MOLECULAR INVESTIGATION OF MULTIPLICITY OF INFECTIONS AND DRUG RESISTANCE TO SULPHADOXINE-PYRIMETHAMINE (SP) IN *PLASMODIUM FALCIPARUM* MALARIA IN MLIMBA, TANZANIA

BY

ERASTO MBUGI

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY MEDICINE OF SOKOINE UNIVERSITY OF AGRICULTURE.

MOROGORO, TANZANIA.

2004

ABSTRACