3%)
PCR amplified *dhfr*	549	683	687	404	488	686
PCR amplified *dhps*	521	592	725	444	347	720
Single or majority *dhfr*	455	420	527	376	238	489
Single or majority *dhps*	417	519	596	365	294	603
Single or majority *dhfr+dhps*	288	278	404	190	138	381

4.1.1 Allelic Haplotypes found at *dhfr* and *dhps* genes

The point mutations found at *dhfr* gene were N51I, C59R and S108N. They were found in the following haplotypic arrangements CNCSI, CNCNI, CNRNI, CICNI and CIRNI which are common throughout East Africa and have previously been reported in Tanzania (Alifrangis *et al.*, 2003b; Pearce *et al.*, 2003), Malawi (Bwijo *et al.*, 2003) Kenya (Nzila *et al.*, 2000a, Omar *et al.*, 2001), Uganda (Kyabayinze *et al.*, 2003, Sendagire *et al.*, 2005). Two rare combinations of mutations were found; N51I with C59R (CIRSI) was found in a single individual (previously reported in Uganda (Kyabayinze *et al.*, 2003), and C59R alone (CNRSI) which was also found once. Five *dhps* mutations (S436A, S436C, S436F, A437G and K540E) were found, with nine distinct haplotypic arrangements; SAKAA, AAKAA, SGEAA, SGKAA, and SAEAA have been described previously in isolates from East Africa (Nzila *et al.*, 2000a; Omar *et al.*, 2001; Kyabayinze *et al.*, 2003; Pearce *et al.*, 2003, Sendagire *et al.*, 2005) while the remaining four CAKAA, FAKAA, AAEAA, and FAEAA were found in extremely low frequency (Appendix I) and have not been reported before. This is a reflection of the larger sample size and the fact that some alternative typing methodologies do not have the capacity to detect most of the codon 436 SNP's.

The *dhfr* CIRN (N51I+C59R+S108N) haplotype and the *dhps* SGEAA (A437G+K540E) are the two alleles with greatest significance for SP efficacy and their frequency in the three successive surveys in Kilombero / Ulanga and Rufiji are shown in Figures 6A and B.

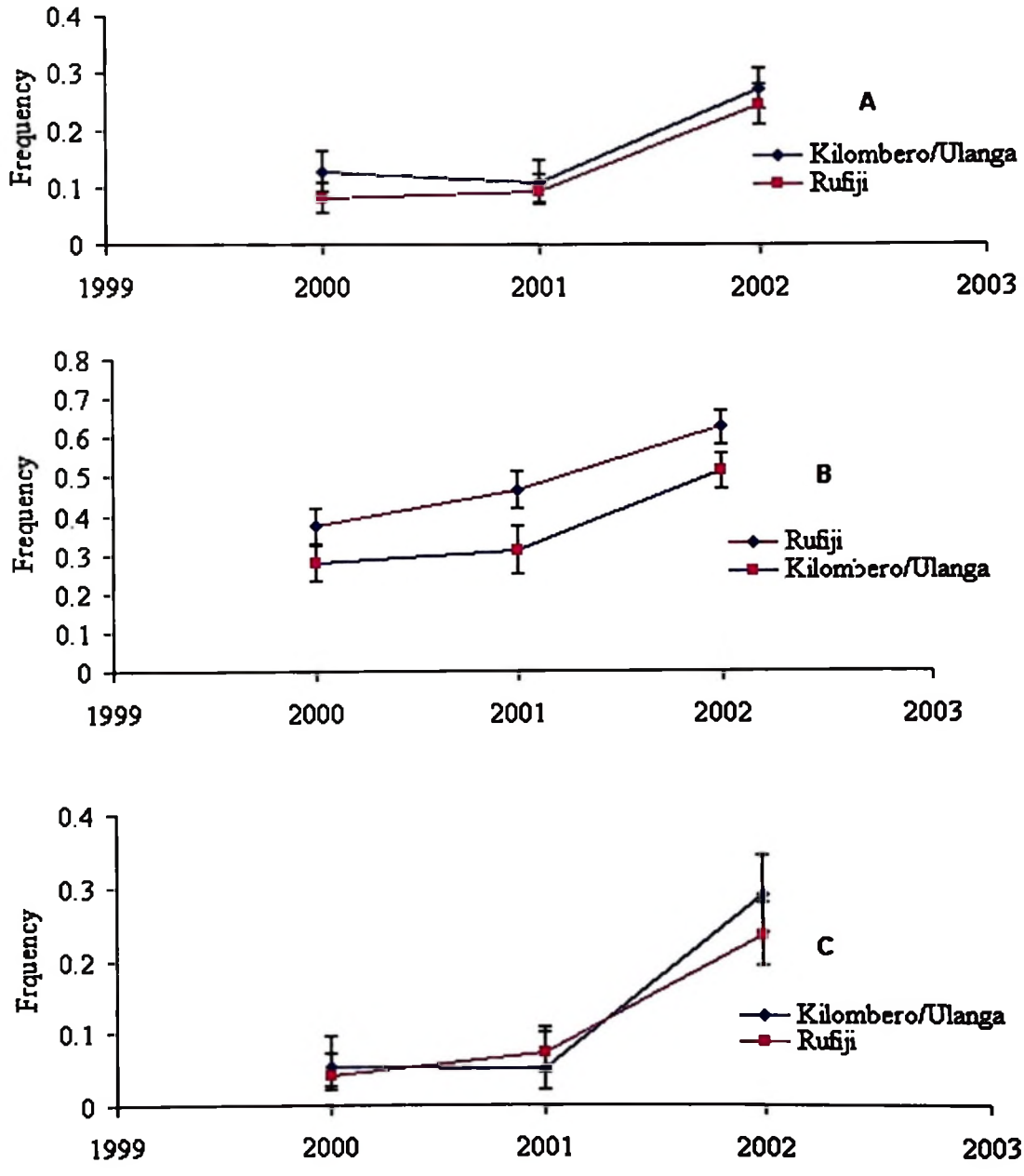

Figure 6. Changes in the frequency of SP resistance genes in Kilombero/Ulanga and Rufiji in cross sectional surveys in 2000, 2001 and 2002. A) The frequency of the *dhps* double mutant allele. B) The frequency of *dhfr* triple mutant alleles. C) The frequency of triple mutant *dhfr* + double mutant *dhps* genotype.

Changes occurring under CQ first line (2000 to 2001) are markedly different to those under SP use (2001 - 2002). The frequency of the *dhps* double mutant A437G+K540E (Figure 6A) did not change significantly between 2000 and 2001 in either Rufiji ($p=0.411$) or in Kilombero/Ulanga ($p=0.497$). Contrastingly, there was a highly significant change between 2001 and 2002 in both Rufiji ($P\leq0.0001$) and Kilombero/Ulanga ($P\leq0.0001$) (95% confidence intervals were calculated by binomial exact). The frequency of the *dhps* double mutant haplotype was remarkably similar in Kilombero/Ulanga and Rufiji and there were no significant differences between the two districts at any time point. Changes in the frequency of the *dhfr* triple mutant allele N51I+C59R+S108N are shown in Figure 6B was significantly higher in Rufiji than in Kilombero/Ulanga at all timepoints. Between 2000 and 2001 there was a non-significant trend towards increase in both Kilombero/Ulanga ($P\geq0.05$) and Rufiji ($P\geq0.05$), while between 2001 and 2002 the increase was highly significant in Kilombero/Ulanga ($P\leq0.0001$) and Rufiji ($P\leq0.0001$)

4.1.2 Two locus genotypes

In a further subset of samples for which *dhfr* and *dhps* haplotypes could both be conclusively resolved it was possible to measure the frequency of two locus genotypes. In Figure 6C the frequency of the triple *dhfr*-double *dhps* mutant genotype in the two populations was compared. The frequency estimates are similar in the two districts. In both districts there was no change between 2000 and 2001 (Kilombero/Ulanga, $P\geq0.5$

and Rufiji, $P \geq 0.5$) but a highly significant 4-fold increase in frequency between 2001 and 2002 in Kilombero/Ulanga ($P \leq 0.0001$) and Rufiji ($P \leq 0.0001$).

4.1.3 Haplotypes conferring intermediate levels of resistance

In addition to the highly resistant *dhps* A437G+K540E double mutant a number of single 436 mutant alleles were recorded. Among these by far the most common was the S436A which was consistently found at frequencies of 10%-20% throughout. Figure 7 shows the frequencies of the sensitive, S436A single mutant and A437G+K540E double mutant alleles over time in both districts. This data shows that the significant increase of the double mutant allele between 2001 - 2002 displaced the sensitive allele which decreased significantly in Rufiji (Figure 7A) ($P \leq 0.0001$) and Kilombero/Ulanga (Figure 7B) ($P \leq 0.01$). Interestingly, the frequency of the A436A allele was static throughout.

The effect of policy upon the *dhfr* sensitive and double mutant alleles are shown in Figure 8. The increase of the triple mutant allele acted to displace sensitive alleles, which showed a substantial decline in Kilombero/Ulanga 2000 - 2001 ($P \geq 0.5$), 2001-2002 ($P \leq 0.0001$) and Rufiji 2000 - 2001 ($P \geq 0.5$), 2001 - 2002 ($P \leq 0.0001$) period. The double mutant *dhfr* alleles which confer intermediate levels of resistance neither increased nor decreased, remaining at a frequency of around 10% in both districts throughout all surveys.

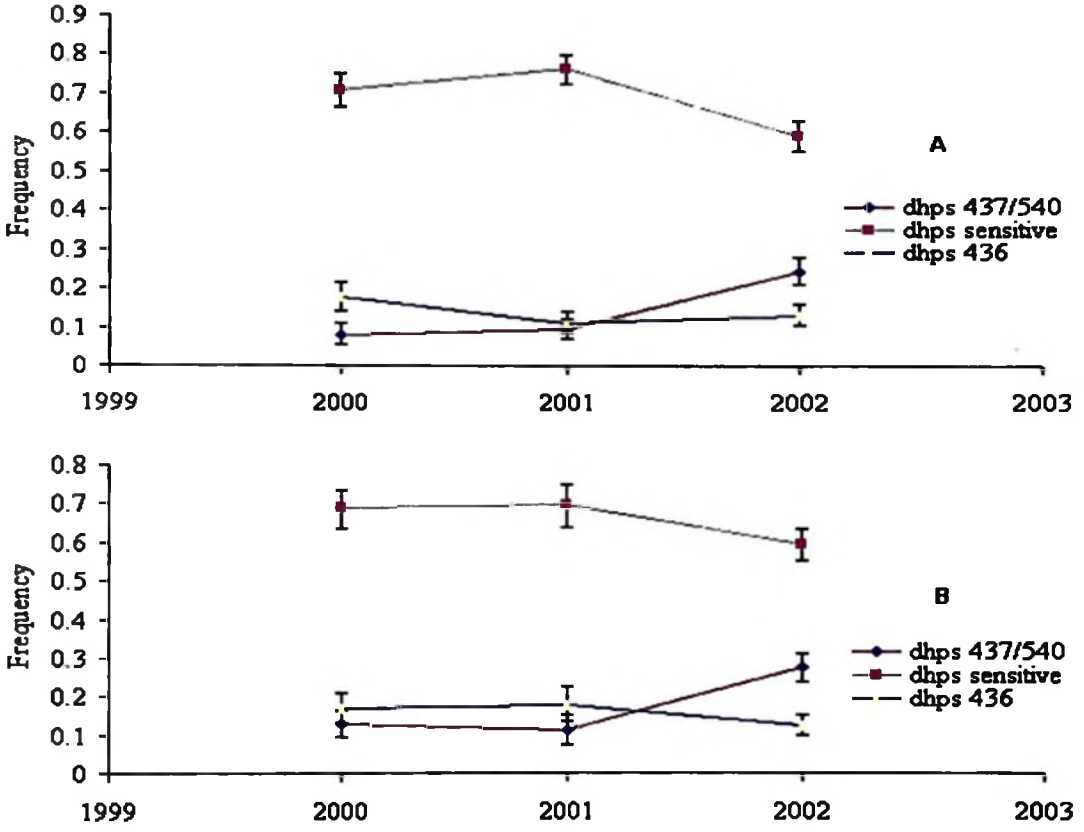

Figure 7. Allele frequency changes at *dhps* in Rufiji (A) and Kilombero/Ulanga (B).

4.1.4 Linkage disequilibrium

To examine the effects of simultaneous selection by pyrimethamine on *dhfr* and sulphadoxine on *dhps*, the two-locus genotypes sampled from both districts in the three successive surveys were analysed. Taking the subset of samples for which point mutation haplotypes could be unequivocally be resolved at both genes, the observed and expected frequencies generated from contingency tables were compared. Significant association between the *dhfr* triple mutant allele and the *dhps* double mutant allele occurred in 2002 in both districts.

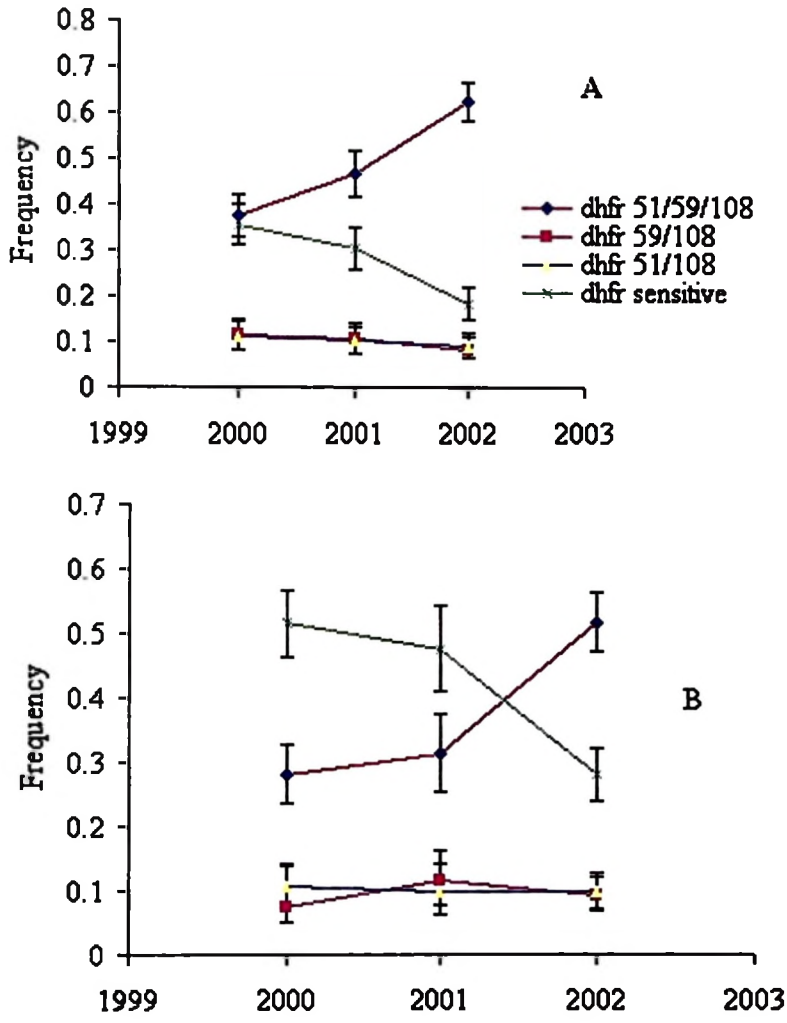

Figure 8. Allele frequency changes at *dhfr* in Rufiji (A) and Kilombero/Ulanga (B). The sensitive allele, the triple mutant N51I+C59R+S108N allele and double mutants C59R+S108N and N51I +S108N.

The d' value an index of linkage disequilibrium are shown in Figure 9 which examines linkage between all *dhfr* alleles and the *dhps* A437G+ K540E double mutant allele, and supplementary information showing observed and expected values for LD is given in Appendix XVI. There were non-significant positive associations between *dhfr* triple and

double mutant alleles and the *dhps* double mutant allele prior to the introduction of SP first line therapy in 2000 and 2001.

The strength of the association between *dhfr* triple mutant and *dhps* double mutant alleles increased in 2002 with d' 0.32 and d'=0.36 in Rufiji and Kilombero/Ulanga respectively and chi square analysis showed this was highly significant (Kilombero/Ulanga $P \leq 0.0001$, Rufiji $P \leq 0.001$). The non-significant positive association between *dhfr* double mutant alleles and *dhps* double mutant alleles during 2000 and 2001 became negative in 2002.

4.1.5 Estimation of haplotype frequencies

Haplotype frequency was calculated as the ratio of allelic haplotypes among those infections where a single or majority haplotype was detected at the resistance locus. Across the study as a whole the proportion of infections included in the frequency calculation was 81% of infections for *dhps* and 71% of infections for *dhfr*. To examine the extent of the underlying rate of mixed infections and test the robustness of the assumption that these are an adequate reflection of the frequencies among mixed infections, unlinked microsatellite markers (*Poly A, Pfpk2 and TA109)* were characterised in 178 and 180 samples from Kilombero/Ulanga and Rufiji, respectively, collected in 2002. The minimum number of co-infecting genotypes in each infection was determined according to the number of alleles detected in each sample this is known as the "multiplicity of infection" or (MOI) (Figure 10)

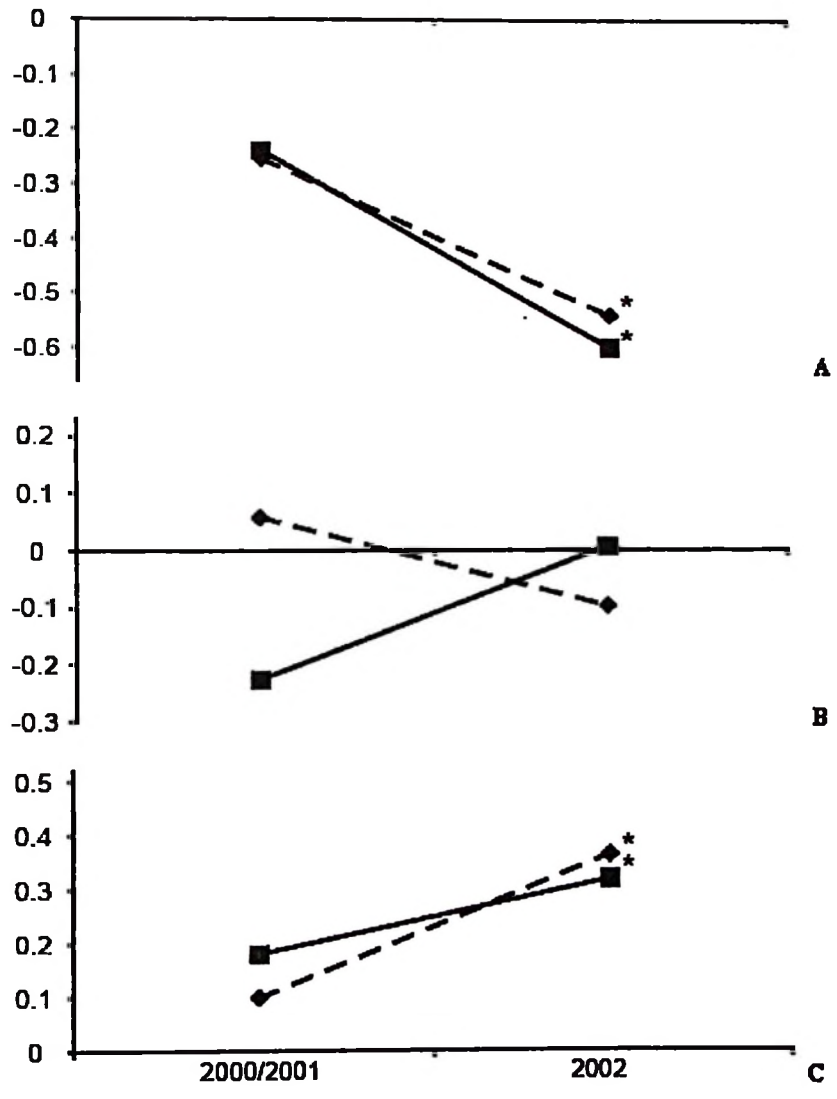

Figure 9. Linkage disequilibrium between the *dhps* A437G+ K540E allele and different alleles at *dhfr* (A) the *dhfr* triple mutant N51I+C59R+S108N (B) *dhfr* double mutants C59R+S108N and N51I+ S108N (C) the *dhfr* sensitive allele. The d' values for 2000 + 2001(Kilombero/Ulanga (dashed line) n=328, Rufiji (solid line) n=566) combined and for 2002 (Kilombero/Ulanga n=381, Rufiji n= 404) are shown. Significant deviation between observed from expected occurred in 2002 indicated by *($P \leq 0.001$).

This was achieved by running all three markers on each sample and an MOI for a particular sample was taken to be the highest number of infection detected in one or more markers. To test the assumption that the distribution of resistance alleles among mixed infections is random, the presence/absence of *dhps* A437G+K540E mutations was recorded in mixed and non-mixed infections. Then the underlying frequency based on the estimates of the multiplicity of infection given above was predicted using the maximum likelihood method of Schneider *et al.* (2002).

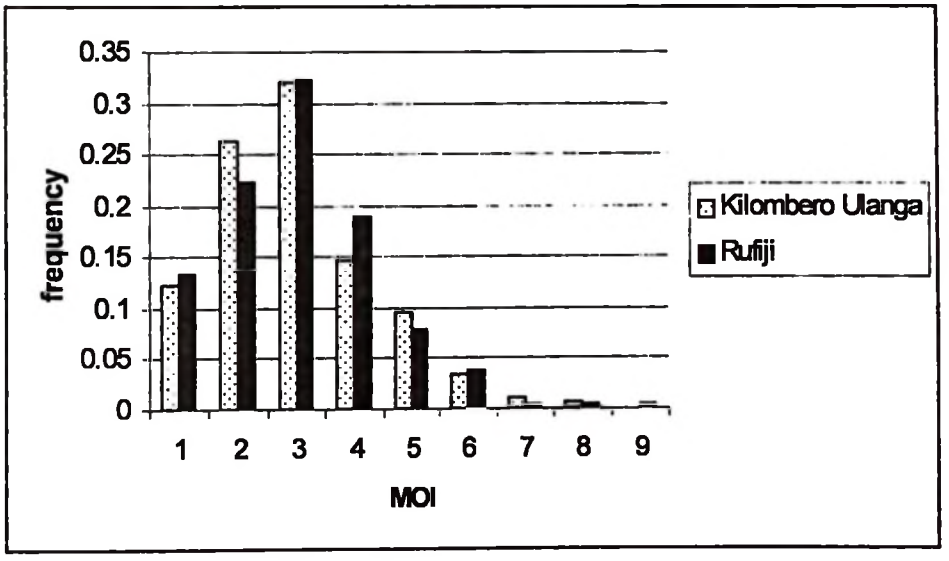

Figure 10. MOI estimated using 3 microsatellite loci (Poly A, Pfpk2 and TA109) in 2002 isolates.

The analysis predicted an underlying frequency of the *dhps* double mutant of 0.276 in Rufiji (where the frequency among single and majority genotype infections was 0.274) and a frequency of 0.262 in Kilombero/Ulanga (where the frequency among single and majority genotype infections was 0.245). The estimates using these two approaches were

very consistent showing that no obvious bias was introduced into the estimation of frequencies by excluding mixed infections.

4.2 Effects of act on resistant *dhfr* and *dhps* alleles in Rufiji

The effect of antimalarial CT of SP and artesunate (SP + ART) in Rufiji on the selection for resistant *dhfr* and *dhps* alleles was measured by comparing two sites with contrasting antimalarial first line treatment policies and hence contrasting selection strengths. In Rufiji, SP + ART combination begun in late 2002 as a first-line therapy for uncomplicated malaria while Kilombero/Ulanga maintained SP monotherapy which begun a year earlier, as the national guideline for the treatment of non severe malaria.

About 34 477 individuals were sampled in a series of 4 annual surveys conducted between 2002 and 2006 in Rufiji (test district) and Kilombero/Ulanga (comparative district) and 6 441 asymptomatic infections identified. DNA was extracted from the 6 441 *P. falciparum* positive bloodspots and PCR of *dhfr* and *dhps* was performed once, giving a combined rate of PCR amplification success of 75% for both genes (Table 6). The amplified products were screened for all the variant sequences as described in Table 3. Out of the 4 690 isolates which amplified for *dhfr* 71% were single or majority genotype infections and the point mutation haplotypes could easily be resolved. On the other hand, 82% of the 4 942 samples which amplified for *dhps,* were single or majority genotype with resolvable haplotypes.

Table 6. Annual survey population, proportions that were malaria positive and their PCR amplification outcome for the period between 2002 – 2006 in Rufiji and Kilombero/Ulanga populations.

	Rufiji				Kilombero/Ulanga			
Year	**2002**	**2004**	**2005**	**2006**	**2002**	**2004**	**2005**	**2006**
Survey population	3 349	3 771	5 516	4 267	4 098	4 122	5 006	4 500
P. falciparum positive	854 (25.5%)	672 (17.8%)	1021 (18.5%)	916 (21.5%)	875 (21.3%)	972 (23.6%)	486 (9.7%)	645 (14.3%)
PCR amplified *dhfr*	687	509	797	683	686	660	374	294
PCR amplified *dhps*	725	505	847	703	720	712	407	323
Single or majority *dhfr*	527	428	805	616	489	540	267	275
Single or majority *dhps*	596	364	755	588	603	562	293	275
Single or majority *dhfr+dhps*	404	263	593	492	381	381	283	206

Allelic haplotype frequencies at dhfr and dhps genes

As observed in section 4.2.1 for the 2000 – 2002 isolates from Rufiji and Kilombero/Ulanga, the same point mutations (N51I, C59R and S108N at the *dhfr* and S436A, S436C, S436F, A437G and K540E at the *dhps*) were observed from the two populations in the 2002 – 2006 isolates. Moreover, the same common haplotypes (CNCSI, CNRNI, CICNI and CIRNI for the *dhfr* and SAKAA, AAKAA, SGEAA, SGKAA, and SAEAA for the *dhps* gene) and rare (CNRSI and CNRSI for the *dhfr* and

CAKAA, FAKAA, AAEAA, and FAEAA for the *dhps* gene) were also observed from Rufiji and Kilombero/Ulanga populations between 2002 – 2006. The only exception is the detection of 164L mutation in a single isolate at the *dhfr* gene. Mutation at codon 164 (I164L) was detected in only one of the 4 690 isolates, which amplified at *dhfr* gene, and it occurred as a mix of minority mutant in a sensitive majority.

Frequencies of common and rare haplotypes detected in all 11 390 isolates genotyped between 2000 – 2006 are given in the Appendix I. The 2002 isolates provided starting frequencies at both *dhfr* and *dhps* genes in both Rufiji and Kilombero/Ulanga sites which were used as a baseline for ACT monitoring. Evaluation of the effectiveness of the ACT in Rufiji is therefore based on the monitoring of the trend of *dhfr* and *dhps* haplotypes in Rufiji relative to Kilombero/Ulanga. Figures 11 and 12 show the effects of different treatment policy between Rufiji (with SP + ART and hence a putatively reduced SP selection strength) and Kilombero/Ulanga (with SP monotherapy and hence sustained maximum selection strength).

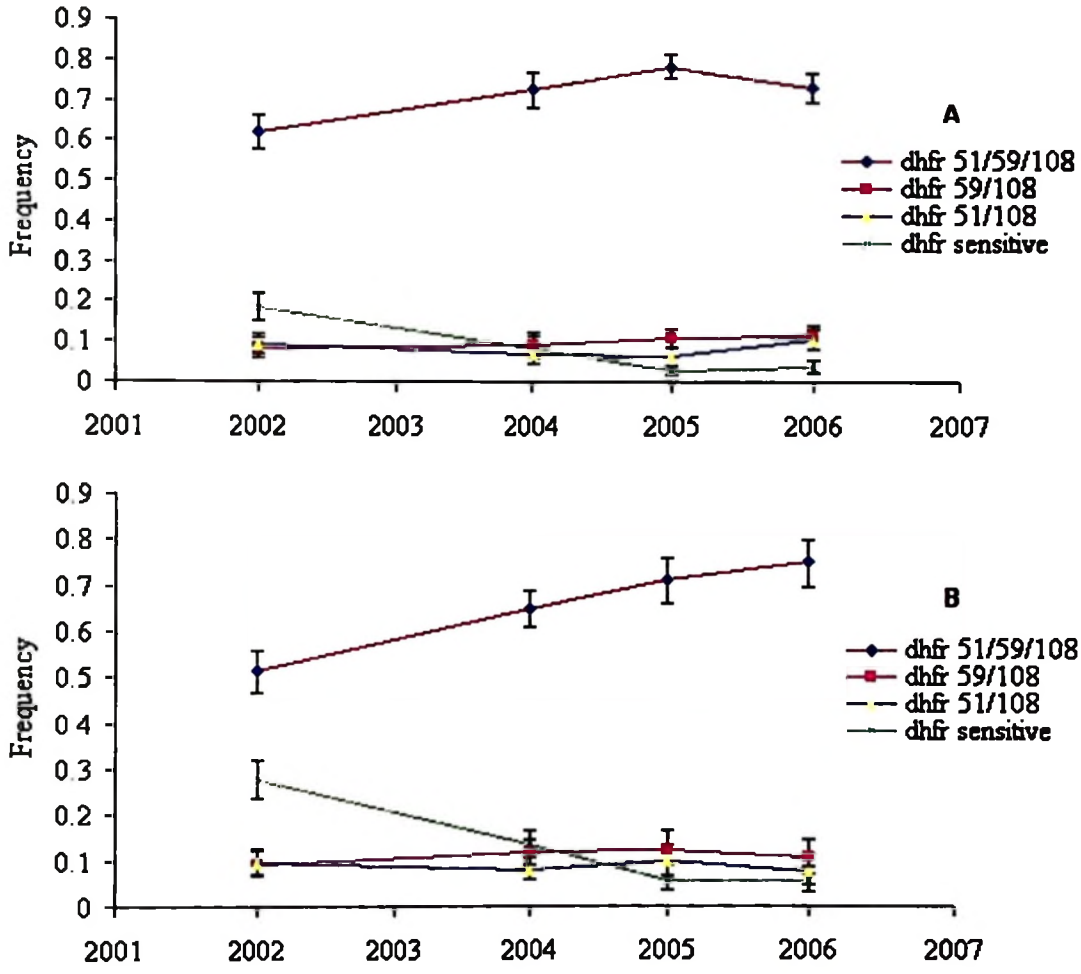

Figure 11. Changes in frequency of *dhfr* allelic haplotypes in Rufiji (A) and Kilombero/Ulanga (B). Frequency values are shown with 95% confidence interval calculated by binomial exact method.

(i) Starting (baseline) frequency of the allelic haplotypes

The baseline *dhfr* and *dhps* allelic haplotypes detected in both Kilombero/Ulanga and Rufiji districts before the start of pilot ACT in Rufiji in late 2002; provide an important landmark for monitoring the effect of the ACT intervention in Rufiji.

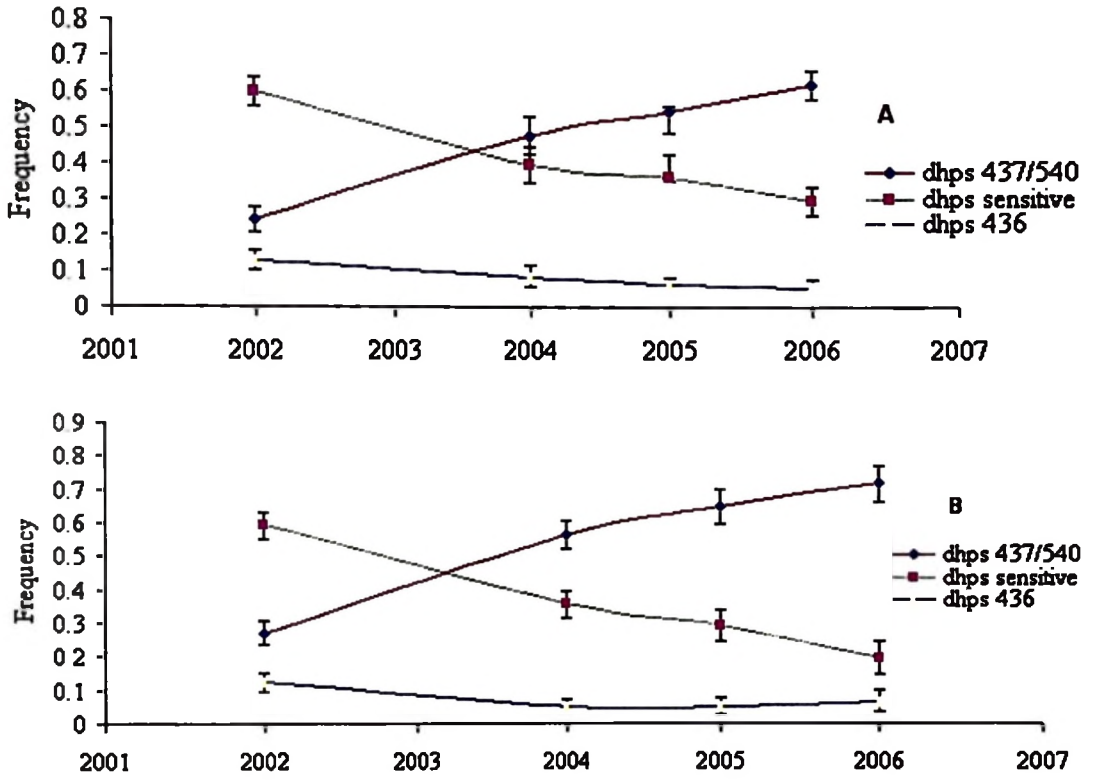

Figure 12. Allele frequency changes at *dhps* in Rufiji (A) and Kilombero/Ulanga (B). Frequency values are shown with 95% confidence interval calculated by binomial exact method.

This is because for the benefit of combination therapy to be maximised, all drugs contained in the combination must be highly efficacious. Since sampling in all of the surveys were done at a latter part of high malaria transmission season (between June and September) and the ACT in Rufiji begun between December 2002 and January 2003 (Kachur *et al.*, 2004), the 2002 genotype data provide a timely starting (baseline) frequency just before the intervention. Figures 11 and 12 show the frequency change of the allelic haplotypes at both *dhfr* and *dhps* genes occurring between 2002 - 2006 in Rufiji and Kilombero/Ulanga. As shown previously the *dhfr* triple mutant (N51I+C59R+

S108N), *dhps* double mutant (A437G+ K540E) and their combination (triple *dhfr*-double *dhps*) are the three alleles which have the greatest significance for SP efficacy. The starting frequency of the *dhfr* triple mutant (N51I+C59R+ S108N) allelic haplotype was significantly higher in Rufiji (63%) than Kilombero/Ulanga (52%) ($P \leq 0.001$) (Figure 13A). The triple mutant *dhfr* allele continued to rise in Kilombero/Ulanga and Rufiji displacing the sensitive *dhfr* alleles while double mutant *dhfr* remained static. At the *dhps* locus the starting frequency of the double mutant (A437G+ K540E) allelic haplotype was higher in Kilombero/Ulanga (27%) than Rufiji (25%) though not statistically significant ($P \geq 0.05$) (Figure 13B). In the absence of I164L mutation, isolates bearing the combination of triple *dhfr* and double *dhps* mutations bears the greatest statistical association with failure to clear parasitaemia after SP treatment (Kublin *et al.*, 2002) and hence holds the greatest predictive value for the outcome following SP administration.

The study has detected higher starting frequency of the triple *dhfr*-double *dhps* mutant isolates in Kilombero/Ulanga (29%) than Rufiji (23%) ($P \geq 0.05$) (Figure 13C). There is scientific evidence suggesting that, once established drug resistance determinants to pyrimethamine and sulfadoxine are highly mobile (Roper *et al.*, 2003; Roper *et al.*, 2004). Generally the starting frequencies of both triple *dhfr* and double *dhps* resistance alleles and their combinations were too high for SP to be an ideal partner for the combination with artesunate.

(ii) Effects of treatment policy in Rufiji and Kilombero/Ulanga

Figure 13 compares changes of allelic haplotype frequencies of the alleles which have been shown to have greatest significance for SP efficacy between Rufiji (SP + ART) and Kilombero/Ulanga (SP monotherapy). As shown earlier, at the *dhfr* gene, the baseline frequency of the triple mutant allele was significantly higher in Rufiji (63%) than Kilombero/Ulanga (52%) ($P \leq 0.001$). As the SP drug pressure increased in Kilombero/Ulanga a steady increase in the frequency of the *dhfr* triple mutant along with a simultaneous slight decline in the frequency of the allele in Rufiji was observed, as ACT took force. However, during the year 2006, a reversal of the trend was observed, where the frequency of the triple mutant *dhfr* allele became higher in Kilombero/Ulanga (75%) than Rufiji (74%) (Figure 13A), even though the difference was not significant ($P \geq 0.05$). The effect of ACT on the double *dhps* alleles in Rufiji (Figure 13 B) showed a similar trend as the triple mutant *dhfr* alleles. However, the double mutant *dhps* curves show gradual divergence between Rufiji and Kilombero/Ulanga because as opposed to the triple mutant *dhfr* allele the starting frequency of double *dhps* allele was higher in Kilombero/Ulanga (27%) than Rufiji (25%) ($P \geq 0.05$). The differences in the double *dhps* allele between Rufiji and Kilombero/Ulanga continued to widen gradually as ACT and SP drug pressure increases in Rufiji and Kilombero/Ulanga, respectively reaching a frequency of 62% in Rufiji and 72% in Kilombero/Ulanga in 2006.

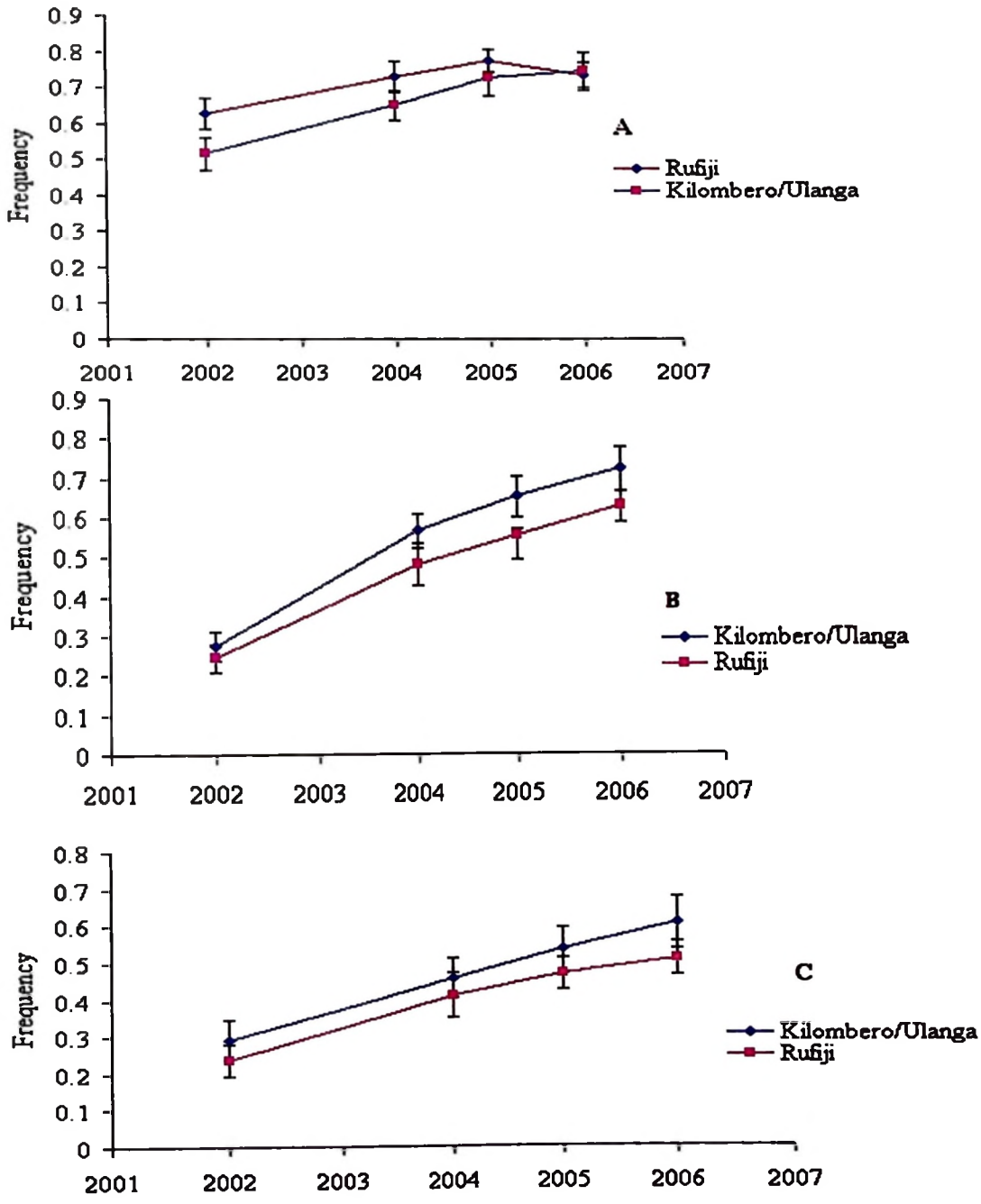

Figure 13. ***Dhfr*** **and** ***dhps*** **allelic frequency change between 2002 and 2006 in Rufiji and Kilombero/Ulanga districts (A) Triple mutant** ***dhfr*** **allele (B) double mutant** ***dhps*** **allele (C) triple** ***dhfr*****-double** ***dhps*** **allele.**

In other words, the ACT in Rufiji appeared to slow the rate of increase of double *dhps* alleles, reducing their frequency relative to that in Kilombero/Ulanga by 8%. By measuring directly the frequency of the highly resistant two locus genotype combinations consisting of double *dhps* and triple *dhfr* mutant alleles, their frequencies were compared between Rufiji and Kilombero/Ulanga. A trend very similar to that displayed by the double *dhps* allele was observed in both Rufiji and Kilombero/Ulanga populations (Figure 13C). The starting frequency was higher in Kilombero/Ulanga (29%) than in Rufiji (23%) ($P \geq 0.05$). While the SP drug pressure maintained a steady increase of the starting frequency in Kilombero/Ulanga, the ACT slightly slowed the selection of the double *dhps*-triple *dhfr* allele in Rufiji, such that by 2006 the frequencies were 60% in Kilombero/Ulanga and 50% in Rufiji ($P \leq 0.05$). Likewise, the ACT in Rufiji achieved the slowing of the rate of selection of the highly resistant two locus genotype combination consisting of double *dhps* and triple *dhfr* mutant alleles by 4%.

(iii) Alleles conferring intermediate levels of resistance

Both *dhfr* (N51I + S108N) and (C59R + S108N) double mutant alleles showed irregular slight rise and fall of frequency in both Rufiji and Kilombero/Ulanga between 2002 – 2006, maintaining an average frequency of around 10% (Figure 11). This trend is similar to the one described in Section 4.2.3 for the same alleles between 2000 – 2002, a time marked by the escalation of SP drug pressure following national policy change from SP second line to first line antimalarial treatment (Figure 8). In the absence of better explanation, the apparent lack of response to the intensifying drug selective pressure

suggests these alleles plays a peripheral role in conferring SP resistance. At the *dhps* locus, most of the single mutant alleles such as S436C and S436F were rare and their presence or absence at a particular survey was purely stochastic. Consequent to this it was difficult to understand their contribution in sulfadoxine resistance. Interestingly, the S436A single mutant *dhps* allele was present in all the surveys starting with an average frequency of 13% in both Kilombero/Ulanga and Rufiji populations. The frequency decreased gradually in both Rufiji and Kilombero/Ulanga populations and by 2006 the frequency had reached an average of 6% in both populations. These findings indicate there was no specific effect of ACT on single mutant *dhps* allele (S436A) but the question still remains, does this mutation have a role in sulfadoxine resistance? It could be speculated that, since as the double mutant *dhps* inreased the single mutant *dhps* allele was displaced in much the same way that the sensitive allele was, it is likely that the allele shares similarities with the sensitive *dhps* allele.

(iv) Sensitive alleles

Figure 14 depicts *dhfr* and *dhps* sensitive allele frequencies over time. The starting frequencies of the sensitive *dhfr* allele in the year 2002 was significantly higher in Kilombero/Ulanga (28%) than Rufiji (18%) ($P \leq 0.001$). The rate of decline of the sensitive *dhfr* allelic haplotype frequency was slowed by the use of ACT in Rufiji such that by 2004, the difference was less significant between Kilombero/Ulanga (13%) and Rufiji (8%) populations ($P \leq 0.05$).

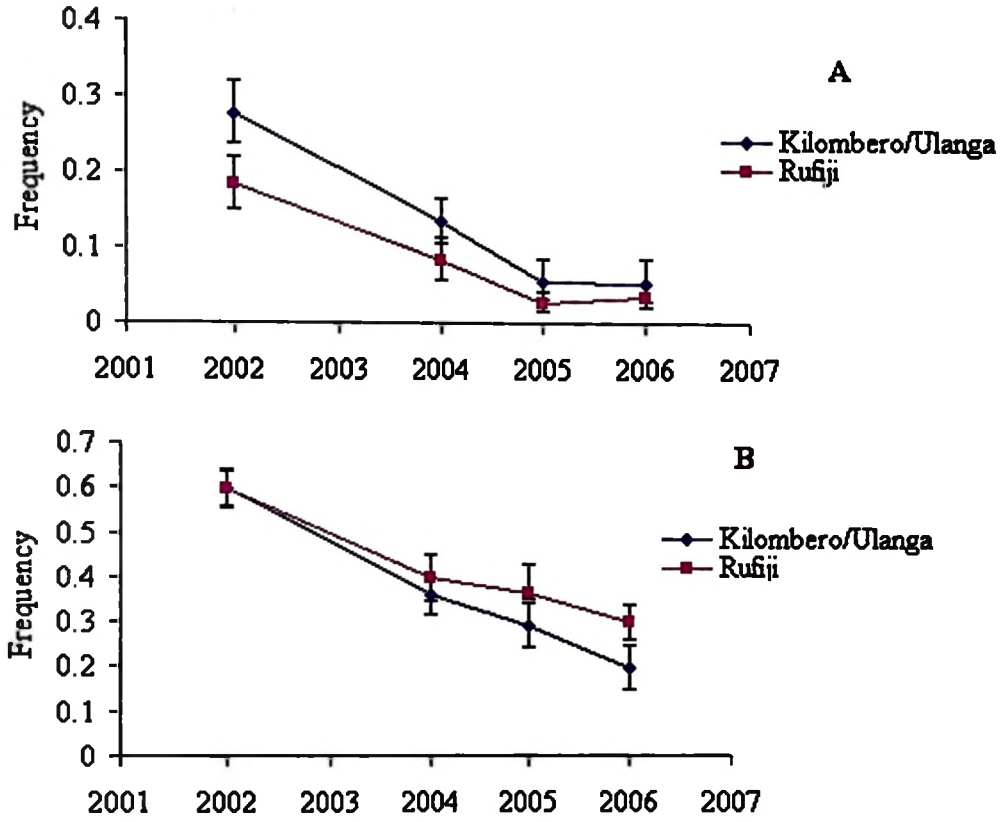

Figure 14. Sensitive *dhfr* and *dhps* allelic frequency change between 2002 and 2006 in Rufiji and Kilombero/Ulanga districts. Sensitive *dhfr* allele (A) Sensitive *dhps* allele (B).

The frequency of the allele continued to decline faster in Kilombero/Ulanga than Rufiji progressively narrowing the gap to 1% (5% in Kilombero/Ulanga and 4% in Rufiji) in the year 2006. While the declining sensitive allele frequency in *dhfr* displayed converging curves, an opposite trend in the decline of the *dhps* sensitive alleles was observed portraying diverging curves. Both populations started with an equal frequency of 60% but Kilombero/Ulanga kept with faster declining rate than Rufiji where the rate of decline was slowed by the ACT effect (Figure 14B). The difference in the rate of

deline between the two populations continued to widen and by 2006 ACT had achieved a significant effect in stabilizing the sensitive *dhfr* alleles in Rufiji ($P \leq 0.05$)

(v) Effects of ACT on linkage disequilibrium (LD)

As previously described in Section 4.2.4 the same procedure was used to measure the effect of ACT on simultaneous selection by pyrimethamine on *dhfr* and sulfadoxine on *dhps* by comparing two-locus genotype combinations (triple *dhfr*-double *dhps*) sampled from both districts at the time of implementation of ACT in Rufiji (2002 - 2006). A highly significant LD in Kilombero/Ulanga, D'=0.36, $P \leq 0.00001$ and a very significant LD but slightly reduced strength of association in Rufiji D'=0.32, $P \leq 0.001$ was observed at the start of pilot ACT in Rufiji indicating that in both populations selection pressure was sufficiently higher to overcome the effect of recombination (Table 7).

By 2004, LD in Rufiji was non significant (D'=0.197, $P \geq 0.05$) while a highly significant LD in Kilombero/Ulanga (D'=0.3, $P \leq 0.00001$) was maintained, indicating that the combination of SP + ART in Rufiji brought about the disruption of the association between *dhps* double mutant and *dhfr* triple mutant alleles. Disruption of association between double *dhps* and triple *dhfr* alleles continued in Rufiji in 2005 (D'=0.11, $P \geq 0.05$) while in Kilombero/Ulanga the observed strength of association between the two alleles was maintained in 2005 (D'=0.26, $P \leq 0.0001$).

Table 7. The trend of the D' values (an index of linkage disequilibrium) and their P values in the period between 2002 – 2006 in Rufiji and Kilombero/Ulanga populations.

Year of survey	Rufiji		Kilombero/Ulanga	
	D'	P	D'	P
2002	0.3184	0.0012**	0.3633	0.00001****
2004	0.1967	0.0673	0.2948	0.00001****
2005	0.1076	0.121	0.2595	0.0001***
2006	0.1921	0.0008**	0.0814	0.2686

*Indicates degree of significance

In 2006 however, unexpected shift in the trend occured leading to re-gaining of association between double *dhps* and triple *dhfr* alleles in Rufiji (D'=0.19, P≤0.001) and disruption of the association between the two alleles in Kilombero/Ulanga (D'=0.08, P≥0.05).

4.3 Spatial distribution of *dhfr* and *dhps* resistance alleles

To examine the spatial distribution of *dhfr* and *dhps* alleles in the two populations of Rufiji and Kilombero/Ulanga, the wards from which sampled households belong, were used to define sub-populations within each district. In Kilombero/Ulanga, seven wards were sampled, Idete, Iragua, Kichangani, Lupiro, Mbingu, Mchombe and Minepa while four, Bungu, Ikwiriri, Kibiti and Mchukwi were sampled from Rufiji. As observed at district level, a similar number of both common and rare *dhfr* and *dhps* allelic haplotypes, which were distributed differently across ward subpopulations, were found in both Rufiji and Kilombero/Ulanga districts and their frequency are shown in

Appendix VII. As expected, most rare alleles were observed in the surveys with larger sample size. The 2000, 2002 and 2004 annual surveys had relatively larger sample sizes and hence more rare alleles than 2001, 2005 and 2006 (Appendix VII). It was also noted that in those surveys with fewer samples, rare alleles were not observed in some of the sub-populations in the Kilombero/Ulanga districts (Iragua, Lupiro and Minepa in 2001; Idete, Iragua, Kichagani, Mchombe and Minepa in 2005 and Kichangani and Lupiro in 2006, Appendix VII). During the survey an equal sample size was collected in both Rufiji and Kilombero/Ulanga populations, but on microscopy Rufiji had larger positive sample size than Kilombero/Ulanga, suggesting high malaria prevalence in Rufiji than Kilombero/Ulanga.

For the first time a non synonymous mutation was observed at codon 164 in 2006 cross sectional survey in Rufiji. Mutation at codon 164 causes an amino acid change from isoleucine to leucine. This mutation was detected in only a single sample from Ikwiriri sub population as a mixture of minority mutant in *dhfr* majority sensitive isolate. This mutation is believed to confer high levels of SP resisistance. Untill now, the I164L mutation remain extremely rare in African population while remaining very common in South East Asia and South American populations. Figure 15 show temporal distribution of the major resistant *dhfr* (triple mutant allele) and *dhps* (double mutant allele) and their combination (triple *dhfr*-double *dhps* mutant allele) in Rufiji and Kilombero/Ulanga wards. The trend was similar in the two districts and the overall pattern described at the district level was also seen at the ward sub-populations.

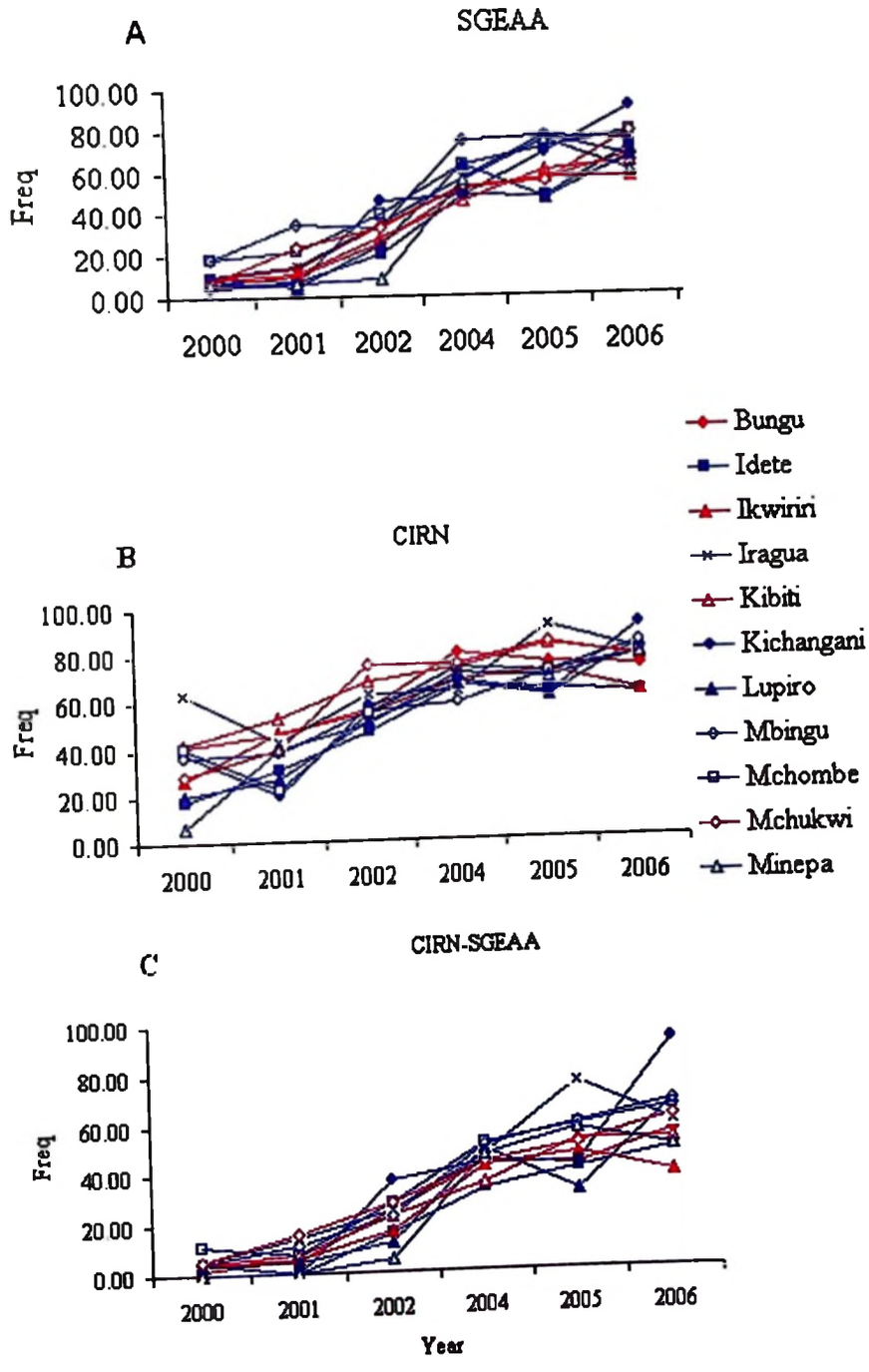

Figure 15. Resistance allele frequency distribution in Rufiji (red) and Kilombero/Ulanga (blue) wards. A is double mutant *dhps*, B is triple mutant *dhfr* and C is combination of the two (trple *dhfr*-double *dhps*).

Despite this trend of allele distribution, a significant sub-population differentiation was observed between wards of Rufiji and Kilombero/Ulanga and this differentiation varied between surveys (Appendix VIII). To test whether there was significant differences between wards, the frequencies of *dhfr* and *dhps* alleles were compared using Wright's fixation index and the significant values for these pairwise comparison are highlighted with shed (Appendix VIII).

The Fst pairwise comparison computed for each survey (2000 – 2006) within and between Kilombero/Ulanga and Rufiji sub-populations suggest significant differences in the frequency distribution of *dhfr* and *dhps* alleles. The heterogeneity in the frequency distribution beteen sub-populations was particularly prominent on the *dhfr*-triple and *dhps* double mutant alleles (Appendix VII). Therefore, the observed differences in the resistant *dhfr* and *dhps* alleles which were also confirmed by significant Fst pairwise comparison were due to heterogeneity in the drug selection strengths between sub-populations. Generally, pairwise population differentiation as revealed by significant Fst values were mainly due to differences in the double-*dhps* and triple-*dhfr* mutant proportions between populations.

To confirm whether the pairwise population differentiation revealed by significant Fst values were due to frequency differences of two most resistant alleles, the triple *dhfr* and double *dhps*, these alleles were excluded and Fst test was performed on the mild and intermediate resistant *dhfr* and *dhps* alleles alone (Appendix IX). The results showed

that there was no significant population differentiation of Fsts obtained from the mild and intermediate resistant *dhfr* and *dhps* alleles, thus confirming the observation.

4.4 *Dhfr* flanking microsatellite loci

In the current study, 728 isolates (CNCS = 126, CNRN + CICN = 346, and CIRN = 256) of *P. falciparum* carrying resistant or the sensitive *dhfr* chromosomes were genotyped. Although all these samples were unmixed based on the PCR-SSOP point mutation genotype data, 99 (14%) isolates contained >1 allele at one or more microsatellite loci, indicating existence of multiple *P. falciparum* clones while also highlighting higher sensitivity of microsatellite in detecting polyclonality compared to conventional methods. A further 183 (25%) isolates did not amplify at one or more microsatellite loci making construction of haplotype incomplete. Both the polyclonal, and isolates with incomplete microsatellite data were excluded from haplotype construction and any further analysis. Overall, 446 (61%) isolates, were included in haplotype construction and analysis of diversity values; 78 were the *dhfr* sensitive, 184 double mutant *dhfr* (C59R + S108N or N51I + S108N) and 184 the triple mutant *dhfr* (N51I + C59R + S108N).

Polymorphisms at microsatellite loci were measured by recording microsatellite allele length variation at each locus which is due to differences in the number of sequence repeats (Table 8). Numerous alleles at the 3 dhfr linked microsatellite loci were identified. The -0.3 kb locus had 11 alleles of 85-115 bp, the -4.4 kb locus had 19

alleles of 156-199 bp and the -5.3 kb locus had 18 alleles of 190-221bp, and these were evenly distributed among the two regions.

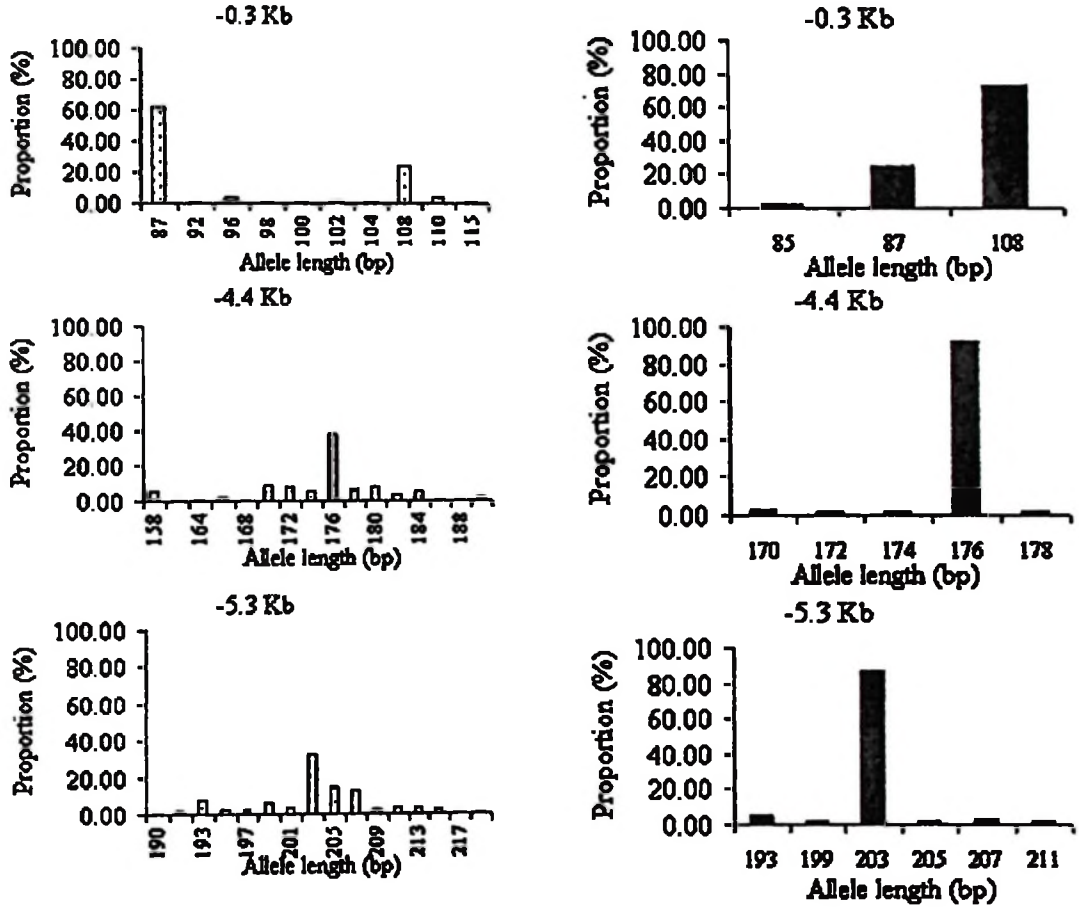

Figure 16. Frequency and distribution of microsatellite allele at the 3 loci upstream of *dhfr* gene. Sensitive (white) on the left and triple mutant *dhfr* (black) on the right.

Complete frequency and allele distribution of the three microsatellite loci within all forms of the *dhfr*-mutant and sensitive are given in Appendix II. Frequency of allelelic polymorphism within the sensitive and triple-mutant dhfr allele groups are presented in Figure 16. In the sensitive type *dhfr*, many variable alleles were detected at each microsatellite locus demonstrating high diversity in the sensitive *dhfr* group while in the *dhfr* triple-mutant group, at each locus, a few low-frequency alleles and a single predominant allele were found in most isolates; the 176 bp allele at -4.4 Kb and the 203 bp at the -5.3 Kb demonstrating the amount of lost diversity. This allelic pattern likely reflects recent evolution by mutation (Anderson *et al.*, 2000b; Anderson, 2004) and/or possibly recombination of the predominant allele that has previously been selected.

Three-locus microsatellite haplotypes were constructed from the 446 unmixed isolates, in the sensitive, double mutant (C59R+S108N), double mutant (N51I+S108N) and triple mutant *dhfr* groups (Table 9). A total of 104 different haplotypes were identified, ranked and numbered from H1-H104 according to size of allele at -0.3 Kb then at -4.4 Kb and finally at -5.3 Kb locus. Of the 104 different microsatellite haplotypes, 56 were identified among the 78 sensitive isolates, 19 among the 87 C59R+S108N double mutant isolates, 26 among the 97 N51I+S108N double mutant isolates and 21 were identified among the 184 triple mutant *dhfr* isolates. Nine (H16, H22, H32, H35, H36, H39, H47, H51 and H53) of the 56-sensitive haplotypes were shared among double mutant and triple mutant *dhfr* isolates. Two others (H45 and H52) found among 19 C59R+S108N double mutant isolates were shared with the N51I+S108N double mutant

dhfr. Haplotype H90 was in the majority of the triple mutant *dhfr* isolates (135 of 184) but was also found in 2 of 106 among N51I+S108N double mutant *dhfr* isolates. Haplotype H89 was shared (2 of 106 against 2 of 184) among the N51I+S108N double mutant and the triple mutant *dhfr* isolates, respectively. This large extent of haplotype sharing is probably a reflection of high degree of recombination in this area of stable high malaria transmission.

There was a clear difference in the haplotypic diversity between the sensitive and all resistant forms of the *dhfr* chromosomes. While the sensitive forms revealed high diversity with all the 56 haplotypes among 78 isolates contributing on average equally to the gene pool, the resistant forms had just a few predominant haplotypes, with the number of dominant haplotypes decreasing with increasing level of resistance (Figure 17). The triple mutant *dhfr* chromosome had a single dominant haplotype (H90) which was identical to the Asian origin type described in Roper *et al.* (2004), and later reported to be wide-spread in Africa; Tanzania, Mozambique and South Africa (Pearce *et al.*, 2005), Senegal (Ndiaye *et al.*, 2006), Kenya (McCollum *et al.*, 2005), Benin, Cameroon, Comoros, Congo-Brazaville, Ivory coast, Gabon, Ghana, Guinea, Mali, Senegal and Uganda (Maiga *et al.*, 2007; Lynch *et al.*, 2008). Seventy three percent (135) of the triple mutant *dhfr* isolates analysed here were found to carry this haplotype.

Table 8. Number of alleles (A) and allele length range at the three microsatellite loci in Kilombero/Ulanga and Rufiji populations

Locus	Kilombero/Ulanga		Rufiji		Overall	
	A	Size range (bp)	A	Size range (bp)	A	Size range (bp)
-0.3	10	85-115	9	87-115	11	85-115
-4.4	17	158-193	17	156-199	19	156-199
-5.3	15	190-219	13	190-221	18	190-221

Beside the predominant haplotype H90, the triple mutant *dhfr* had 21 other microsatellite haplotypes, and these differed at one or two of the three flanking microsatellite loci. These alternative haplotypes were generally of low frequency of <2% (except haplotype 21 which occurred at 11% frequency) and most likely to be relatives of the dominant haplotype with the different microsatellite allele(s) possibly introduced by recombination with the double *dhfr* mutant types. An exception however, are the four haplotypes (H32, H35, H18 and H38) which had the 87 bp allele at the closest *dhfr* microsatellite loci (-0.3 Kb). The 87 bp allele at -0.3 Kb loci is a common allele for the sensitives and double *dhfr* mutants, while 108 bp is for the triple *dhfr* mutants, thus there is a possibility that these haplotypes represent cases where double mutants have acquired an extra mutation transforming to triple *dhfr* mutant types. Two novel haplotypes (H29 and H58) associated with the triple mutant type were identified and differed by allele length with the dominant (Asian type) haplotype at all three microsatellite loci. They have not been described before and in this study each was present in one isolate only.

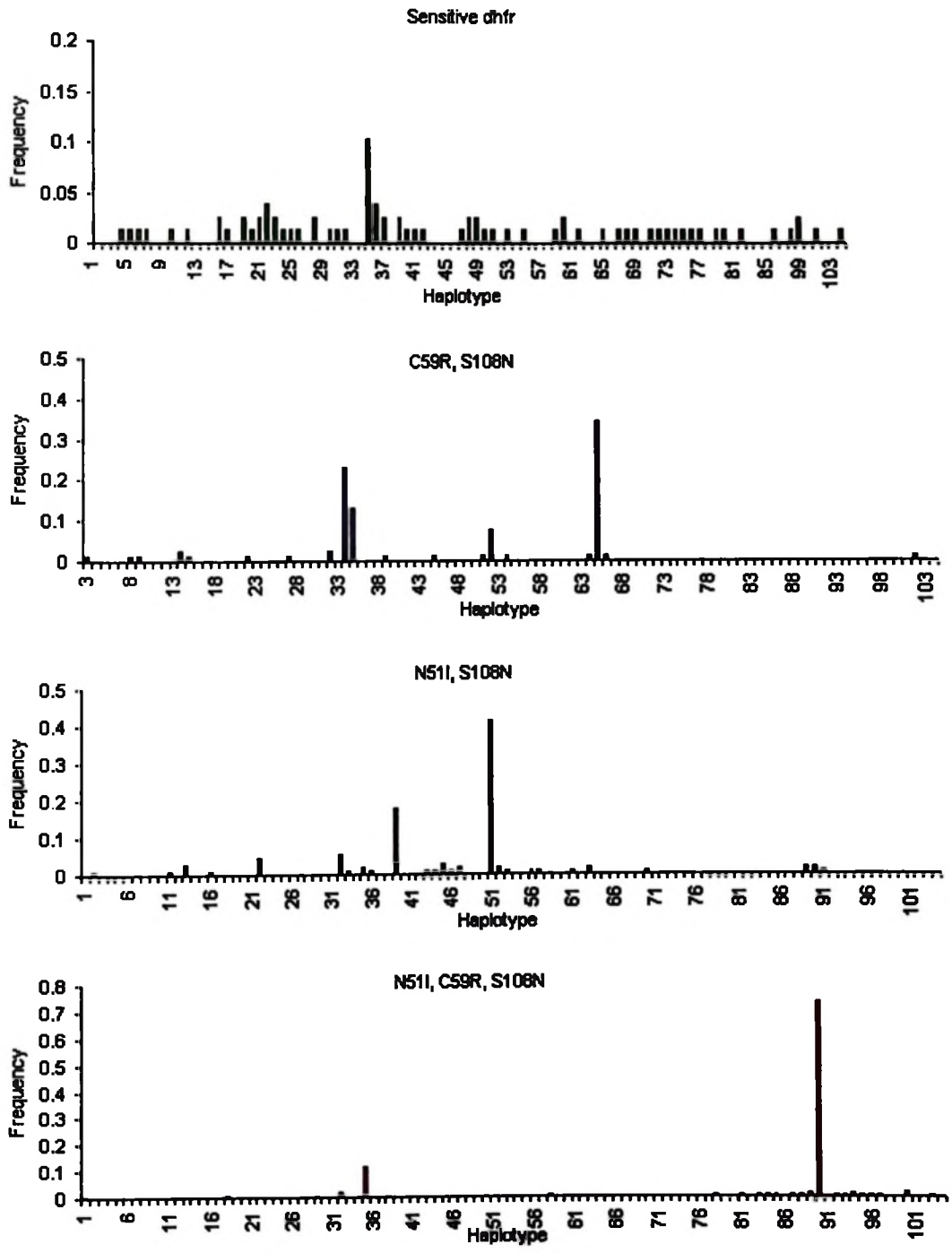

Figure 17. Allele associated microsatellite polymorphisms in the flanking region upstream of *dhfr*.

The haplotypic diversity displayed by the double mutant *dhfr* isolates was intermediate between that found in the sensitive and triple mutant *dhfr* types. Both double mutant *dhfr* alleles were associated with four common haplotypes, the C59R+S108N with the H35, H51, H34 and H64 and N51I+S108N with H22, H32, H39 and H51. Haplotype H64 was the most common for the C59R+S108N double mutant, while H51 was the most common for the N51I+S108N double mutant *dhfr*. Both haplotypes were reported in Tanzania and South Africa (Roper *et al.*, 2003) and later found in other African *P. falciparum* populations (McCollum *et al.*, 2005; Pearce *et al.*, 2005; Ndiaye *et al.*, 2006; Maiga *et al.*, 2007; Lynch *et al.*, 2008). To examine if there were any temporal or geographical trends in the extent of diversity flanking the major resistance alleles, isolates from two populations of Kilombero/Ulanga and Rufiji at two time points, 2001/2002 and 2006 were compared (Appendix II).

Generally, it was observed that the common microsatellite haplotypes associated with resistant *dhfr* chromosomes were broadly consistent at both surveys (2001/2002 and 2006) (Appendix II). By contrast, the rare haplotypes were very elusive; with good proportion present in one of the two sampling time point but not the other. For instance, the rare microsatellite haplotypes associated with triple mutant *dhfr* chromosomes constituted 13% of the triple mutant haplotype pool. One third of these rare haplotypes were only observed in the 2001/2002 while the remaining two third were only observed in 2006 (see Appendix II).

Table 9. Flanking *dhfr* microsatellite polymorphisms detected in two cross sectional srveys of 2001/2002 and 2006 in Rufiji and Kilombero/Ulanga A) Sensitive allele (N51, C59, S108) B), Double mutant (C59R, S108N) allele, C) Double mutant (N51I, S108N) allele and D) Triple mutant (N51I, C59R, S108N) allele.

A) Sensitive allele (N51, C59, S108)

Haplotype	n	-0.3	-4.4	-5.3
H4	1	87	158	205
H5	1	87	158	207
H6	1	87	158	215
H7	1	87	160	199
H10	1	87	164	211
H12	1	87	166	209
H16	2	87	170	193
H17	1	87	170	197
H19	2	87	170	207
H20	1	87	170	211
H21	2	87	170	213
H22	3	87	172	205
H23	2	87	172	207
H24	1	87	172	219
H25	1	87	174	190
H26	1	87	174	195
H28	2	87	174	201
H30	1	87	174	211
H31	1	87	176	191
H32	1	87	176	193
H35	8	87	176	203
H36	3	87	176	205
H37	2	87	176	207
H39	2	87	178	193
H40	1	87	178	197
H41	1	87	178	199
H42	1	87	178	207
H47	1	87	180	203
H48	2	87	180	205
H49	2	87	180	207
H50	1	87	180	213
H51	1	87	182	193
H53	1	87	182	205
H55	1	87	182	209
H59	1	87	184	201
H60	2	87	184	207
H62	1	87	184	215

Sensitive allele (N51, C59, S108) continued

Haplotype	n	-0.3	-4.4	-5.3
H65	1	87	186	205
H67	1	87	188	209
H68	1	87	193	193
H69	1	87	193	195
H71	1	92	166	199
H72	1	96	178	205
H73	1	96	180	207
H74	1	96	180	211
H75	1	96	182	199
H76	1	98	178	215
H77	1	100	174	213
H79	1	102	172	217
H80	1	102	176	201
H82	1	104	168	203
H86	1	108	172	195
H98	1	110	158	191
H99	2	110	158	205
H101	1	110	184	197
H104	1	115	184	199

B) Double mutant *dhfr* (C59R, S108N) allele

Haplotype	n	-0.3	-4.4	-5.3
H3	1	87	156	203
H8	1	87	162	199
H9	1	87	164	199
H14	2	87	168	199
H15	1	87	168	205
H22	1	87	172	205
H27	1	87	174	199
H32	2	87	176	193
H34	18	87	176	199
H35	10	87	176	203
H39	1	87	178	193
H45	1	87	180	193
H51[a]	6	87	182	193
H52	1	87	182	199
H54	1	87	182	207
H64[a]	27	87	186	199
H65	1	87	186	205
H66	1	87	188	199
H102	1	110	186	199

[a]Reported previously Roper *et al.* (2003)

C) Double mutant *dhfr* (N51I, S108N) allele

Haplotype	n	-0.3	-4.4	-5.3
H2	1	85	184	190
H11	1	87	166	193
H13	3	87	168	193
H16	1	87	170	193
H22	5	87	172	205
H32	6	87	176	193
H33	1	87	176	198
H35	2	87	176	203
H36	1	87	176	205
H39[b]	19	87	178	193
H43	1	87	178	209
H44	1	87	178	211
H45	3	87	180	193
H46	1	87	180	201
H47	2	87	180	203
H51[a]	44	87	182	193
H52	2	87	182	199
H53	1	87	182	205
H56	1	87	184	193
H57	1	87	184	199
H61	1	87	184	209
H63	2	87	186	193
H70	1	87	199	207
H89	2	108	176	193
H90	2	108	176	203
H91	1	108	176	205

Reported previously [a]Roper *et al.* (2003), [b]Lynch *et al.* (2008)

D) Triple mutant *dhfr* (N51I, C59R, and S108N) allele

Haplotype	n	-0.3	-4.4	-5.3
H1	1	85	176	203
H18	1	87	170	203
H29	1	87	174	207
H32	3	87	176	193
H35	21	87	176	203
H38	1	87	176	211
H58	1	87	184	199
H78	1	102	162	203
H81	1	102	176	203
H83	1	104	176	203
H84	1	108	166	203
H85	1	108	170	205
H87	1	108	172	199
H88	1	108	172	203
H89	2	108	176	193
H90[a]	135	108	176	203
H92	1	108	176	207
H93	1	108	178	201
H94	2	108	178	203
H95	1	108	178	207
H96	1	108	182	221
H97	1	108	186	203
H100	3	110	176	203
H103	1	115	176	203

[a]Roper *et al.* (2003)

	Common allele for C59R+S108N *dhfr* double mutant parasite
	Common allele for N51I+S108N *dhfr* double mutant parasite
	Common allele for N51I+ C59R+S108N *dhfr* triple mutant parasite

A similar trend was also seen for the double mutant *dhfr* chromosomes, although the proportions of the rare haplotypes were higher (23% and 27% for the C59R+S108N and N51I+S108N double mutant *dhfr* chromosomes, respectively). Frequency and distribution of allele length polymorphisms at the three *dhfr*-linked microsatellite loci, among two populations of Rufiji and Kilombero/Ulanga at two time points is presented in Appendix II while microsatellite haplotype heterozygosity comparing the two

populations over time for each *dhfr* allele are given in Appendix III. Distribution of both common haplotypes and heterozygisity values at two survey points and between the two populations indicated that the two populations were very similar.

To further examine levels of gene diversity, haplotype-based heterozygosity values were computed for each *dhfr* allele (Figure 18). The sensitive *dhfr* allele had highest He of 0.99 and 0.97 in Kilombero/Ulanga and Rufiji), respectively, reflecting its ancestral state (Roper *et al.,* 2003). By contrast, the resistant *dhfr* alleles showed a progressive decrease of diversity with increasing number of mutation; the *dhfr* double mutant C59R+S108N, He = 0.863, 0.8309 and N51I+S108N, He = 0.8262, 0.7978 in Kilombero/Ulanga and Rufiji, respectively. Most striking was the amount of reduced diversity detected in the triple mutant *dhfr* indicating more than a half of heterozygosity has disappeared, He = 0.4411 and 0.4828, in Kilombero/Ulanga and Rufiji, respectively, when compared with sensitive *dhfr* alleles. The heterozygosity values for the resistant *dhfr* alleles were compared between the 2001/2002 and 2006 at the two populations of Rufiji and Kilombero/Ulanga (Figure 19). Both *dhfr* double mutant alleles heterozygosity values were almost similar (C59R+S108N; He = 0.800 in 2001/2002 and 0.86 in 2006 in Rufiji and 0.85 in 2001/2002 and 0.87 in 2006 in Kilombero/Ulanga, and N51I+S108N; He = 0.81 in 2001/2002 and 0.78 in 2006 in Rufiji and 0.85 in 2001/2002 and 0.79 in 2006 in Kilombero/Ulanga) in both populations indicating little change in diversity over time.

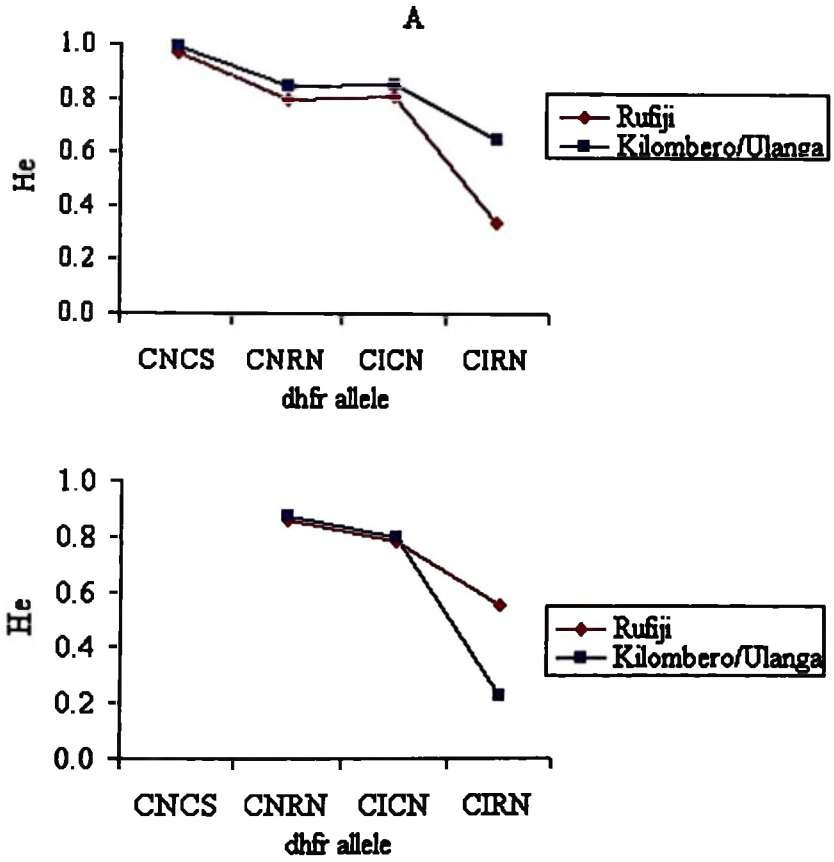

Figure 18. *Dhfr* flanking microsatellite heterozygosity. A) 2001 and B) 2006. Note: in B the CNCS was already too rare to get sufficient samples.

By contrast, the triple mutant *dhfr* allele heterozygosity values revealed a contrasting pattern between Kilombero/Ulanga and Rufiji populations. In Rufiji, He was 0.41 in 2001/2002 but increased slightly to 0.55 in the 2006 while in Kilombero/Ulanga, He was 0.65 in 2001/2002 but decreased dramatically to 0.23 in 2006.

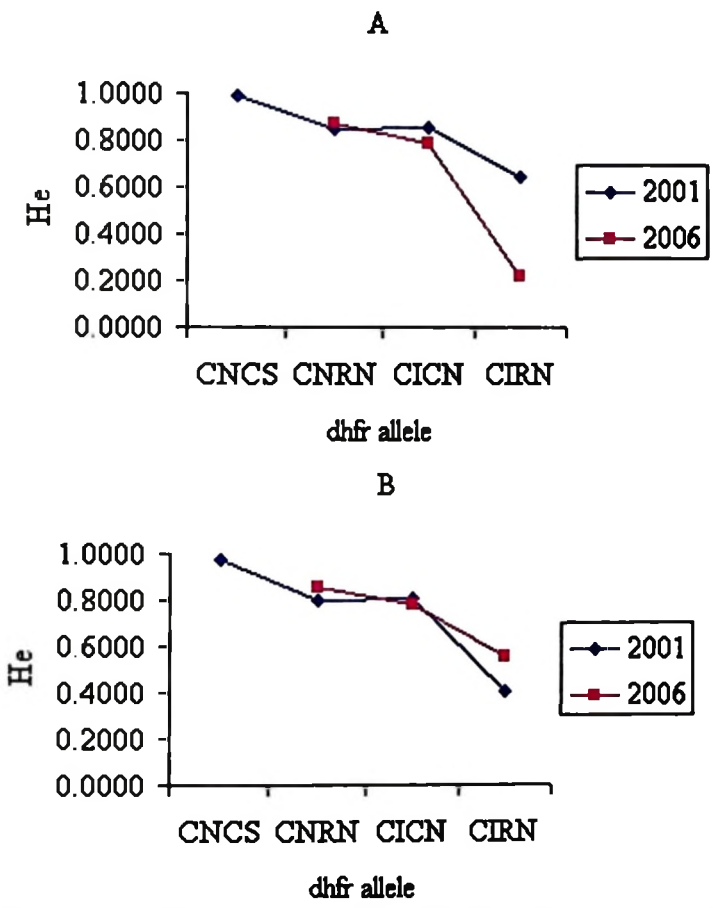

Figure 19. ***Dhfr*** **flanking microsatellite heterozygosity. A) Kilombero/Ulanga B) Rufiji. Note: in 2006 the CNCS was already too rare to get sufficient samples.**

4.5 Selectively neutral microsatellite loci

To assess population differentiation at neutral polymorphic markers, a subset of samples from 2002 survey (n=182 from each of Rufiji and Kilombero/Ulanga) was selected to genotype the three microsatellite loci, Poly A, PfPk2 and TA109 (Anderson *et al.* 1999). To avoid sampling bias that might result from changes in population structure associated with drug selection, the samples were selected irrespective of the *dhfr* and *dhps* allele

present or the number of parasite detected per sample. As *P. falciparum* is haploid in nature, appearance of multiple bands indicates presence of more than one genetically distinct coinfecting parasites and the number of bands points to the number of genotypes per sample. The three neutral microsatellite loci used in this study differ in their degree of polymorphism. Here the number of alleles in each particular isolate was counted and hence the number of sample is different for each neutral microsatellite loci reflecting differences in sensitivity for detecting policlonality.

The three microsatellite loci used are distributed throughout the genome and therefore unlikely to be biased by a loss of diversity. Table 10 shows the chromosomal location and expected heterozygosity of the three neutral loci used in the current study. The TA109 and PfPk2 are found on chromosomes 6 and 12, respectively, a very far distance from both *dhfr* and *dhps* genes, which are located at chromosomes 4 and 8, respectively. Poly A microsatellite however, is located on the same chromosome 4 as *dhfr* gene, but is sufficiently distant from the *dhfr* (>215Kb), making reduction in gene diversity at the locus as a result of drug selection on *dhfr* unlikely.

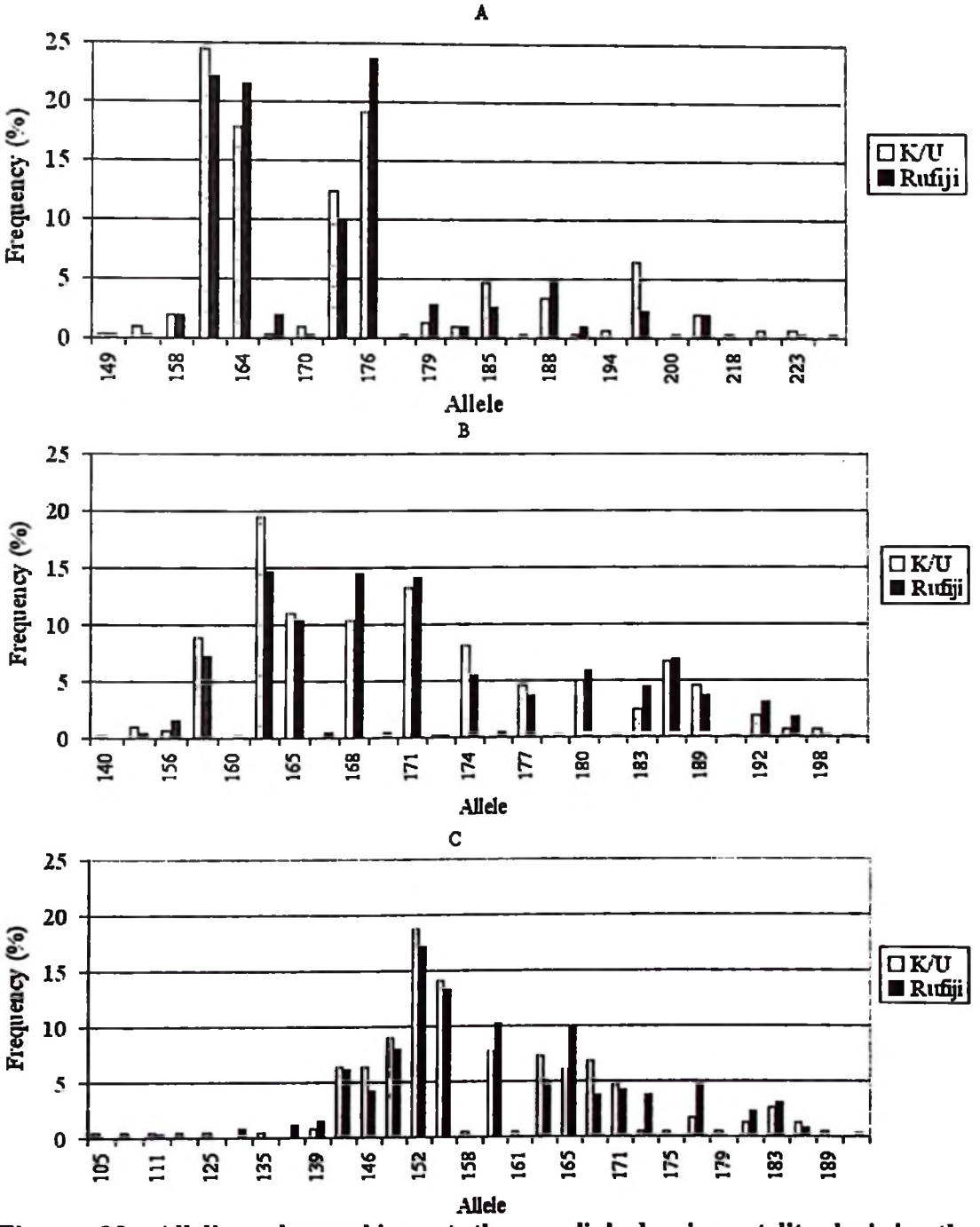

Figure 20. Allelic polymorphism at three unlinked microsatelite loci in the populations of Kilombero/Ulanga and Rufiji. A) TA109 B) PfPk2 C) Poly A. K/U = Kilombero/Ulanga.

Table 10. Number of alleles (A) and and allele size range at neutral microsatellite loci detected in Kilombero/Ulanga and Rufiji populations.

Marker	Cromosome	Kilombero/Ulanga			Rufiji		
		A	Size range	He	A	Size range	He
Poly A	4	27	105-189	0.9091	20	111-195	0.9136
TA109	6	20	149-223	0.8503	21	149-245	0.8358
PfPk2	12	19	140-200	0.8973	24	144-198	0.9062

Polymorphisms at the three neutral microsatellite markers were determined by recording allelelic variations at each microsatellite loci which is due to differences in the number of sequence repeats (Table 10). All three microsatellite loci were highly polymorphic in all isolates and this was consistent in the two populations. The poly A locus had 27 difeferent alleles of 105-189 bp in Kilombero/Ulanga and 20 alleles of 111-195 bp in Rufiji, the TA109 had 20 alleles of 149-223 bp in Kilombero/Ulanga and 21 alleles of 149-245 bp in Rufiji and the PfPk2 had 19 allele of 140-200 bp in Kilombero/Ulanga and 24 alleles of 144-198 bp in Rufiji.

The diversity of the two populations was also compared based on the distribution of alleles at the three neutral microsatellite loci. The allele distribution at all three microsatellite loci were broadly similar in both populations with the major alleles at each locus showing even distribution in these populations (Figure 20). This indicates the two populations are equally diverse and could not be differentiated at the neutral microsatellite loci. The diversity of the two populations was also assessed using expected heterozygosity values at each of the three nutral microsatellite markers. The

poly A locus was the most polymorphic with a common He of 0.91for both populations, PfPk2 had itermediate polymorphism with He of 0.90 in both populations and TA109 was the least polymorphic with He of 0.85 in Kilombero/Ulanga and 0.84 in Rufiji. Consistent with allele distribution, the He values indicate the two populations were indifferent.

Multiplicity of infection (MOI)

The number of distinct *P. falciparum* genotypes detectable per sample is defined as the 'multiplicity of infection' (MOI). To test the assumption that ACT reduce gametocytaemia thus limiting malaria transmission, the unlinked microsatellite markers (PolyA, PfPk2 and TA109) were also analysed in a sub set of 2006 isolates (n = 182) from each of Kilombero/Ulanga and Rufiji, to compare the underlying rate of mixed infections between the two populations at the two time points (2002 and 2006). This was done by counting the number of alleles observed in each isolate and taking the highest value.

The polymorphic microsatellite markers are very sensitive for detecting coinfections and in the current study a maximum of 9 coinfections per patient was detected in each of Kilombero/Ulanga and Rufiji populations (Figure 21). In the year 2002, isolates from both Rufiji and Kilombero/Ulanga districts had remarkably similar distribution of MOI (Figure 21). The most common MOI was 3 in both districts, and an estimated 280 of 875

(32%) of patients from Kilombero/Ulanga and 281 of 854 (33%) patients from Rufiji were found to be coinfected with 3 genetically distinct *P. falciparum* parasites.

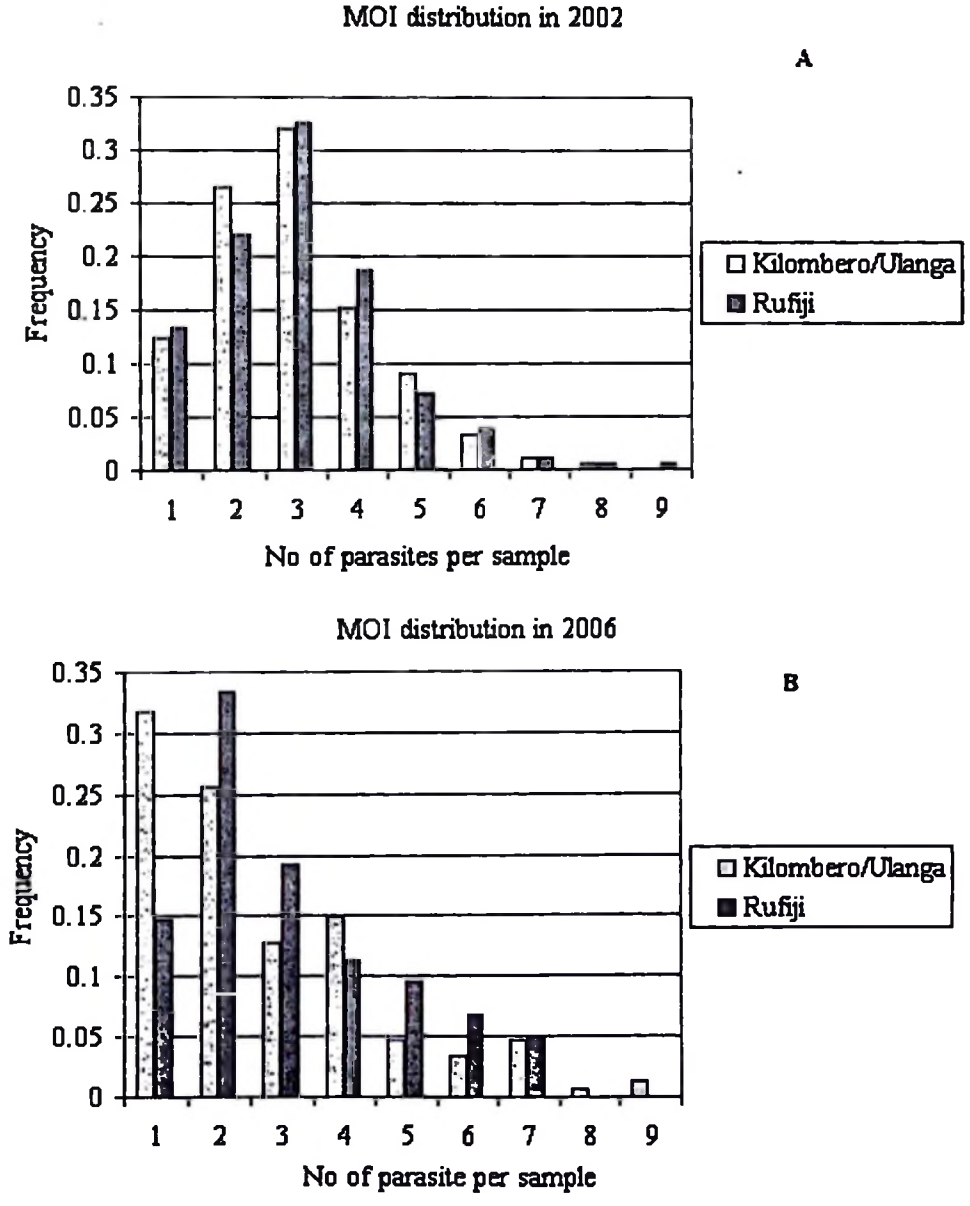

Figure 21. Multiplicity of infection in the 2002 and 2006 isolates from Rufiji and Kilombero/Ulanga populations.

By contrast, a decrease of most common MOI from 3 to 1 in Kilombero/Ulanga and from 3 to 2 in Rufiji was observed in 2006 (Figure 21), indicating there was a trend towards decrease in malaria transmission over time in both populations which was more pronounced in Kilombero/Ulanga than Rufiji. This observation is consistent with prevalence of malaria infections detected in the present study, indicating reduction of malaria prevalence from 27.4% in 2000 to 21.3% in 2006 in Rufiji compared to 29.0% in 2000 to 14.3% in 2006 in Kilombero/Ulanga (Table 5 and 6).

CHAPTER FIVE

5.0 DISCUSSION

This study has generated results of six years follow up of *dhfr* and *dhps* genetic changes in the *P. falciparum* in three rural districts of south eastern Tanzania. A high throughput sequence specific olignucleotide method was used to genotype point mutations present at the SP resistance genes, the *dhfr* and *dhps* in population samples of *P. falciparum* infections collected annually from year 2000 to 2006 in Rufiji, and the combined districts of Kilombero and Ulanga. Significant increase of the frequencies of resistant *dhfr* and *dhps* haplotypes was observed in both the study districts after the change of the National antimalarial guidelines from first line CQ to first line SP. Although the introduction of SP + ART combination therapy was delayed until the genetic resistance to SP was already high, its use in Rufiji successfully disrupted a significant association between the double *dhps* and triple *dhfr* (A437G + K540E – N51I +C59R + S108N), leading to absence of linkage disequilibrium. Clearly, changes of drug use policies implemented during the conduct of this study had impact on the genetic determinants of SP resistance.

The first section of this chapter (5.1) discusses *dhfr* and *dhps* frequency changes occurring in Kilombero/Ulanga and Rufiji populations one year before and after policy change from first line CQ to first line SP. The second section (5.2) compares *dhfr* and *dhps* frequency changes occurring in Kilombero/Ulanga where SP monotherapy was a recommended first line antimalarial therapy with those occurring in Rufiji populations

during the time of SP + ART combination therapy implementation in Rufiji population between 2002-2006. Section 5.3 discusses important findings from analysis of spatial distribution of the *dhfr* and *dhps* alleles in sub populations of Kilombero/Ulanga and Rufiji. The next two sections (5.4 and 5.5) discusses results of the microsatellite analysis, section 5.4 the *dhfr* flanking microsatellite loci and 5.5 the selectively neutral microsatellite loci.

5.1 Effects of national policy change on *dhfr* and *dhps* alleles

5.1.1 Policy and genetic change

The study has demonstrated that the influence of the national antimalarial treatment policy change on the genetic composition of the malaria parasite population was profound. *dhfr* and *dhps* allele frequency did not change significantly during 12 months (year 2000 to 2001) of first line CQ with second line SP, but after the switch to first line SP treatment (year 2001 to 2002), the frequency of the triple mutant *dhfr* allele increased by 37% - 63% and the frequency of the double mutant *dhps* allele increased 2-3 fold. A combination of these alleles is predictive of *in vivo* failure of SP treatment (Omar *et al.*, 2001, Kublin *et al.*, 2002, Staedke *et al.*, 2004), and the rapid increase of this genotype from 5% - 25% between 2001 - 2002 suggests that the outlook for SP efficacy in this region is poor. In fact this observation is bourne out by the results of SP treatment efficacy monitoring in Southeastern Tanzania during 2003 which found that 49% of SP treatments failed by day 28 (Mugittu *et al.*, 2005). Due to this high rate of SP resistance,

national policy decision was made to switch the recommended first line treatment to Artemether + Lumefantrine in 2006.

5.1.2 Correlation between the trend of SP usage and the levels of *dhfr* and *dhps* resistance alleles

Prior to the introduction of SP as first line therapy (between 2000 and 2001), SP was widely available for self treatment via purchase from shops (Goodman *et al.*, 2004) as well as through formal health facilities as the second line treatment. The SP drug pressure exerted during this period was only sufficient to maintain constant *dhfr* and *dhps* resistance alleles. By contrast, following the introduction of SP as firstline in 2001 there was dramatic increase in SP use (Goodman *et al.*, 2004) leading to intense SP pressure and escalation of *dhfr* and *dhps* resistance alleles. Although SP is currently being replaced as a first line drug for treatment of malaria in Tanzania and much of the rest of Africa, it will continue to be used in intermittent preventive treatment of infants and pregnant women (National Antimalarial guidelines). The drug pressure applied when SP is used solely in intermittent programmes of treatment in infants and pregnant women will probably more closely resemble the situation observed between 2000 - 2001 when SP was secondline treatment than during the period it was firstline. The consequences of more restricted use for future efficacy of SP are therefore two fold. Firstly rates of resistance allele frequency change are likely to stabilise as a result of reduction of SP selection pressure. Second the reduction of selection is predicted to disassociate the triple mutant *dhfr* allele and double mutant *dhps* allele which will reduce

the frequency of highly pyrimethamine and sulphadoxine resistant parasites in the *P. falciparum* population at large.

5.1.3 The spread of resistance

For the successful spread of a resistance, the mutants must be transmitted at a faster rate than the sensitive forms. This occurs in the presence of drug because of the differential survival and reproduction rates conferred by these alleles (Anderson *et al.*, 1989). The population–wide rates of change of resistant *dhfr* and *dhps* alleles observed are attributable to changes in SP coverage during 2001 - 2002 period. In order to quantify drug pressure under a specific policy intervention its effect upon the relative abundance, or frequency, of every haplotype needs to be measured because each allele is subject to a differing selection pressure by virtue of its differing resistance properties. The frequencies of each allelic haplotype were measured through time and this can be used to estimate the selection coefficient (s). The selection coefficient describes the relative survival of resistant and non-resistant parasites in each generation. It was estimated that during first line SP use in Kilombero/Ulanga and Rufiji populations a selection coefficient of 0.16 operated on the *dhps* double mutant and a selection coefficient of 0.07 operated on the *dhfr* triple mutant. When s=0.16 the relative fitness (w) of resistant parasites is s+1 (ie 1.16) and they survive on average 16% better than sensitives.

As observed in this study, the finding that first line use of SP brought about the same genetic changes independently in Kilombero/Ulanga and in Rufiji indicates they are

likely to have broad applicability elsewhere. However, direct transposition to predict rates of genetic changes in other settings should take account of differences in access to drugs and in the infrastructure for provision of healthcare services which differs significantly from country to country and sometimes even between regions within the same country. Importantly, from a molecular genetic perspective, although in this study the frequency of the *dhps* A437G + K540E double mutant allele was as high as 25% on average in Kilombero/Ulanga and Rufiji, recent studies in west and central African countries still show this allele to be absent or very rare (Marks *et al.*, 2005; Menard *et al.*, 2006). The rates of genetic change observed in the current study will only be predictive for such populations once these alleles are established.

5.1.4 Evolutionary history of resistance alleles

By following haplotype frequencies over time, this study has described the contrasting behaviours of mutant alleles which confer intermediate resistance and those which are highly resistant. The frequency of weakly resistant alleles such as the *dhfr* double mutant alleles and the *dhps* single mutant did not increase in response to the increase in SP use in both the Kilombero/Ulanga and Rufiji populations. During the course of the three annual surveys each of the two *dhfr* double mutant alleles (N51I + S108N and C59R + S108N) maintained a steady frequency of around 10%, while the *dhps* single mutant maintained a frequency of around 15% (see Figure 7 and 8) . They were neither displaced by the growth of the highly resistant alleles, as the sensitive alleles were, nor did they increase in frequency in response to elevated drug pressure.

Double mutant *dhfr* alleles are often regarded as precursors of the triple mutant since they can potentially convert to the highly resistant triple mutant allele by the simple acquisition of a single point mutation. Somewhat surprisingly flanking microsatellite analysis in both Kilombero/Ulanga and Rufiji populations (Results Chapter section 4.4) has confirmed previous reports showing that the triple mutants in Southeast Africa (Roper *et al.*, 2003), and in the Kilombero/Ulanga region specifically (Pearce *et al.*, 2005), belong to a single lineage which originated in Asia (Roper *et al.*, 2004). The double mutant *dhfr* alleles found in this region belong to a restricted number of independently derived lineages (Roper *et al.*, 2003) which are in all probability of African origin. So while the growth of triple mutant allele in Kilombero/Ulanga and Rufiji can be explained by the expansion of the Asian derived lineage under selection by SP treatment, the steady persistence of the mildly resistant double mutant *dhfr* lineages at around 10% requires some further explanation. The existence of conserved flanking region around them is evidence of selection (Roper *et al.*, 2003) and there is further evidence that double mutant *dhfr* alleles can gain fitness advantage under specific circumstances. They have been associated with treatment failure in individuals who have not had sufficient exposure to malaria to have acquired immunity (Staedke *et al.*, 2004; Khalil *et al.*, 2002). In Colombia the N51I+S108N double mutant, while not affecting clinical efficacy, was associated with prolonged parasite clearance times and with gametocytes 14 and 28 days after treatment, indicating they confer survival and reproductive advantages (Mendez *et al.*, 2002). It has also been proposed that exposure to sub-therapeutic drug levels might apply selection for weakly resistant parasites which

are newly innoculated into people who recently received treatment (Hastings, 2002). Jawetz *et al.* (1991) proposed that sub-optimal treatments tends to give intermediate phenotypes a selective advantage rather than killing them. An alternative explanation for the continuing existence of *dhfr* double mutant alleles is that they pre-date the use of SP. Pyrimethamine was used as monotherapy during the 1950s and 60s and numerous studies showed that resistance quickly arose locally in response to drug pressure (Avery-Jones, 1958; Clyde, 1967; Peters, 1970). Double mutant *dhfr* alleles could have been the basis of these early reports of pyrimethamine resistant malaria and may well have preceded the arrival of the *dhfr* triple mutant. Supporting evidence comes from studies in Kenya where double mutant alleles were in the majority in a panel of isolates collected as early as 1981, N51I+S108N; 21% and C59R+S108N; 47% (Khan *et al.*, 1997). Reports of double mutant *dhfr* are concurrent with reports of intermediate resistance around 20% in Kilifi between 1984 and 1989. This rose to 92% between 1993 and 1995 (Watkins *et al.*, 1997), by which time the triple mutant in Kilifi was more prevalent (Wang *et al.*, 1997a).

5.1.5 Evolution of multi-drug resistance

By combining *dhfr* and *dhps* data the frequency of the highly resistant combined *dhfr-dhps* genotype was estimated in each of three timepoints (2000, 2001 and 2002). The frequency of the mutant triple *dhfr*-double *dhps* genotype more than quadrupled between 2001 and 2002 in Kilombero/Ulanga and Rufiji, and it also was found greatly in excess of the numbers expected based on random association. Such population-wide

associations between unlinked resistance genes are expected to occur when two drugs with independent resistance mechanisms are combined, or where resistance to a single drug is controlled by more than one gene (Anderson *et al.*, 1989). This association occurs because of the combined effects of two selection processes which occur simultaneously. The double mutant resistant parasites survive treatment while genotypes with only one resistance gene do not. In addition to that the drug treatment itself, by purging the co-infecting sensitive genotypes, promotes assortative mating among resistant survivors of treatment.

Recombination which occurs between gametocytes taken up in the same blood meal, is a force which can disrupt these associations and the high rates of recombination in the high transmission setting of Ifakara was once proposed to be responsible for the relatively slow spread of CQ resistance in that area (Paul *et al.*, 1995). Models have shown that recombination is insufficiently strong to counteract the effect of drug pressure unless the two resistance alleles are rare, or drug pressure is low (Dye and Williams, 1997; Hastings, 1997; Hastings and Mackinnon, 1998; Mackinnon and Hastings, 1998). The genetic evidence obtained from this study supports this conclusion since the population-wide association of *dhfr* triple and *dhps* double mutant alleles was not apparent when drug pressure was relatively low but rapidly became apparent once drug pressure increased. This is a significant consideration for all combination treatments for malaria and most particularly for those combinations for which resistance to one or both of the component drugs is already established. It suggests that in high

transmission settings there may be value in using a suite of antimalarial drugs rather than blanket use of the same drug for all programmes of treatment (Intemittent Preventive Treatment of Infants-IPTI, Intermittent Preventive Treatment of Pregnant women-IPTP as well as direct therapy for malaria). It is widely agreed that malaria treatments should be used in a manner which will minimise drug pressure and limit the growth of resistance, the difficulty lies in gathering empirical support for specific policy alternatives in order to achieve this. The current study was designed and applied a methodology which successfully compared drug pressure applied through use of SP for first and second line treatment of malaria in the field. This study provides empirical evidence from the field by measuring the relationship between treatment policy and drug pressure. The demonstration of a strong link between treatment policy and drug pressure will undoubtedly assist regional evaluations of treatment interventions and supports the view that change in treatment policy and practice have the potential if used properly to prolong the useful life of antimalarial drugs.

5.2 Effects of act on the *dhfr* and *dhps* alleles in Rufiji

The SP+ART combination antimalarial therapy study in Rufiji has demonstrated the ability of the combination to disrupt the association of the triple *dhfr* and the double *dhps* alleles removing the linkage disequlibrium between them. The combination genotype represents the most highly SP resistant form of the African *P. falciparum* malaria parasite and its existence has been shown to predict *in vivo* SP treatment failure (Omar *et al.*, 2001; Kublin *et al.*, 2002; Steadke *et al.*, 2004). Therefore this disruption

brought about by the ACT was an important step of reducing high SP resistance in the population. Furthermore, although the combination therapy trial did not halt completely the growth of SP resistance, the study has shown that its use in Rufiji slowed the rate of increase of triple mutant *dhfr* and double mutant *dhps* alleles. The frequency of the triple mutant *dhfr* was 10% higher in Rufiji than Kilombeo-Ulanga ($P \leq 0.004$) at the start of ACT in Rufiji (in late 2002) but during the course of the intervention the difference narrowed, and by 2006 the frequency was 1% higher in Kilombero/Ulanga than Rufiji ($P \geq 0.6936$), suggesting that ACT in Rufiji slowed down the growth in frequency of the triple *dhfr* allele relative to Kilombero/Ulanga by a factor of 11%. A similar effect was also observed at the *dhps* locus, where frequencies in Rufiji and Kilombero/Ulanga were initially the same but subsequent increases were significantly higher in Kilombero/Ulanga. This suggests ACT in Rufiji retarded growth in frequency of the double *dhps* resistant allele relative to Kilombero/Ulanga by 8%.

Comparison of the frequencies of the highly resistant two locus genotype consisting of the double *dhps* with triple *dhfr* resistant allele in Rufiji and Kilombero/Ulanga revealed ACT in Rufiji slowed its rate of increase by a factor of 4% by 2006. Despite all the achievements attained by the use of ACT in Rufiji, its failure to completely halt the growth and dissemination of resistant *dhfr* and *dhps* alleles in Rufiji requires some explanations. There are several key ways in which the application of drug pressure solely through provision of SP+ART through government health facilities could have been undermined. Firstly, in Tanzania the availability of drugs is unregulated and

varieties of antimalarial drugs were widely available in shops and kiosks for self medication during the time of this study (Goodman *et al.*, 2004). Secondly, the unmatched half-life of 5-10 days for the SP and 45 minutes for the ART leaves little chance for two component drugs to work synergistically as a combination. Consequently, any new infection acquired after ART is cleared in the blood will likely be exposed to SP monotherapy providing opportunity for SP selection.

Thirdly, access to treatment was imperfect, only a proportion of infections were treated as a result of high proportions of untreated asymptomatic infections. In this study, the results suggests on average 20% of healthy looking individuals were infected with *P. falciparum* malaria without expressing clinical symptoms of the disease (Tables 5 and 6). This provides a very significant reservoir of parasite populations that are not exposed to chemotherapy (Smith, 1990; Anderson and May, 1991). The effect of the reservoir is that, it provides refugia for parasites which are untreated and which compete with those exposed to ACT.

Linkage disequilibrium (LD) is an indicator of simultaneous selection of both triple *dhfr* and double *dhps* alleles. The LD between the *dhfr* triple and *dhps* double mutants is an indirect measure of effective selection pressure in a population. The combination of the two alleles confers greater fitness to the parasite than each allele separately, thus enhancing the chance of better survival of the parasite in the face of SP drug pressure. With the exception of the recently observed I164L *dhfr* mutant which is still very rare in

Africa, the combination of the *dhfr* triple mutant and *dhps* double mutant is the highest form of SP resistance present in African *P. falciparum*. In a population where selection is intense these two alleles although physically unlinked (placed far from each other, *dhfr* at chromosome 4 and *dhps* at chromosome 8 in the genome) will become associated through co-selection and assortative mating. Consequently, the presence of LD between these two alleles in a population indicates that an association is being maintained through intense selection.

In Rufiji, there was a dramatic shift in LD from highly significant ($P \leq 0.0001$) at both Rufiji and Kilombero/Ulanga populations in 2002 the time when at both populations SP was the first line, to a non significant LD in 2004 and 2005, the time when ACT took force in Rufiji. This suggests that, either selection was not occurring in Rufiji at this time or at least selection was not sufficiently intense in the population to maintain an association between the two loci. By contrast the highly significant LD ($P \leq 0.0001$) observed in Kilombero/Ulanga in 2002 was maintained throughout 2004 and 2005 suggesting intense SP selection continued to operate in Kilombero/Ulanga population while selection was reduced in Rufiji. One big question remaining though is if selection was not occurring in Rufiji during ACT implementation what caused the observed high frequencies of *dhfr* and *dhps* resistance alleles in the population during that time? One possible explanation is that geneflow from the surrounding areas was a contributory factor. The role of gene flow may not be underestimated, as the population of Rufiji is not isolated from the rest of neighbouring populations where SP firt line remained the

National antimalarial treatment guideline. This argument is strongly supported by the findings of the present study on the analysis of the microsatellite flanking the *dhfr* gene which indicated that there was extensive genetic exchange among populations of Rufiji and Kilombero/Ulanga. It is therefore most likely that, while National treatment policy change was applied everywhere, the switch to ACT was applied in one district only, and genetic exchange with surrounding areas which were still subject to selection by SP monotherapy may have diluted the effects of the intervention.

5.2.1 The geography of antifolate selection in Tanzania

Although Tanzania only started using SP nation-wide as first line policy for the treatment of uncomplicated form of malaria in late 2001, SP had been the second line drug for the past 18 years (since 1983) for the treatment of cases where CQ failed and as an alternative treatment for those patients whose CQ reactions were sufficiently intense to prompt contraindication. The selection history is even longer in the Northern part of Tanzania. Use of pyrimethamine for the treatment of malaria goes back to 1950s, where according to Clyde, (1967), semi immune individuals in Muheza district in Tanga region were treated with monthly doses of pyrimethamine. This provided a nucleus for the rapid development and expansion of resistance out of the area to the sites where selection (use of pyrimethamine) was absent. In another study using *dhfr* and *dhps* linked microsatellite markers (Roper *et al.*, 2003; Roper *et al.*, 2004), a widespread migration of *dhfr* resistance alleles was observed at long distances (from Southeast Asia to East and Southern Africa) by means of geneflow. Consequently, by the time ACT was

implemented in Rufiji in late 2002, the *dhfr* triple mutant allele frequency was already 52% and 62% in Kilombero/Ulanga and Rufiji, respectively, compromising the possibility of mutual protection of the component drugs in the SP and ART combination. Direct observation such as plasma drug levels in the patient would give the best direct measure of drug selection in the population and it would be interesting to examine this in comparison to the LD measures.

If LD gives a good in-direct indication of SP selection pressure, the lack of significant LD in Rufiji in 2004 and 2005 clearly indicates weak SP selection pressure, yet the re-appearance of significant LD in Rufiji in the 2006 when ACT was still in force and its disappearance in Kilombero/Ulanga population where SP monotherapy was still in use is curious. Clearly, the re-surfacing of significant LD in Rufiji at the time when ACT was still in use in the population suggests SP selection pressure re-emerged in the population maintaining the association between triple *dhfr* and double *dhps* mutant alleles. However, what might have caused this re-surgence of SP selection pressure in the population remains purely a matter of speculation the most likely factors being firstly, that access to ACT was imperfect and due to high proportion of untreated asymptomatic infections in the population only a subset of infections were treated. Secondly, a variety of drugs including SP were widely available in the population (Goodman *et al.*, 2004) and self medication without prescription was very common. Thirdly, Rufiji was not isolated from the rest of Tanzania and gene flow from nearby populations was highly likely. It is possible that all these factors were compounded by out-stocking of ACT in

Rufiji (Mukondya, J. personal communication, 2007) as the pilot implementation was phasing out in the 2006, leading to more people reverting to the SP monotherapy, significantly revitalising SP drug selection pressure. Direct observation such as plasma drug levels in the patient would give the best direct measures of drug selection in the population and it would be interesting to examine this in comparison to the LD measures.

The disappearance of LD in Kilombero/Ulanga population in 2006 where SP monotherapy was still the first line antimalarial treatment policy was also a curious observation, which seems to indicate that, the strength of selection became insufficiently intense to overcome the force of recombination to keep *dhps* double and *dhfr* triple mutant alleles in association.

The frequency changes previously described in section 5.1 (and in Figure 22), show that during the period between 2001-2002 (the first year of SP first line use) there was dramatic increase of both the *dhfr* triple and *dhps* double mutant alleles along with equivalent drop in the *dhfr* and *dhps* sensitive alleles. However they also seem to indicate that following this period of intense SP selection there followed a gradual reduction of SP selection strength between 2002- 2006 which was characterised by slower rates of genetic change in the parasite population. To consider why this might have occurred it is necessary to review the process of policy change and the public response to changes in government treatment guidelines.

5.2.2 Changes in Government guidelines and the public response

In order to explain the underlying cause of reduction of SP selection strength just one year after SP first line adoption in Tanzania, this study retrospectively reviewed the process of policy change from CQ to SP first-line. The study also examined retrospectively the public perception and the role of media as key informant to the public, who are the end point consumers of the policy. The National Task Force on Antimalarial Drug Policy was formulated in May 1999 as a sub-committee of the National Malaria Advisory Committee (NMAC) in consultation with the East African Network for Monitoring Antimalarial Treatments (EANMAT) and WHO country office in Dar es Salaam (Mubyazi and Gonzalez-Block, 2005).

On 23 July 1999, the Task Force developed a three-page summary, drawing on evidence from clinical trials in the sentinel sites. It was recommended that the decision to change the policy should be interim because of increasing evidence of high SP resistance in various parts of the country such as Muheza and Kilombero districts (Mubyazi, 2003). After the presentation of the new policy by the Task Force, a series of news papers and some private radio stations began to inform the public that CQ was no longer a recommended drug for malaria treatment and that the Government was considering replacing it with a new drug. This information caused public concern and debates erupted in different parts of the country about the rationality for the change (Eriksen *et al.*, 2005; Mubyazi and Gonzalez-Block, 2005; Nsimba, 2006). Those involved were the

general public, the research community, traders, the pharmaceutical industry, and health-care providers in both the public and private health facilities.

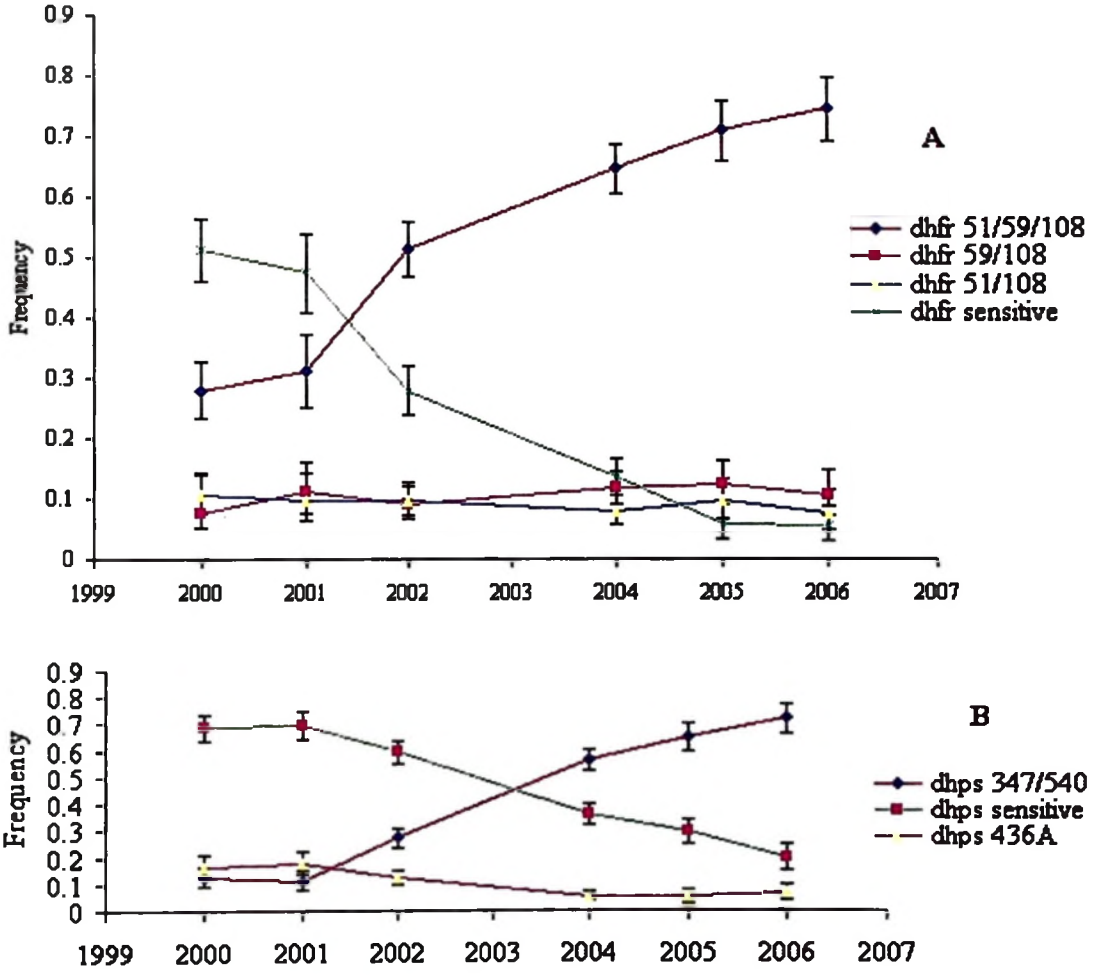

Figure 22. Response of Sulfadoxine and Pyrimethamine resistance alleles in Kilombero/Ulanga in a six year period between 2000 to 2006. A) *dhfr* alleles B) *dhps* alleles.

In an attempt to maintain public confidence, the Government through the Ministry of Health gave out a press release that indicated its stand concerning the treatment guidelines to be followed while strategies were underway to make an appropriate

decision. Anecdotal evidence indicates that many health professionals were unaware of the extent of resistance to CQ and did not perceive an urgent need for change (Mubyazi and Gonzalez-Block, 2005).

While WHO recommends change to an alternative drug when the treatment failure rates with the first-line drug reach 25%, evidence from different sentinel sites in the country indicated that up to the time of policy change, CQ treatment failure rate had already reached 52% (ranging 28–72%), 9.5% for SP (ranging 6–32%), while treatment failure to other drugs such as amodiaquine (AQ) and quinine was less than 4.6% (ranging 3.5% – 6%) (Abdulla *et al.*, 2000). More criticism ensued between the Ministry of health, the local newspapers and the research community on the effectiveness of CQ. While the public were advised to remain patient until the government gathered sufficient evidence, researchers continued to disseminate information indicating high levels of resistance and suggesting for ways of finding out a more suitable drug (Mubyazi and Gonzalez-Block, 2005). The government's official announcement of the policy change came out of the media in 2000, although the actual implementation officially started on 1st August 2001.

Clearly, there was a big gap between different actors which spurred a great deal of dilemma in the long term uptake and support of the new policy down to the communities. The study conducted in the year 2002 (one year of policy adoption) reported widespread fear and negative perceptions about the use of SP (Nsimba, 2006). Instigated by lack of educational campaign from the Ministry of Health and blown up by

the media domination with the reports of adverse SP reaction, there was a growing lack confidence in SP use as demonstrated by the public (Eriksen *et al.*, 2005; Nsimba, 2006). The trend of events in the policy adoption and implementation described here corroborates well with the genetic events described in Figure 22. Within the first year of policy implementation, usage of SP was highest (intense selection pressure with significant positive LD in the population) but as cases of serious SP reaction continued to mount and blown up by media, SP usage gradually became unpopular. It is also very possible that as SP resistance intensified over time, an increasing number of SP users reverted to alternative antimalarials again reducing SP selection pressure.

5.3 Spatial distribution of *dhfr* and *dhps* alleles

Allelic haplotypes, consisting of different configurations of point mutations in *dhfr* and *dhps* have been shown to confer known differences in levels of drug resistance as previously confirmed by *in vitro* studies (Wu *et al.*, 1996) and later *in vivo* efficacy studies (Kublin *et al.*, 2002; Omar *et al.*, 2001). For example, the triple mutant *dhfr* alleles comprised of point mutations N51I+C59R+S108N is 1600 times more tolerant to pyrimethamine than the sensitive *dhfr* allele *in vitro* (Wu *et al.*, 1996) and a combination of three point mutations on the *dhfr* gene and two on the *dhps* gene of the same parasite is predictive of SP failure in clearing such parasite *in vivo* in African settings (Kublin *et al.*, 2002). The frequencies of the different SP resistant alleles present in the field samples collected from 11 wards, 7 from Kilombero/Ulanga and 4 from Rufiji districts were characterised. Remarkably, the frequencies of both the common and rare

haplotypes at *dhfr* and *dhps* loci at the level of ward-sub population were highly consistent with those reported at district level and described under Section 4.2.1.

Pair-wise FST comparing frequencies of resistance alleles across the wards indicated significant population differentiation. The observed heterogeneity was due to differences in the distribution of triple mutant *dhfr* and double mutant *dhps* alleles in those sub populations, - even those under the same operating treatment policy. This could be due to uneven distribution of drugs by relevant national authorities along with unmatched number of private sector shops and pharmaceuticals stocking and selling drugs. It is also obvious that the wards differ greatly in terms of location whereby the most remotely located wards lacks some crucial infrastructures to permit provision of health services including accessible roads which is key for the transportation and supply of drugs. The former is especially important given the previously documented treatment seeking behaviour suggesting in these populations, where variety of drugs were available in the private sector including drug shops, kiosks and pharmacies for self medication (Goodman *et al.*, 2004).

Comparison of the inter- and intra-district allele frequency across wards revealed remarkable homogeneity with common allelic haplotypes found throughout all wards at similar frequencies. This highlights the level of inter-population genetic exchange in the region and underlines the uniformity of the observed frequency changes at the district

level across all the wards within those districts (Figure 15), indicating that similar evolutionary events were happening at all levels of population.

5.4 *Dhfr* flanking microsatellite loci

The present study have examined the evolutionary origin of the resistant *dhfr* alleles associated with pyrimethamine resistance in *P. falciparum* isolates from an area of high malaria transmission in two districts of south eastern part of Tanzania. It confirms some previous findings but also generated new observations which are important for understanding the evolution of resistance at *dhfr* in African populations. High genetic diversity was detected in the sensitive *dhfr* allele supporting the view that this is the ancestral form of *dhfr* (Roper *et al.*, 2003; Roper *et al.*, 2004). The predominant triple mutant *dhfr* flanking microsatellite haplotype observed in this study (haplotype H90: 108 bp/176 bp/203 bp) was identical to the Asian type haplotype reported in Roper *et al.* (2004) and subsequent studies indicate it is widespread in Africa (McCollum *et al.*, 2005; Pearce *et al.*, 2005; Ndiaye *et al.*, 2006; Maiga *et al.*, 2007; Lynch *et al.*, 2008).

The 25% triple mutant *dhfr* flanking microsatellite haplotypes shared similar allele length with haplotype H90: 108 bp/176 bp/203 bp at one or two microsatellite loci, indicating that the variation observed are possibly explained by the evolution of haplotype H90: 108 bp/176 bp/203 bp through replication errors (mutation) (Anderson *et al.*, 2004) or recombination as reported in Roper *et al.*, (2003) rather than because of a local independent origin. Two haplotypes (H29: 87 bp/174 bp/207 bp and H58: 87

bp/184 bp/199 bp), had completely different allele length at all three microsatellite loci and exhibited variation by 1 or 10 repeats compared to haplotype H90: 108 bp/176 bp/203 bp, and in this case it does suggest they have arisen independently. Both haplotypes were carrying an allele which is characteristically associated with the dhfr double mutant at the closest microsatellite (-0.3 Kb) loci, and this indicates that these haplotypes may have arisen through recombination between *dhfr* double mutant and dhfr triple mutant flanking sequences after the unique *dhfr* haplotype H90: 108 bp/176 bp/203 bp had undergone series of replication errors at the -4.4 Kb and -5.3 Kb loci leading to the 1 or 2 repeat variations observed at the two distant microsatellite loci.

Confirmation of this interpretation may require genotyping of these isolates at other polymorphic sequences that are closely linked to the *dhfr* gene. Assessment of the *dhfr* double mutant flanking microsatellite sequences revealed intermediate levels of diversity with sequence variation being more conserved than the sensitives but more diverse than the triple mutant. The two *dhfr* double mutant alleles (C59R+S108N and N51I+S108N), each had four different common haplotypes indicating that each has arisen independently at least four times. A caution however, has to be taken when interpreting haplotype H51 (87 bp/182 bp/193 bp) which was common to both C59R+S108N and N51I+S108N *dhfr* double mutant alleles. This haplotype was the most common for the N51I+S108N *dhfr* double mutant allele in the current study and is consistent with earlier findings in the Tanzanian parasite population (Roper *et al.*, 2003). It could be a result of mutation, recombination or both involving the two distant loci (-4.4 Kb and -5.3 Kb) of

the C59R+S108N *dhfr* double mutant flanking microsatellite sequences. Generally, there was a high degree of sharing of both allele length at different microsatellite loci among the *dhfr* double and triple mutant alleles, highlighting high levels of recombination and errors of replication associated with extreme malaria transmission pattern of the study area.

Taken together, data from this study show that all 184 *dhfr* triple mutant isolates typed (perhaps with 2 possible exceptions) are related to the Asian ancestral type previously described in Roper *et al,* (2003) and later found to be dispersed widely in Africa (Roper *et al.*, 2004; McCollum *et al.*, 2005; Pearce *et al.*, 2005; Ndiaye *et al.*, 2006; Maiga *et al.*, 2007; Lynch *et al.*, 2008). Recent study in Western Kenya has also found additional *dhfr* triple mutant novel haplotypes suggesting multiple origin of the *dhfr* triple mutant allele (McCollum *et al.*, 2006). Further studies may be necessary to explore the possibility of replication of Kenyan findings. The findings of the present study however, was very similar to reports of recent study in Uganda (Lynch *et al.*, 2008) and that of Maiga and co-workers involving samples from 11-sub Saharan African countries with different levels of malaria transmission (Maiga *et al.*, 2007), showing the broad application throughout Africa.

To further explore the issue of genetic diversity around *dhfr* flanking microsatellite heterozygosity values were assessed. At microsatellites linked to the sensitive allele there was large amount of genetic variation with average gene diversity values of 0.52,

0.71 and 0.73 for the -0.3 Kb, -4.4 Kb and -5.3 Kb loci, respectively. Allele length polymorphisms (A) the -4.4 Kb loci and -5.3 Kb loci revealed higher allele length polymorphisms (19 and 18) than -0.3 Kb which showed 11 allele length polymorphisms. The -0.3 Kb locus therefore was less polymorphic possibly because of low mutation rate. These findings, when considered in the context of recent studies, shows far higher *dhfr* flanking microsatellite polymorphism than report of studies in Senegal (A= 8, 7 and 9 for the -0.3 Kb, -4.4 Kb and -5.3 Kb loci, respectively) (Ndiaye *et al.*, 2006) and in Kenya (A= 5, 9 and 3 for the -0.3 Kb, -4.4 Kb and -5.3 Kb loci, respectively) (McCollum *et al.*, 2006). The higher diversity in the present study is likely to be explained by many factors including high malaria transmission and large size of samples analysed from an area covering about 29,400 km^2 and spanning sites up to 300 km apart.

Theory suggests SP drug pressure is the driving force behind evolution of its resistance. Positive selective pressure act to increase the frequency of favoured allele meanwhile creating association with the sequences immediately flanking the gene. Initially there is generalized association but as frequency of the favoured allele increases recombination breaks down the more distant associations retaining only associations of the allele with sequences immediately flanking it, the hitchhiker (Smith and Haigh, 1974; Kaplan *et al.*, 1989). Ultimately, the result of selection is a pattern of reduced gene diversity or expected heterozygosity, He and the loss of diversity is described as selective sweep.

Theoretically, it was expected that combination of SP+ART in Rufiji would reduce SP drug pressure and hence reduce evolution of its resistance. By comparing heterozygosity values for the highly resistant triple mutant *dhfr allele* (C59R+N51I+S108N) after 4 years of SP+ART implementation in Rufiji and 4 years of SP monotherapy in Kilombero/Ulanga it was observed that greatest reduction in gene diversity was found in Kilombero/Ulanga. The diversity values dropped from 0.65 to 0.23 in Kilombero/Ulanga population between 2002 and 2006. While in Rufiji diversity around the *dhfr* triple mutant (C59R+N51I+S108N) was stable being 0.41 to 0.55 in 2002 to 2006 possibly reflecting the reduced SP pressure due to use of SP+ART during that time. This finding is consistent with previous reports in parasites from south east Africa showing significant loss of diversity across a region of 70 kb around the most resistant *dhfr* allele as an evidence of a selective sweep attributable to selection through widespread use of SP for the treatment of malaria (Pearce *et al.*, 2005).

5.5 Selectively neutral microsatellite

The gene diversity values observed in this study for the 3 selectively neutral microsatellites loci analysed (PfPk2, TA109 and Poly A) were higher and broadly similar between the two populations of Rufiji and Kilombero/Ulanga, with the average of 0.885 across all markers in both populations. The poly A locus had a slightly higher gene diversity values of 0.91 in both Kilombero/Ulanga and Rufiji, compared to the PfPk2 0.90 in both populations and TA109 (0.85 and 0.84) in Kilombero/Ulanga and Rufiji, respectively. These findings are comparable with previous estimates elsewhere,

DRC Congo 0.9305 (Durand *et al.*, 2003), Vietnam 0.91 (Ferreira *et al.*, 2002). Both the TA109 and PfPk2 loci exhibited gene diversity values comparable to the previously reports in DRC Congo (Durand *et al.*, 2003), of 0.8593 and 0.8886 for TA109 and PfPk2, respectively. These values however, were different from those described previously in Papua New Guinea (Anderson *et al.*, 1999), of 0.21 and 0.68 for the TA109 and PfPk2 loci, respectively. These results are expected in an area of intense malaria transmission and corroborate an earlier report by Anderson *et al.* (2000a) describing random association among loci, high genetic diversity and minimal geographical differentiation as features of large regions of Africa, where transmission is intense.

By using data obtained for the 3 selectively neutral microsatellites to assess differentiation between Kilombero/Ulanga and Rufiji parasite populations, it was observed that at both inter and intra district comparisons there was no evidence of population subdivision. This contrasts with the same measures using resistance loci in the different subpopulations supporting the view that the heterogeneity in the frequency of resistant alleles is due to heterogeneity in the strength of drug selection pressure in the different wards.

The three microsatellite loci typed are distributed throughout the genome and unlikely to be biased by a loss of diversity in any segment of the genome. The data obtained from the three microsatellite markers strongly suggest extensive genetic exchange indicating

that the parasite population in the two districts of Rufiji and Kilombero/Ulanga were undoubtedly mixing freely. This finding corroborates earlier studies conducted in three sites namely Kilombero/Ulanga in Tanzania, Mozambique and South Africa (Pearce *et al.*, 2005). By using 8 selectively neutral microsatellites, Pearce *et al.* (2005) concluded that the parasite population in the three Southeast African countries named above were panmictic and that a great deal of mixing was occurring in the parasite population across the three countries such that, resistant alleles could diffuse from a site where the drug was in use to sites where drug pressure were officially absent.

Similar findings were reported by Clyde (1967) from a study conducted in Muheza, Tanzania in the 1950s. According to Clyde (1967) by giving monthly prophylactic dose of pyrimethamine to semi immune individuals, resistance quickly developed and diffused out of the area to sites where selection was absent up to more than 100 miles away.

Transmission intensity

In areas of high malaria transmission, infections tend to be composed of multiple genetically distinct parasite lines (Babiker *et al.*, 1999). This may occur in two ways through super infection of a host following receiving multiple infective bites or through the bites of a mosquito carrying a mixture of genotypes within its saliva inoculum. One of the attributes of ACT is that it acts so rapidly killing the parasites massively irrespective of their resistance properties hence reducing gametocyte carriage rates and

in so doing limiting malaria transmission (Price *et al.*, 1996; White *et al.*, 1998). Transmission intensity is measured by Entomological Inoculation Rate (EIR), which is an estimation of the number of infective bites per person per annum. However, the transmission intensity can indirectly be measured by using highly polymorphic markers such as Merozoite Surface Proteins (MSP 1&2) or selectively neutral microsatellite markers. In this case, the transmission intensity can be indicated by the number of genetically distinct parasite lines identified per person (multiplicity of infection, MOI) in which case the higher the degree of mixture of infection the higher the transmission intensity and vice versa.

In the current study, three selectively neutral microsatellite markers were used to estimate the MOI in Rufiji and Kilombero/Ulanga populations in the 2002 and compare with the MOI estimated for the same populations in 2006. The results showed a marked decline in MOI after 2002. In 2002 the most common MOI was 3 in both populations yet by 2006 the most common MOI in Rufiji was 2 and Kilombero/Ulanga was 1. This finding indicate there was a trend towards decrease in malaria transmission over time in both populations, which was apparently independent of SP+ART intervention in Rufiji and in fact rather surprisingly was more pronounced in Kilombero/Ulanga than Rufiji. This observation is consistent with the overall prevalence of malaria infections detected in the cross sectional surveys which also declined by 6.1% in Rufiji and 14.7% in Kilombero/Ulanga, between 2000-06. Recent studies have reported a similar trend of decreasing malaria transmission in Tanzania (Killeen *et al.*, 2007) and in Kenya (Okiro

et al., 2007) attributing the reduction to the effect of transmission blocking interventions. This effect was directly measured by Alifrangis *et al.* (2003a) in northern Tanzania where the effect of ITNs on *P. falciparum* resistance genes was assessed by monitoring the prevalence of *dhfr* and *dhps* genotypes in children less than five years old living in the village of Magoda from 1998 to 2000. In 2000, after two years of bed net use, the prevalence of wild types in codon 51, 59, and 108 of *dhfr* increased significantly in Magoda compared with previous years.

In Kilombero/Ulanga in particular, an area-wide evaluation of malaria transmission and the impact of high coverage with bed nets (Killeen *et al.*, 2007) reported that bed nets were common place in the area and coverage levels in the whole population, rather than just target group, exceeded the thresholds required to achieve community-level suppression of transmission with insecticidal nets. This remarkable net coverage in Kilombero/Ulanga population most likely was due to success of the previous bed net promotion in the district, implemented from 1996 onwards (Schellenberg *et al.*, 1999) and probably exceeds the coverage in Rufiji population. While ACT in Rufiji might have contributed to the reduced malaria transmission observed, it is equally possible that the reduction is due to increased bed net coverage.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

6.1.1 Drug pressure

The National policy change brought about an immediate shift in treatment practice and this in turn had a highly significant impact on drug pressure. This shows that even in rural areas where access to treatment is imperfect and treatment coverage relatively low, first line SP selection is sufficiently strong to rapidly change the genetic composition of the parasite population in one year. Furthermore selection applied was strong enough to overcome recombination pressure and create linkage disequilibrium between the unlinked genetic determinants of resistance to the two co-administered drugs (Sulfadoxine and Pyrimethamine), showing that recombination is not a barrier to the evolution of multidrug resistance in high endemicity settings.

6.1.2 Effect of SP + ART combination therapy in Rufiji

SP + ART combination therapy in Rufiji had a small but measurable impact on the frequencies of the resistant *dhfr* and *dhps* mutant alleles. It appeared to reduce the rate of increase of both triple mutant *dhfr* and double mutant *dhps* in the Rufiji population compared to Kilombero/Ulanga. The combination therapy also, disrupted the association between *dhps* double and *dhfr* triple mutant alleles (which was created earlier in the population by high SP drug pressure following shift of policy from first-line CQ to SP in 2001) to a non-significant level, equivalent to when SP was a second line antimalarial

treatment. This is a significant finding as it suggests that the combination therapy reduced the SP selection pressure to the level where it was insufficient to keep the two alleles in combination, although it is somewhat confounded by the observations in 2006 where the reverse trend was seen.

6.1.3 Why did combination therapy not halt the increase in these alleles?

The use of SP monotherapy was not completely halted in Rufiji as it continued to be used for IPTP and by individuals seeking treatment from outlets other than the government health facilities through which the ACT was supplied. In addition, the SP + ART constitute two drugs with completely unmatched half-life, namely 5-10 days for the SP and 45 minutes for the Artesunate. While artesunate is a very short-acting antimalarial drug with a half-life that does not exceed 1 h (White, 1997), SP has a contrastingly long half-life such that, following SP administration, for about 15–52 days, there is a strong selective pressure for pyrimethamine resistance (Watkins and Mosobo, 1993). When given as a 3-day course with single dose of SP, the combination most likely will clear SP resistant parasites (*dhfr* triple mutants) in the initial 3 days and thus extend the UTL of SP. However, as suggested elsewhere (Nzila *et al.*, 2000b), during new infections the long-acting property of SP most likely selectively clear only the sensitives, concentrating parasites with resistant *dhfr* and *dhps* properties. The unmatched half-lives of SP + ART in the combination imply little synergistic protection between each other leaving the partner drug exposed or acting as monotherapy for much of the time of the treatment course.

6.1.4 The effect of gene flow

Gene flow has been identified as an important means for the dissemination and establishment of resistance. Throughout Rufiji and Kilombero/Ulanga populations there was clear evidence of its action. Microsatellite analysis of the two parasite populations of Rufiji and Kilombero/Ulanga found a non significant population differentiation between them indicating the heterogeneity in the resistance allele frequencies were due to differences in the intensity of selection in the two populations. The findings of the current study support previous findings describing intercontinental gene flow (Roper *et al.*, 2004) showing that the Asian triple mutant *dhfr* was abundant in the area and was the allele most strongly selected by SP use in the community. Clearly, the role of gene flow in the dissemination and establishment of drug resistance has a clear implication for treatment policy at a regional level and this observation may be important for policy makers for future policy improvement.

A unilateral policy in a region may imply large scale gene flow is ensured when selection occurs in one or few state(s), limiting the usefulness of the drug for the neighbouring states. Furthermore, adoption of a drug policy which has been abandoned by a neighbouring state will be a truly poor decision as the UTL of the adopted drug policy will most likely be very short. For the successful protection of the next generation of antimalarial drugs (ACTs), multilateral antimalarial policies need to be adopted to limit the extent to which gene flow from neighbouring countries might undermine the successful deployment of a particular treatment.

6.1.5 Transmission intensity

There was a trend towards decrease of malaria transmission in both Rufiji and Kilombero/Ulanga parasite populations as meassured by MOI using selectively neutral microsatellite markers. The decrease was more pronounced in Kilombero/Ulanga than Rufiji which is contrary to the expectation that SP + ART combination would reduce malaria transmission. It is not clear why there was more reduced malaria transmission in Kilombero/Ulanga (SP first-line) than Rufiji (SP + ART) but it is speculated that concurrent interventions such as social marketing and scaling up of the use of insecticide treated nets (ITNs) could have brought about this effect. It is also worth noting that the baseline frequency of genetic resistance to SP in the study population at the start of pilot ACT in Rufiji had already reached >60% which would negatively affect the performance of ACT.

6.1.6 Selective sweep

SP monotherapy in Kilombero-Ulaga increased the selective sweep around drug resistant *dhfr* triple mutant chromosomes by narrowing the diversity values from 0.65 to 0.23, while ACT in Rufiji stabilized and slightly decreased it by increasing the diversity values from 0.41 to 0.55.

6.2 Recommendations

6.2.1 Policy change

Policy change brings about shift in drug use practices due to frequent and widespread use of the newly introduced replacement drug. Experience suggests frequent and widespread use of drug is a very potent selection pressure in favour of parasite genotypes expressing resistance to the effect of the drug (Medley, 1994; this study). Resistance has several consequences, the most important of which is that only a limited number of drugs are available, and once resistance has developed to all of them, clinical disease caused by parasites becomes untreatable. The current study has demonstrated a strong link between treatment policy and drug pressure in field settings. It is widely agreed that parasitic drug treatments should be used in a manner which will minimise drug pressure to limit the growth of resistance. In the light of the current study, as a consideration for future policy formulation, it is suggested here that in high transmission settings there may be value in using a suite of antimalarial drugs rather than blanket use of the same drug for all programmes of treatment (IPTI, IPTP prophalaxis as well as direct therapy for malaria). This will significantly limit the extent of drug pressure thereby prolonging the UTL of the drugs in use.

6.2.2. Choice of the ACT

The success of any ACT intervention largely depends upon the choice of combination therapy adopted. Ideally, a potentially successful combination therapy should have its properties similar or close to the following: good efficacy and safety profile, high degree

of acceptability and ease of administration, capable of being used in special groups e. g. pregnant women and infants, affordability, little or no reported resistance or cross resistance and should also have predicted longer UTL. In this study, SP + ART pilot combination therapy was introduced in Rufiji at a time when genetic resistance to SP was well above 60%. This rate of resistance is by any standard sufficient to rule out its use in communities with such level of baseline frequency of resistance to either one or both component drugs in the combination. It was not surprising therefore to see the combination failing to halt resistance to SP as it is predicted in the literature. The findings of this study emphasise the importance of selection of ACT for which resistance is not established in any of the two component drugs. As a general rule, unless is intended for a specific policy intervention, which is unlikely to drive drug pressure significantly, combinations of drugs for which resistance to one or both of the components is already established, should be avoided. In fact this recommendation is bourne out by the results of population-wide association of *dhfr* triple and *dhps* double mutant examined in the current study, which showed an apparent association of the two alleles when drug pressure was relatively low but rapidly became apparent once drug pressure increased. Likewise blanket use of ACTs is predicted to trigger high drug pressure which is the main driving force behind selection for resistance.

6.2.3 Panmixia of parasite population

Results of three selectively neutral microsatellite markers suggest parasite populations were mixing freely between Kilombero/Ulanga and Rufiji populations. Previous studies

using similar microsatellite markers identified panmixia of parasite populations in three countries of East and Southern African region, namely Tanzania, Mozambique and South Africa (Pearce *et al.*, 2005). Earlier in 1960s, Clyde (1967) reported that by giving monthly prophylactic dose of pyrimethamine to semi immune individuals in Muheza district in Tanzania, resistance quickly developed and diffused out of the area to sites where selection was absent up to more than 100 miles away. Clearly, this suggests resistance is very mobile with no regard to national boundaries. This has a major implication in the policy formulation process since policy decisions made by a neighbouring country will inevitably affect the surrounding nations. It may be useful in the future to consider planning regional policies rather than each country independently plan its national guidelines. In doing so the issue of gene flow will have been taken on board and the policies designed while taking note of the influence of gene flow are likely to be more optimal while retaining longer UTL.

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APPENDICES

Appendix I. Allele frequencies of the most common *dhfr* and *dhps* haplotypes

		DHFR						*DHPS*				
Year	**Locality**	**CNCS**	**CNRN**	**CICN**	**CIRN**	**Rare***	**N**	**SAKA A**	**AAKA A**	**SGEA A**	**Rare***	**N**
2000	K/U	0.513	0.074	0.106	0.279	0.028	**376**	0.688	0.167	0.126	0.019	**365**
	Rufiji	0.359	0.116	0.114	0.374	0.037	**457**	0.712	0.177	0.079	0.032	**417**
	Average	**0.436**	**0.095**	**0.11**	**0.327**	**0.033**		**0.70**	**0.172**	**0.103**	**0.026**	
2001	K/U	0.475	0.113	0.097	0.311	0.005	**238**	0.697	0.177	0.109	0.017	**294**
	Rufiji	0.302	0.110	0.102	0.467	0.019	**420**	0.771	0.112	0.094	0.023	**519**
	Average	**0.389**	**0.112**	**0.10**	**0.389**	**0.012**		**0.734**	**0.145**	**0.102**	**0.02**	
2002	K/U	0.278	0.092	0.096	0.515	0.019	**489**	0.595	0.124	0.274	0.007	**603**
	Rufiji	0.184	0.084	0.091	0.626	0.015	**527**	0.599	0.131	0.245	0.025	**596**
	Average	**0.231**	**0.088**	**0.094**	**0.57**	**0.017**		**0.597**	**0.128**	**0.26**	**0.016**	
2004	K/U	0.135	0.117	0.08	0.652	0.016	**540**	0.358	0.055	0.566	0.021	**562**
	Rufiji	0.084	0.091	0.07	0.734	0.021	**428**	0.398	0.085	0.481	0.036	**364**
	Average	**0.110**	**0.104**	**0.075**	**0.693**	**0.019**		**0.378**	**0.07**	**0.524**	**0.029**	
2005	K/U	0.056	0.124	0.098	0.716	0.006	**338**	0.293	0.057	0.647	0.003	**351**
	Rufiji	0.029	0.11	0.065	0.792	0.004	**734**	0.366	0.063	0.551	0.02	**697**
	Average	**0.043**	**0.175**	**0.082**	**0.754**	**0.005**		**0.33**	**0.06**	**0.60**	**0.012**	
2006	K/U	0.055	0.106	0.076	0.753	0.001	**275**	0.196	0.066	0.723	0.015	**275**
	Rufiji	0.036	0.115	0.107	0.74	0.002	**616**	0.299	0.056	0.628	0.017	**588**
	Average	**0.046**	**0.111**	**0.092**	**0.747**	**0.002**		**0.248**	**0.061**	**0.676**	**0.016**	

K/U = Kilombero/Ulanga, N= Number of samples, Rare* = Haplotypes with frequency <5%

Appendix II. Distribution of the common and rare (bold) *dhfr* flanking microsatelite haplotypes detected in 2001/2002 and 2006 cross sectional surveys in Rufiji and Kilombero/Ulanga

i) Triple *dhfr* (CIRN)

Sample	-5.3 Kb	-4.4 Kb	-0.3 Kb	51 Kb	59	108	164	2001/2002		2006	
								Ru	KiU	Ru	KiU
N=134	203	176	108	I	R	N	I	22	17	38	57
N=21	203	176	87	I	R	N	I	1	9	10	1
N=3	193	176	87	I	R	N	I	1	1	1	
N=2	193	176	108	I	R	N	I		1		1
N=1	**207**	**174**	**87**	**I**	**R**	**N**	**I**		**1**		
N=1	**211**	**176**	**87**	**I**	**R**	**N**	**I**		**1**		
N=1	**205**	**170**	**108**	**I**	**R**	**N**	**I**	**1**			
N=1	**199**	**172**	**108**	**I**	**R**	**N**	**I**	**1**			
N=1	**207**	**178**	**108**	**I**	**R**	**N**	**I**		**1**		
N=1	**203**	**176**	**85**	**I**	**R**	**N**	**I**		**1**		
N=1	**203**	**170**	**87**	**I**	**R**	**N**	**I**	**1**			
N=3	**203**	**176**	**110**	**I**	**R**	**N**	**I**			**3**	
N=1	**199**	**184**	**87**	**I**	**R**	**N**	**I**			**1**	
N=1	**203**	**176**	**102**	**I**	**R**	**N**	**I**			**1**	
N=1	**203**	**162**	**102**	**I**	**R**	**N**	**I**				**1**
N=1	**203**	**176**	**104**	**I**	**R**	**N**	**I**			**1**	
N=1	**221**	**182**	**108**	**I**	**R**	**N**	**I**			**1**	
N=1	**201**	**178**	**108**	**I**	**R**	**N**	**I**			**1**	
N=1	**203**	**176**	**115**	**I**	**R**	**N**	**I**			**1**	
N=2	**203**	**178**	**108**	**I**	**R**	**N**	**I**				**2**
N=1	**203**	**172**	**108**	**I**	**R**	**N**	**I**			**1**	
N=1	**203**	**166**	**108**	**I**	**R**	**N**	**I**				**1**
N=1	**203**	**186**	**108**	**I**	**R**	**N**	**I**				**1**
N=1	**207**	**176**	**108**	**I**	**R**	**N**	**I**				**1**

Rare haplotypes shown by bold

Indicate common allele

ii) Double *dhfr* (CICN)

Sample	-5.3 Kb	-4.4 Kb	-0.3 Kb	51	59	108	164	2001/2002		2006	
								Ru	KiU	Ru	KiU
N=44	193	182	87	I	C	N	I	1	12	16	15
N=19	193	178	87	I	C	N	I	1	5	4	9
N=6	193	176	87	I	C	N	I		3	1	2
N=4	205	172	87	I	C	N	I		1	2	1
N=3	193	168	87	I	C	N	I		1		2
N=2	199	182	87	I	C	N	I	1		1	
N=3	193	180	87	I	C	N	I		1		2
N=2	203	176	87	I	C	N	I	1			1
N=1	**193**	**166**	**87**	**I**	**C**	**N**	**I**	**1**			
N=1	**193**	**170**	**87**	**I**	**C**	**N**	**I**		**1**		
N=1	**201**	**180**	**87**	**I**	**C**	**N**	**I**	**1**			
N=1	**209**	**184**	**87**	**I**	**C**	**N**	**I**		**1**		
N=1	**205**	**176**	**87**	**I**	**C**	**N**	**I**		**1**		
N=2	**193**	**186**	**87**	**I**	**C**	**N**	**I**			**1**	**1**
N=1	**207**	**199**	**87**	**I**	**C**	**N**	**I**			**1**	
N=1	**193**	**184**	**87**	**I**	**C**	**N**	**I**			**1**	
N=1	**211**	**178**	**87**	**I**	**C**	**N**	**I**			**1**	
N=1	**209**	**178**	**87**	**I**	**C**	**N**	**I**				**1**
N=2	**203**	**180**	**87**	**I**	**C**	**N**	**I**			**2**	
N=1	**199**	**184**	**87**	**I**	**C**	**N**	**I**				**1**
N=1	**198**	**176**	**87**	**I**	**C**	**N**	**I**				**1**
N=1	**205**	**182**	**87**	**I**	**C**	**N**	**I**			**1**	
N=1	**190**	**184**	**85**	**I**	**C**	**N**	**I**				**1**
N=2	**193**	**176**	**108**	**I**	**C**	**N**	**I**			**1**	**1**
N=1	**205**	**176**	**108**	**I**	**C**	**N**	**I**			**1**	
N=2	**203**	**176**	**108**	**I**	**C**	**N**	**I**			**2**	

Rare haplotypes shown by bold

Indicate common allele

iii) Double *dhfr* (CNRN)

Sample	-5.3 Kb	-4.4 Kb	-0.3 Kb	51	59	108	164	2001/2002		2006	
								Ru	KiU	Ru	KiU
N=27	199	186	87	N	R	N	I	2	5	9	11
N=18	199	176	87	N	R	N	I	2	8	5	3
N=6	193	182	87	N	R	N	I		3	1	2
N=2	199	168	87	N	R	N	I		1	1	
N=2	193	176	87	N	R	N	I		1		1
N=10	203	176	87	N	R	N	I		1	3	6
N=1	**199**	**172**	**87**	**N**	**R**	**N**	**I**		**1**		
N=1	**207**	**182**	**87**	**N**	**R**	**N**	**I**		**1**		
N=1	**203**	**156**	**87**	**N**	**R**	**N**	**I**	**1**			
N=1	**193**	**178**	**87**	**N**	**R**	**N**	**I**		**1**		
N=1	**193**	**180**	**87**	**N**	**R**	**N**	**I**		**1**		
N=1	**205**	**172**	**87**	**N**	**R**	**N**	**I**		**1**		
N=1	**199**	**174**	**87**	**N**	**R**	**N**	**I**				**1**
N=1	**199**	**182**	**87**	**N**	**R**	**N**	**I**			**1**	
N=1	**199**	**162**	**87**	**N**	**R**	**N**	**I**			**1**	
N=1	**205**	**186**	**87**	**N**	**R**	**N**	**I**			**1**	
N=1	**203**	**176**	**108**	**N**	**R**	**N**	**I**				**1**
N=1	**199**	**186**	**110**	**N**	**R**	**N**	**I**			**1**	
N=1	**199**	**164**	**87**	**N**	**R**	**N**	**I**				**1**
N=1	**199**	**188**	**87**	**N**	**R**	**N**	**I**				**1**
N=1	**205**	**168**	**87**	**N**	**R**	**N**	**I**			**1**	

Rare haplotypes shown by bold

Indicate common allele

Appendix III. *Dhfr* flanking microsatellite diversity values for sensitive and resistant chromosomes recorded in Kilombero/Ulanga and Rufiji populations in 2001 and 2006 isolates.

Year	*Dhfr* Haplotype	Kilombero/Ulanga				Rufiji			
		Marker	N	A	He	Marker	N	A	He
2001	CIRNI	-0.3	32	3	0.52	-0.3	27	2	0.20
		-4.4	32	3	0.12	-4.4	27	3	0.21
		-5.3	32	4	0.29	-5.3	27	4	0.21
		MEAN	**32**	**3.3**	**0.31**		**27**	**3**	**0.21**
	CNCSI	-0.3	46	8	0.42	-0.3	60	6	0.57
		-4.4	46	15	0.92	-4.4	33	11	0.89
		-5.3	46	13	0.92	-5.3	33	10	0.86
		MEAN	**46**	**12**	**0.75**		**42**	**9**	**0.77**
	CICNI	-0.3	26	1	0	-0.3	6	1	0
		-4.4	26	8	0.75	-4.4	6	5	0.93
		-5.3	26	3	0.22	-5.3	6	4	0.8
		MEAN	**26**	**4**	**0.32**		**6**	**3.3**	**0.58**
	CNRNI	-0.3	24	1	0	-0.3	5	1	0
		-4.4	24	7	0.78	-4.4	5	3	0.8
		-5.3	24	5	0.57	-5.3	5	2	0.4
		MEAN	**24**	**4.3**	**0.45**		**5**	**2**	**0.4**
2006	CIRNI	-0.3	65	3	0.06	-0.3	59	7	0.48
		-4.4	65	5	0.15	-4.4	59	5	0.16
		-5.3	65	3	0.06	-5.3	59	5	0.13
		MEAN	**65**	**3.7**	**0.09**		**59**	**5.7**	**0.26**
	CICNI	-0.3	38	3	0.10	-0.3	34	2	0.21
		-4.4	38	8	0.77	-4.4	34	8	0.72
		-5.3	38	7	0.29	-5.3	34	6	0.53
		MEAN	**38**	**6**	**0.39**		**34**	**5.3**	**0.49**
	CNRNI	-0.3	27	2	0.07	-0.3	24	2	0.08
		-4.4	27	7	0.77	-4.4	24	6	0.79
		-5.3	27	3	0.54	-5.3	24	4	0.43
		MEAN	**27**	**4**	**0.46**		**24**	**4**	**0.44**

Appendix IV. Allele frequencies (%) at 3 unlinked microsatellite loci

1. TA109

Allele	Kilombero/Ulanga frequncy	Rufiji frequency
149	0.34	0.32
152	1.01	0.32
158	2.01	1.92
161	24.50	22.12
164	17.79	21.47
167	0.34	1.92
170	1.01	0.32
173	12.42	9.94
176	19.13	23.72
177		0.32
179	1.34	2.88
182	1.01	0.96
185	4.70	2.56
186		0.32
188	3.36	4.81
191	0.34	0.96
194	0.67	
197	6.38	2.24
200		0.32
209	2.01	1.92
218	0.34	
221	0.67	
223	0.67	0.32
245		0.32
	N=273	N=282

2. PfPk2

Allele	Kilombero/Ulanga frequency	Rufiji frequency
140	0.24	
144	0.96	0.39
156	0.72	1.56
159	8.87	7.23
160		0.20
162	19.42	14.65
165	11.03	10.35
167		0.39
168	10.31	14.45
169		0.39
171	13.19	14.06
172	0.24	0.20
174	8.15	5.47
175		0.39
177	4.56	3.71
179		0.20
180	5.04	5.86
181		0.20
183	2.40	4.49
186	6.71	6.84
189	4.56	3.71
191		0.20
192	1.92	3.13
195	0.72	1.76
198	0.72	0.20
200	0.24	
	N=416	N=512

3. Poly A

Allele	Kilombero/Ulanga frequency	Rufiji frequency
105	0.43	
108	0.43	
111	0.43	0.38
120	0.43	
125	0.43	
133		0.76
135	0.43	
136		1.14
139	0.85	1.52
142	6.41	6.08
146	6.41	4.18
149	8.97	7.98
152	18.80	17.11
156	14.10	13.31
158	0.43	
159	7.69	10.27
161	0.43	
162	7.26	4.56
165	5.98	9.89
168	6.84	3.80
171	4.70	4.18
174	0.43	3.80
175	0.43	
177	1.71	4.56
179	0.43	
180	1.28	2.28
183	2.56	3.04
186	1.28	0.76
189	0.43	
195		0.38
	N=234	N=264

Appendix V. Allele frequencies (%) at each of the 3 *dhfr* flanking microsatellite loci among *dhfr* sensitive and triple mutant alleles

(A) 2001/2002 Survey

1. -0.3 Kb

Allele	Kilombero/Ulanga		Rufiji	
	Sensitive	CIRN	Sensitive	CIRN
85		3		
87	76	38	85	11
92	2			
96	7		3	
98	2			
100			3	
102	2		3	
104	2			
108		59	3	89
110	7		3	
115	2			
	N=46	N=32	N=33	

2. -4.4 Kb

Allele	Kilombero/Ulanga		Rufiji	
	Sensitive	CIRN	Sensitive	CIRN
190			3	
191	4			
193	13	6	3	4
195			9	
197	2		6	
199	11			4
201	7		3	
203	11	84	15	89
205	15		21	4
207	11	6	27	
209	7			
211	7	3	3	
213	4		6	
215	7			
217			3	
219	2			
	N=46	N=32	N=33	N=27

3. -5.3 Kb

Allele	Kilombero/Ulanga		Rufiji	
	Sensitive	CIRN	Sensitive	CIRN
158	9		3	
160	2			
164	2			
166	4			
168	2			
170	13		6	7
172	9		13	4
174	4	3	13	
176	20	94	22	89
178	11	3	6	
180	4		22	
182	7		3	
184	9		6	
186			3	
188	2			
193	33		3	
	N=46	N=32	N=32	N=27

(B) 2006 Survey

1. -0.3 Kb

Allele	Kilombero/Ulanga CIRN	Rufiji CIRN
87	2%	20%
102	2%	2%
104		2%
108	97%	69%
110		3%
111		2%
115		2%
	N=65	N=59

2. -4.4 Kb

Allele	Kilombero/Ulanga CIRN	Rufiji CIRN
193	2%	2%
199		2%
201		2%
203	97%	93%
207	2%	
221		2%
	N=65	N=59

3. -5.3 Kb

Allele	Kilombero/Ulanga CIRN	Rufiji CIRN
162	2%	
166	2%	
172		2%
176	92%	92%
178	3%	3%
182		2%
184		2%
186	2%	
	N=65	N=59

Appendix VI. Allele frequencies (%) at each *dhfr* flanking microsatellite loci among *dhfr* double mutant alleles

(A) 2001/2002 Survey

1. CICN

1. 1 -0.3 Kb

Allele	Kilombero/Ulanga CICN	Rufiji CICN
87	100	100
	N=26	N=6

1.2 -4.4 Kb

Allele	Kilombero/Ulanga CICN	Rufiji CICN
193	88	50
199		17
201		17
203		17
205	8	
209	4	
	N=26	N=6

1.3 -5.3 Kb

Allele	Kilombero/Ulanga CICN	Rufiji CICN
166		17
168	4	
170	4	
172	4	
176	15	17
178	19	17
180	4	17
182	46	33
184	4	
	N=26	N=6

2. CNRN

2.1 -0.3 Kb

Allele	Kilombero/Ulanga CNRN	Rufiji CNRN
87	100	100
	N=24	N=5

2.2 -4.4 Kb

Allele	Kilombero/Ulanga CNRN	Rufiji CNRN
193	25	
199	63	80
203	4	20
205	4	
207	4	
	N=24	N=5

2.3 -5.3 Kb

Allele	Kilombero/Ulanga CNRN	Rufiji CNRN
156		20
168	4	
172	8	
176	42	40
178	4	
180	4	
182	17	
186	21	40
	N=24	N=5

(B) 2006 Survey

1. CICN

1.1 -0.3 Kb

Allele	Kilombero/Ulanga CICN	Rufiji CICN
85	3	
87	95	88
108	3	12
	N=38	N=34

1.2 -4.4 Kb

Allele	Kilombero/Ulanga CICN	Rufiji CICN
190	3	
193	84	68
198	3	
199	3	3
203	3	12
205	3	12
207		3
209	3	
211		3
	N=38	N=34

1.3 -5.3 Kb

Allele	Kilombero/Ulanga CICN	Rufiji CICN
168	5	
172	3	
176	13	6
178	26	15
180	5	15
182	39	6
184	5	50
186	3	3
191		3
	N=38	N=34

2. CNRN

2.1 -0.3 Kb

Allele	Kilombero/Ulanga CNRN	Rufiji CNRN
87	96	96
108	4	
110		4
	N=27 .	N=24

2.2 -4.4 Kb

Allele	Kilombero/Ulanga CNRN	Rufiji CNRN
193	11	4
199	63	75
203	26	13
205		8
	N=27	N=24

2.3 -5.3 Kb

Allele	Kilombero/Ulanga CNRN	Rufiji CNRN
162		4
164	4	
168		8
174	4	
176	41	33
182	7	8
184	22	29
186	19	17
188	4	
	N=27	N=24

Appendix VII. Spatial distribution of *dhfr* and *dhps* alleles among Rufiji and Kilombero/Ulanga wards in annual surveys between 2000 and 2006.

2000 Frequencies

	dhfr						*dhps*				
Locality	CNCS	CNRN	CICN	CIRN	RARE	N=	SAKAA	AAKAA	SGEAA	RARE	N=
Bungu	0.37	0.12	0.09	0.39	0.03	117	0.72	0.19	0.07	0.02	111
Idete	0.59	0.07	0.05	0.27	0.02	56	0.61	0.25	0.14		44
Ikwiriri	0.46	0.10	0.09	0.30	0.04	99	0.74	0.15	0.06	0.04	95
Iragua	0.42	0.03	0.09	0.39	0.04	33	0.63	0.24	0.13		38
Kibiti	0.28	0.11	0.1	0.42	0.03	166	0.71	0.19	0.07	0.03	150
Kchangani	0.37	0.17	0.04	0.33	0.04	46	0.79	0.13	0.05	0.03	39
Lupiro	0.49	0.03	0.24	0.22	0.02	59	0.81	0.09	0.09		64
Mbingu	0.54	0.06	0.12	0.26	0.02	50	0.55	0.26	0.15	0.04	53
Mchombe	0.53	0.05	0.09	0.32	0.01	99	0.65	0.15	0.17	0.03	89
Mchukwi	0.33	0.14	0.08	0.37	0.04	63	0.76	0.14	0.10		51
Minepa	0.64	0.15	0.09	0.12		33	0.72	0.22	0.03	0.03	36

2001 Frequencies

	dhfr						*dhps*				
Locality	CNCS	CNRN	CICN	CIRN	RARE	N=	SAKAA	AAKAA	SGEAA	RARE	N=
Bungu	0.29	0.12	0.12	0.46	0.02	125	0.71	0.15	0.12	0.02	160
Idete	0.49	0.10	0.10	0.31		51	0.63	0.27	0.07	0.03	60
Ikwiriri	0.38	0.08	0.09	0.45		91	0.85	0.07	0.05	0.02	96
Iragua	0.25	0.17	0.08	0.50		12	0.73	0.18	0.09		22
Kibiti	0.25	0.13	0.10	0.50	0.02	158	0.79	0.08	0.09	0.03	201
Kchangani	0.50	0.18	0.05	0.23	0.04	22	0.87	0.10	0.03		31
Lupiro	0.41	0.06	0.21	0.32		34	0.77	0.15	0.08		48
Mbingu	0.55	0.08	0.03	0.34		38	0.58	0.17	0.23	0.02	52
Mchombe	0.43	0.17	0.11	0.28		53	0.69	0.10	0.17	0.03	58
Mchukwi	0.29	0.10	0.10	0.45	0.04	42	0.74	0.16	0.10		57
Minepa	0.57	0.07	0.07	0.29		28	0.73	0.24	0.03		37

2002 Frequencies

	dhfr						*dhps*				
Locality	CNCS	CNRN	CICN	CIRN	RARE	N=	SAKAA	AAKAA	SGEAA	RARE	N=
Bungu	0.24	0.10	0.11	0.54	0.01	157	0.57	0.16	0.25	0.02	195
Idete	0.43	0.05	0.06	0.44	0.02	80	0.62	0.14	0.20	0.03	98
Ikwiriri	0.22	0.10	0.12	0.55	0.01	83	0.67	0.08	0.21	0.04	107
Iragua	0.12	0.18	0.06	0.64		50	0.54	0.14	0.30	0.02	63
Kibiti	0.16	0.07	0.05	0.71	0.01	181	0.61	0.13	0.23	0.03	195
Kchangani	0.20	0.12	0.08	0.58	0.02	65	0.60	0.10	0.30	0.01	94
Lupiro	0.25	0.09	0.14	0.50	0.02	56	0.73	0.10	0.17		78
Mbingu	0.30	0.13	0.06	0.51		47	0.46	0.16	0.39		70
Mchombe	0.28	0.07	0.13	0.50	0.01	148	0.53	0.11	0.36		155
Mchukwi	0.10	0.07	0.09	0.71	0.04	92	0.58	0.11	0.29	0.02	93
Minepa	0.31	0.08	0.08	0.49	0.04	49	0.76	0.16	0.08		51

2004 Frequencies

	dhfr						*dhps*				
Locality	CNCS	CNRN	CICN	CIRN	RARE	N=	SAKAA	AAKAA	SGEAA	RARE	N=
Bungu	0.07	0.10	0.06	0.75	0.02	153	0.40	0.09	0.48	0.03	145
Idete	0.17	0.10	0.06	0.64	0.02	81	0.41	0.09	0.49	0.01	80
Ikwiriri	0.10	0.09	0.08	0.70	0.03	66	0.45	0.09	0.43	0.03	56
Iragua	0.08	0.18	0.10	0.64		90	0.36	0.03	0.8	0.03	105
Kibiti	0.09	0.10	0.07	0.72	0.02	121	0.40	0.10	0.47	0.03	102
Kchangani	0.10	0.19	0.03	0.67	0.01	70	0.49	0.05	0.42	0.04	81
Lupiro	0.09	0.13	0.07	0.64	0.04	55	0.29	0.08	0.63		62
Mbingu	0.25	0.09	0.11	0.55		65	0.24	0.03	0.71	0.02	62
Mchombe	0.13	0.07	0.05	0.73	0.02	128	0.27	0.07	0.63	0.03	122
Mchukwi	0.08	0.08	0.07	0.77		86	0.33	0.05	0.55	0.04	60
Minepa	0.14	0.08	0.14	0.64		50	0.51	0.02	0.47		49

2005 Frequencies

	dhfr						*dhps*				
Locality	CNCS	CNRN	CICN	CIRN	RARE	N=	SAKAA	AAKAA	SGEAA	RARE	N=
Bungu	0.03	0.12	0.08	0.76	0.01	292	0.38	0.06	0.55	0.01	272
Idete		0.21	0.09	0.70		33	0.47	0.06	0.47		34
Ikwiriri	0.05	0.12	0.09	0.75		103	0.40	0.05	0.54	0.01	98
Iragua		0.07	0.02	0.91		45	0.29	0.02	0.69		48
Kibiti	0.02	0.10	0.05	0.83	0.01	208	0.35	0.07	0.56	0.02	208
Kchangani		0.22	0.16	0.63		32	0.28	0.06	0.66		32
Lupiro	0.11	0.19	0.08	0.58	0.04	53	0.37	0.14	0.49		51
Mbingu	0.05	0.08	0.13	0.74		61	0.16	0.08	0.74	0.02	62
Mchombe	0.08	0.09	0.10	0.74		91	0.29	0.03	0.68		100
Mchukwi	0.02	0.10	0.05	0.83		130	0.32	0.06	0.56	0.05	117
Minepa	0.13	0.09	0.13	0.65		23	0.26		0.74		23

2006 Frequencies

	dhfr						*dhps*				
Locality	CNCS	CNRN	CICN	CIRN	RARE	N=	SAKAA	AAKAA	SGEAA	RARE	N=
Bungu	0.03	0.11	0.13	0.73		231	0.29	0.04	0.65	0.02	222
Idete	0.08	0.28	0.04	0.60		25	0.23	0.10	0.63	0.03	30
Ikwiriri	0.05	0.15	0.13	0.67		101	0.36	0.07	0.56	0.01	95
Iragua	0.04	0.06	0.06	0.80	0.04	51	0.20	0.09	0.72		46
Kibiti	0.04	0.11	0.07	0.77		190	0.30	0.07	0.62	0.01	181
Kchangani	0.13	0.06		0.81		16	0.08		0.92		13
Lupiro	0.06		0.19	0.75		16	0.30		0.70		10
Mbingu	0.09	0.15	0.03	0.73		33	0.24	0.03	0.70	0.03	37
Mchombe	0.05	0.09	0.11	0.76		111	0.17	0.03	0.78	0.02	118
Mchukwi	0.02	0.11	0.08	0.79	0.01	94	0.24	0.07	0.65	0.03	86
Minepa		0.14	0.05	0.77	0.04	22	0.32	0.23	0.45		22

Appendix VIII. Between Ward population pairwise Fst comparison in Rufiji and Kilombero/Ulanga in the period between 2000 and 2006. Shaded cells indicate significant Fst differences.

2000 Population pairwise Fsts

	Bungu	Idete	Ikwiriri	Iragua	Kibiti	Kicha	Lupiro	Mbingu	Mcho	Mchu	Minepa
Bungu	-										
Idete	0.078	-									
Ikwiriri	0.019	0.006	-								
Irangua	0.012	0.157	0.086	-							
Kibiti	-0.009	0.082	0.030	0.005	-						
Kichangani	-0.008	0.020	-0.013	0.048	0.020	-					
Lupiro	0.041	0.041	0.009	0.144	0.051	0.010	-				
Mbingu	0.029	0.042	0.035	0.034	0.018	0.017	0.070	-			
Mchombe	0.019	0.024	0.005	0.058	0.023	-0.012	0.030	0.004	-		
Mchukwi	-0.007	0.048	0.002	0.046	0.003	-0.017	0.023	0.032	0.017	-	
Minepa	0.092	-0.004	0.019	0.213	0.098	0.018	0.015	0.078	0.043	0.047	-

2001 Population pairwise Fsts

	Bungu	Idete	Ikwi	Iragua	Kibi	Kicha	Lupiro	Mbingu	Mcho	Mchu	Minepa
Bungu	-										
Idete	0.014	-									
Ikwiriri	-0.002	0.003	-								
Irangua	-0.033	-0.035	-0.006	-							
Kibiti	0.001	0.055	0.016	-0.006	-						
Kichangani	0.030	-0.030	0.024	-0.011	0.068	-					
Lupiro	0.006	-0.006	0.001	-0.006	0.030	-0.003	-				
Mbingu	0.024	0.014	0.033	-0.029	0.072	0.030	0.049	-			
Mchombe	0.014	-0.005	0.029	-0.053	0.050	-0.006	-0.005	0.002	-		
Mchukwi	-0.014	0.006	0.003	-0.058	0.005	0.016	-0.004	-0.003	-0.017	-	
Minepa	0.005	-0.036	-0.004	-0.059	0.052	-0.011	0.009	-0.009	0.003	0.004	-

2002 Population pairwise Fsts

	Bungu	Idete	Ikwiriri	Iragua	Kibiti	Kicha	Lupiro	Mbingu	Mcho	Mchu	Minepa
Bungu	-										
Idete	0.018	-									
Ikwiriri	-0.000	0.004	-								
Irangua	-0.006	0.034	0.008	-							
Kibiti	0.001	0.029	0.006	-0.006	-						
Kichangani	0.001	0.041	0.023	-0.008	0.014	-					
Lupiro	0.011	0.009	-0.011	0.030	0.022	0.048	-				
Mbingu	-0.011	0.009	0.001	-0.017	-0.006	-0.007	0.021	-			
Mchombe	-0.005	0.017	0.002	0.000	0.008	-0.003	0.014	-0.008	-		
Mchukwi	0.009	0.055	0.016	0.001	-0.003	0.021	0.030	0.013	0.016	-	
Minepa	0.037	0.005	0.007	0.054	0.039	0.084	-0.004	0.038	0.047	0.060	-

2004 Population pairwise Fsts

	Bungu	Idete	Ikwi	Ira	Kibi	Kich	Lup	Mbingu	Mcho	Mchu	Mine
Bungu	-										
Idete	0.007	-									
Ikwiriri	-0.005	-0.006	-								
Irangua	0.011	0.006	-0.011	-							
Kibiti	-0.002	-0.009	-0.006	0.009	-						
Kichang	0.008	-0.005	-0.007	-0.007	-0.003	-					
Lupiro	0.005	0.007	-0.011	-0.010	0.010	0.003	-				
Mbingu	0.042	0.038	0.014	0.007	0.051	0.034	-0.008	-			
Mchombe	0.002	0.014	-0.000	0.005	0.016	0.010	-0.007	0.009	-		
Mchukwi	-0.005	-0.004	-0.010	-0.001	-0.006	-0.010	-0.002	0.028	-0.003	-	
Minepa	-0.001	-0.008	-0.012	-0.009	-0.007	-0.013	-0.011	0.020	-0.003	-0.014	-

2005 Population pairwise Fsts

	Bungu	Idete	Ikwi	Iragua	Kibiti	Kicha	Lupiro	Mbingu	Mcho	Mchu	Minepa
Bungu	-										
Idete	0.002	-									
Ikwiriri	-0.002	-0.009	-								
Irangua	0.053	0.100	0.043	-							
Kibiti	0.004	0.022	0.002	0.022	-						
Kichangani	0.015	-0.003	0.001	0.071	0.024	-					
Lupiro	0.009	-0.008	0.008	0.114	0.035	0.018	-				
Mbingu	0.047	0.058	0.026	0.026	0.032	-0.002	0.066	-			
Mchombe	0.022	0.032	0.007	0.014	0.011	0.001	0.042	-0.004	-		
Mchukwi	0.002	0.009	-0.003	0.030	-0.005	0.023	0.027	0.036	0.016	-	
Minepa	0.013	0.023	-0.006	0.017	0.007	-0.004	0.018	-0.015	-0.028	0.012	-

2006 Population pairwise Fsts

	Bungu	Idete	Ikwi	Iragua	Kibiti	Kicha	Lupiro	Mbingu	Mcho	Mchu	Minepa
Bungu	-										
Idete	-0.005	-									
Ikwiriri	0.012	-0.012	-								
Irangua	-0.004	-0.004	0.025	-							
Kibiti	-0.002	-0.004	0.009	-0.001	-						
Kichangani	0.052	0.085	0.118	0.028	0.069	-					
Lupiro	-0.133	-0.106	-0.095	-0.144	-0.151	0.018	-				
Mbingu	-0.070	-0.040	-0.035	-0.063	-0.063	0.035	-0.268	-			
Mchombe	-0.004	-0.012	0.030	-0.022	-0.001	0.013	-0.148	-0.069	-		
Mchukwi	0.001	0.005	0.041	-0.012	0.012	0.023	-0.116	-0.052	-0.014	-	
Minepa	-0.003	0.001	0.003	-0.002	-0.007	0.076	-0.151	-0.067	0.007	0.014	-

Appendix IX. Pairwise Fst comparison of populations for alleles conferring mild and intermediate resistance to the *dhfr* and *dhps* genes

2000 Population pairwise Fsts

	Bungu	Ikwi	Iragua	Kibiti	Mbi	Mcho	Mchu	Mine
Bungu	-							
Ikwiriri	-0.167	-						
Irangua	0.333	0.500	-					
Kibiti	-0.166	-0.002	0.210	-				
Mbingu	-0.043	-0.081	-1.000	-0.006	-			
Mchombe	-0.333	-1.000	1.000	-0.107	-1.000	-		
Mchukwi	-0.043	-0.081	-1.000	-0.006	-1.000	-1.000	-	
Minepa	0.467	0.637	1.000	0.385	0.333	1.000	0.333	-

2001 Population pairwise Fsts

	Bungu	Ikwiriri	Iragua	Kibiti	Mchombe	Mchukwi
Bungu	-					
Ikwiriri	-0.556	-				
Irangua	-0.167	1.000	-			
Kibiti	-0.118	0.000	-0.600	-		
Mchombe	-0.481	-1.000	-1.000	-0.469	-	
Mchukwi	-0.167	1.000	1.000	0.200	-0.200	-

2002 Population pairwise Fsts

	Bung	Ikwi	Ira	Kibi	Kich	Lup	Mbi	Mch o	Mch u	Mine
Bungu	-									
Ikwiriri	0.499	-								
Irangua	0.100	0.408	-							
Kibiti	0.060	-0.020	0.000	-						
Kicha	-1.000	0.429	-1.000	-0.333	-					
Lupiro	-0.215	-0.063	-0.143	-0.454	-1.000	-				
Mbingu	0.500	-1.000	0.200	-0.600	1.000	-1.000	-			
Mcho	0.141	-0.011	0.164	-0.173	-0.111	-0.436	-0.667	-		
Mchu	-1.000	0.429	-1.000	-0.333	0.000	-1.000	1.000	-0.111	-	
Minepa	-0.215	-0.063	-0.143	-0.454	-1.000	-1.000	-1.000	-0.436	-1.000	-

2004 Population pairwise Fsts

	Bungu	Idete	Kibiti	Kich	Mcho	Mchu
Bungu	-					
Idete	0.000	-				
Kibiti	-1.000	-1.000	-			
Kichanga	1.000	1.000	-0.500	-		
Mchombe	-0.833	-0.833	-0.203	-0.047	-	
Mchukwi	1.000	1.000	0.250	0.000	0.2845	-

2005 Population pairwise Fsts

	Bungu	Idete	Ikwiriri	Kibiti	Lupiro	Mbingu	Mcho
Bungu	-						
Idete	-1.000	-					
Ikwiriri	0.333	1.000	-				
Kibiti	0.133	0.125	-0.750	-			
Lupiro	-0.333	-1.000	0.333	0.133	-		
Mbingu	-1.000	0.000	1.000	0.125	-1.000	-	
Mchombe	0.529	1.000	0.000	-0.157	0.529	1.000	-

2006 Population pairwise Fsts

	Bungu	Idete	Ikwiriri	Kibiti	Mchukwi
Bungu	-				
Idete	1.000	-			
Ikwiriri	-1.000	0.000	-		
Kibiti	-1.000	-1.000	-0.333	-	
Mchukwi	0.000	1.000	-1.000	-1.000	-

Ikwi = Ikwiriri, Ira = Iragua, Kibi = Kibiti, Kich = Kichangani, Lup = Lupiro, Mbi = Mbingu, Mcho = Mchombe, Mchu = Mchukwi and Mine = Minepa.

Appendix X. Protocol for *P. falciparum* DNA extraction from filter paper

1. Cut a sector (half usually or 3mm x 3mm) from the paper. Use sterile blade for this.
2. Place this in a 1.5ml eppendorf or well of 96 format deep-well plate (1.2ml capacity) with 1ml of 0.5% saponin in 1xPBS (made fresh that day)
3. Leave either at room temp (or 37°C for the more recalcitrant samples) for several hours or overnight. Haemoglobin released into the wash leaving parasite DNA on paper. I leave them overnight.
4. Remove saponin and debris using pipette spin down to *get all* liquid
5. Wash in 1ml 1xPBS
6. Spin and remove liquid and debris. Repeat wash in 1ml 1xPBS.
7. Add 100 µl PCR quality water plus 50ul 20% chelex mix (using trimmed P200 gilson tip)
8. Seal deepwell plate with foil – no need to pierce cap. Boil for 8 minutes
9. Centrifuge for 5 min to spin down chelex
10. Take off DNA supernatant with pipette and discard the chelex and the paper. Or freeze Deep-well plate with DNA, chelex and paper in situ.

Chelex

Chelex-100 resin is from Bio-Rad; 100-200 mesh sodium form; Cat. no. 143-2832.

Make up to (approx) 20% in PCR quality water, pH to 9.5+ by adding 5M NaOH (add 5-10 µl to a 20 ml mixture) and test with pH paper

Appendix XI. Primary PCR protocol for *dhfr* and *dhps* amplification

Reaction conditions	Cocktail
25 µl reaction volumes will contain	
2.5 µl 10 x PCR buffer, 25mM Mg^{2+}	x..... = ... µl 10 x PCR buffer.
2.5 µl dNTP mix (1.25 mM each)	x..... = ... µl dNTP mix.
2.5 µl Forward Primer (10 µM)	x..... = ... µl Forward Primer.
2.5 µl Reverse Primer (10 µM)	x..... = ... µl Forward Primer.
0.2 µl Taq (5U of 5 U/µl)	x..... = ... µl Taq.
13.8 µl PCR – grade H2O	x..... = ... µl PCR – grade H2O.
1.0 µl Template DNA	

1. Add all components except DNA to the universal tube as a cocktail.

2. Vortex thoroughly to mix well

3. Aliquot 24 µl of cocktail into each well of a PCR plate.

4. Add 1.0 µl of DNA to each of the PCR plate well.

5. Cycle 40 times with the following cycle profiles

(i) *Dhfr*: 94^0Cx3 min, 94^0Cx1 min, 52^0Cx2 min, 72^0Cx1 min, 40x, 72^0Cx10 min

(ii) *Dhps*: 94^0Cx3 min, 94^0Cx1 min, 51^0Cx2 min, 72^0Cx1 min, 40x, 72^0Cx10 min

Appendix XII. Nested PCR protocol for *dhfr* and *dhps* amplification

Reaction conditions	Cocktail
25 µl reaction volume will contain	
2.5 µl 10 x PCR buffer, 25mM Mg^{2+}	x..... = ... µl 10 x PCR buffer.
2.5 µl dNTP mix (1.25 mM each)	x..... = ... µl dNTP mix.
2.5 µl Forward Primer (10 µM)	x..... = ... µl Forward Primer.
2.5µl Reverse Primer (10 µM)	x..... = ... µl Forward Primer.
0.2 µl Taq (5U of 5 U/µl)	x..... = ... µl Taq.
13.8 µl PCR – grade H2O	x..... = ... µl PCR – grade H2O.
1.0 µl Primary PCR products	

1. Add all components except Primary PCR products to the universal tube as a cocktail.

2. Vortex thoroughly to mix well

3. Aliquot 24 µl of cocktail into each well of a PCR plate.

4. Add 1.0µl of Primary PCR producs to each of the PCR plate well.

5. Cycle 40 times with the following cycle profiles

(i) *Dhfr*: 94^0Cx3 min, 94^0Cx1 min, 44^0Cx2 min, 72^0Cx1 min, 5x, 94^0Cx1 min, 44^0Cx1min, 72^0Cx1 min, 35x, 72^0Cx10 min

(ii) *Dhps*: 94^0Cx3 min, 94^0Cx1 min, 51^0Cx2 min, 72^0Cx1 min, 40x, 72^0Cx10 min

Appendix XIII. Primary PCR protocol for microsatellite amplification

Reaction conditions	Cocktail
11 µl reaction volume will contain	
1.1 µl 10 x PCR buffer, 25mM Mg^{2+}	x..... = ... µl 10 x PCR buffer.
0.88 µl dNTP mix (1.25 mM each)	x..... = ... µl dNTP mix.
0.44 µl Forward Primer (10 µM)	x..... = ... µl Forward Primer.
0.44 µl Reverse Primer (10 µM)	x..... = ... µl Forward Primer.
0.1 µl Taq (5U of 5 U/µl)	x..... = ... µl Taq.
7.04 µl PCR – grade H2O	x..... = ... µl PCR – grade H2O.
1.0 µl Template DNA	

1. Add all components except DNA to the universal tube as a cocktail.

2. Vortex thoroughly to mix well

3. Aliquot 10 µl of cocktail into each well of a PCR plate.

4. Add 1.0 µl of DNA to each of the PCR plate well.

5. Cycle 25 times with the following cycle profiles

94^0Cx2 min, 94^0Cx30 sec, 42^0Cx30 sec, 40^0Cx30 sec, 65^0Cx40 sec, 25x, 65^0Cx2 min

Appendix XIV. Semi-nested PCR protocol for microsatellite amplification

Reaction conditions	Cocktail
11 µl reaction volumes will contain	
1.1 µl 10 x PCR buffer, 25mM Mg^{2+}	x..... = ... µl 10 x PCR buffer.
0.88 µl dNTP mix (1.25 mM each)	x..... = ... µl dNTP mix.
0.66 µl Forward Primer (10 µM)	x..... = ... µl Forward Primer.
0.66 µl Reverse Primer (10 µM)	x..... = ... µl Forward Primer.
0.1 µl Taq (5U of 5 U/µl)	x..... = ... µl Taq.
6.6 µl PCR – grade H2O	x..... = ... µl PCR – grade H2O.
1.0 µl Template DNA	

1. Add all components except DNA to the universal tube as a cocktail.

2. Vortex thoroughly to mix well

3. Aliquot 10 µl of cocktail into each well of a PCR plate.

4. Add 1.0 µl of DNA to each of the PCR plate well.

5. Cycle 25 times with the following cycle profiles

94^0Cx2 min, 94^0Cx20 sec, 45^0Cx20 sec, 65^0Cx30 sec, 25x, 65^0Cx2 min

Appendix XV. Dot blotting protocol

Preparation of the membranes

1) Cut nitro-cellulose membranes of size 84 mm x 70 mm. Divide the membrane into columns of 7 mm width. Prepare one membrane for each probe that is to be used.
2) Denature the PCR product at 94^0C for 2 minutes.
3) Using a multichannel pipette dot 1.5µl of the PCR products onto the appropriate membranes. Allow the membranes to dry and then cross-link the DNA using a U.V crosslinker.
4) Place the membranes, dot side in with no overlap, in 50 ml centrifuge / falcon tubes.
5) The membranes can then be stored at room temperature until required.

Probing

1) Block membranes in 5ml of blocking solution at 37^0C on a rotisserie in a hybridisation oven for at least 30 mins.
2) Prepare probes. If probes are new then 10µl of labelled probe should be added to 5ml of TMAC prehybridisation solution (2 pmol/ml). If probes are already prepared at this concentration and have been stored in the freezer then they should be refreshed by adding 1µl of stock probe.
3) Place the prepared probes and the prehybridisation solution in a water bath at the hybridisation temperature (e.g. 53^0C) to warm.

4) Pour off the blocking solution and add 5mls of TMAC prehybridisation solution. Place the tubes back on the rotisserie and turn the oven temperature up to the critical hybridisation temperature (e.g. 53^0C).

5) When the hybridisation temperature is reached pour off the TMAC solution and add the relevant probe to each tube. (Work quickly to keep the probes warm).

6) Incubate the tubes on the rotisserie at the hybridisation temperature for $1^1/_2$ hours.

7) Pour probes back into original tubes and turn water bath temperature up to 56^0C to preheat the TMAC for high stringency washes.

8) Remove membranes into low stringency washing solution (2 x SSPE, 0.1% SDS). Wash for 2 x 10 mins on an orbital shaker and make sure that membranes do not stick together. (Use approx. 500 ml for 10 membranes). Transfer each membrane individually between washes.

9) Pour warmed TMAC into a tray in the water bath and quickly transfer membranes individually into the TMAC for the high stringency washes. Ensure a lid is on the tray and use the shaker. Wash 2 x 10mins and check the membranes every 3-4 minutes to ensure they are not sticking together.

10) Rinse membranes in Buffer 1 for 5 mins and blot dry on filter paper. Place the membranes, dot side in and not overlapping, in fresh Falcon tubes. (Detection may proceed immediately or membranes can be stored in tubes for up to 5 days).

Detection

1) Add 5 mls of Buffer 2 (this is Buffer 1 with 1% milk powder added) to each tube and put on rotisserie at 37^0C for at least 30 mins.
2) Add 1µl of anti-digoxigenin antibody Fab fragment conjugated with alkaline phosphatase (Boehringer Mannheim) and put on the rotisserie at 37^0C for at least 40 mins
3) Remove membranes from tubes and wash 3 x 10 mins in Buffer 1 on the orbital shaker. Use approx. 300-500 ml for 10 membranes and check regularly to ensure they do not stick together. Transfer membranes individually between washes.
4) Rinse membranes for 5 mins in Buffer 3 while preparing substrate solution (CSPD).
5) Place 10 ml substrate solution (10 ml Buffer 3 + 100 µl CSPD stock, filtered at 0.2 µm with a syringe and kept protected from the light in a foil covered universal) in a large weighing dish. (The CSPD can be stored for up to one week and should be filtered between each use).
6) Place membranes face down (up to 4 membranes in 10 mls) into the solution ensuring there are no air bubbles and that the surfaces are completely covered. Cover the dish with a box (ice buckets are ideal) and incubate for 5 mins.
7) Blot membranes dry on filter paper and place dot side up in acetate sheets
8) Incubate in oven at 37^0C for at least 15 mins.
9) Expose the membranes to ECL (Enhanced Chemiluminescence) or X-ray film for required time. (Usually 25 – 30 min but considerable longer or shorter times may be required).

10) Develop films and fully mark and label the details of the experiment on the films.

ALTERNATIVELY

If detection is by scaning with phosphorimager scaner, perfom detection up to step 7, but in this case in steps 5 and 6 instead of using CSPD, use enhanced chemofluorescent substrate (ECF). After step 7 scan the memranes on the phosphorimager machine.

Solutions

Blocking Solution (100 ml)

20 x SSPE stock buffer	20 ml	(4 x final concentration)
10% Laurylsarcosine	1 ml	(0.1% final concentration)
Milk Powder	1 g	(1% final concentration)
dH_2O	79 ml	(up to final volume)

TMAC Hybridisation Buffer (1 litre)

5M TMAC stock solution	600 ml	(3M final concentration)
1M Tris (pH 8.0) solution	50 ml	(50mM final concentration)
20% SDS stock solution	5 ml	(0.1% final concentration)
0.5M EDTA (pH 8.0) solution	4 ml	(2 mM final concentration)

2 x SSPE / 0.1% SDS Wash Buffer (1 litre)

20 x SSPE stock buffer	100 ml
20% SDS stock solution	5 ml

Buffer 1 (1 litre of 10x stock)

Tris Base	121.1 g	(1M concentration)
NaCl	87.7 g	(1.5M concentration)

pH to 7.5 and dilute 1/10 before use.

Buffer 2 (100ml)

Buffer 1 (1x)	100 ml
Milk powder	1 g

Buffer 3 (500ml)

1M Tris (pH 9.5) solution	50 ml	(0.1M final concentration)
5M NaCl solution	10 ml	(0.1M final concentration)

20xSSPE (2 litres)

EDTA	14.8 g	(pH to 8.0 to dissolve)
NaCl	350.6 g	
$NaH_2PO_4.H_20$	55.2 g	

pH to 7.4 with NaOH ~ 6.5 ml of 10M NaOH per litre.

Appendix XVI. Supplementary information showing the observed and expected values for linkage disequilibrium analyses.

Two locus genotype tables : frequencies observed/expected
KilomberoUlanga 2000 (N=190)

	SAK	AAK	SGE	rare alleles
CNCS	0.358 0.326	0.100 0.100	0.037 0.060	0.005 0.013
CICN	0.058 0.048	0.000 0.015	0.010 0.009	0.005 0.002
CNRN	0.053 0.051	0.010 0.016	0.016 0.010	0.000 0.002
CIRN	0.179 0.213	0.079 0.065	0.053 0.039	0.016 0.009
rare alleles	0.005 0.003	0.010 0.001	0.005 0.001	0.000 0.0002

KilomberoUlanga 2001 (N=148)

	SAK	AAK	SGE	rare alleles
CNCS	0.351 0.355	0.061 0.066	0.061 0.059	0.013 0.006
CICN	0.061 0.064	0.013 0.012	0.013 0.012	0.000 0.001
CNRN	0.108 0.089	0.013 0.016	0.000 0.015	0.000 0.002
CIRN	0.203 0.217	0.047 0.040	0.047 0.036	0.000 0.004
rare alleles	0.007 0.005	0.000 0.001	0.000 0.001	0.000 0.0001

KilomberoUlanga 2002 (N=358)

	SAK	AAK	SGE	rare alleles
CNCS	0.195 0.151	0.022 0.029	0.036 0.079	0.008 0.004
CICN	0.064 0.060	0.014 0.011	0.025 0.031	0 0.001
CNRN	0.053 0.051	0.011 0.010	0.022 0.027	0.003 0.001
CIRN	0.249 0.302	0.059 0.057	0.215 0.158	0.003 0.007
rare alleles	0.014 0.011	0.003 0.002	0.003 0.006	0 0.0003

Rufiji 2000 (N=285)

	SAK	AAK	SGE	rare alleles
CNCS	0.263 0.256	0.060 0.055	0.017 0.028	0.007 0.008
CICN	0.098 0.093	0.025 0.020	0.000 0.010	0.003 0.003
CNRN	0.081 0.096	0.032 0.020	0.014 0.010	0.003 0.003
CIRN	0.270 0.264	0.035 0.056	0.042 0.029	0.010 0.009
rare alleles	0.025 0.028	0.007 0.006	0.007 0.003	0.000 0.001

Rufiji 2001 (N=282)

	SAK	AAK	SGE	rare alleles
CNCS	0.209 0.218	0.057 0.040	0.025 0.036	0.007 0.004
CICN	0.074 0.070	0.007 0.013	0.014 0.012	0.000 0.001
CNRN	0.082 0.078	0.011 0.014	0.011 0.013	0.003 0.001
CIRN	0.351 0.352	0.060 0.065	0.067 0.058	0.003 0.007
rare alleles	0.014 0.013	0.000 0.002	0.003 0.002	0.000 0.0002

Rufiji 2002 (N=342)

	SAK	AAK	SGE	rare alleles
CNCS	0.108 0.086	0.032 0.021	0.020 0.050	0.003 0.007
CICN	0.053 0.049	0.015 0.012	0.023 0.028	0.003 0.004
CNRN	0.047 0.046	0.015 0.011	0.023 0.027	0.003 0.004
CIRN	0.313 0.340	0.061 0.081	0.237 0.196	0.035 0.026
rare alleles	0.006 0.003	0.003 0.001	0.000 0.002	0.000 0.0003

* significance $p \leq 0.001$

Appendix XVII. Main drugs used to treat malaria

Drug name	Chemical class	Proposed mechanism of action	Resistance reported?
Quinine and derivatives		Interfere with haem detoxification	YES
Quinine	Quinoline methanol		
Amodiaquine	4-aminoquinoline		
Chloroquine	4-aminoquinoline		
Halofantrine	Phenanthrene methanol		
Mefloquine	4-aminoquinoline		
Primaquine	8-aminoquinoline		
Folate antagonists		Inhibition of nucleic acid synthesis	YES
Pyrimethamine	2,4-diaminopyrimidine		
Proguanil	Biguanide derivative		
Sulfonamides		Inhibition of nucleic acid synthesis	YES
Sulfadoxine			
Atovaquone	Hydroxyl naphtoquinone	Interfere with the mitochondrial electron transfer chain	YES
Artemisinin and derivatives		Generation of oxidative stress	Not used as a single agent
Artemisinin	Sesquiterpene trioxane lactone		
Dihydroartemisinin	Reduced artemisinin		
Artesunate	Dihydroartemisinin-β-o hemisuccinate sodium salt		
Artemether	Methyl ether of dihydroartemisinin		
Combination therapy		Synergistic	Yes (for SP and malrone)
Sulfadoxine+Pyrimethamine (SP)			
SP+Artesunate			

Mefloquine+Atemether			
Lumefantrine+Artemether			
Atovaquone+Proguanil (Malarone®)			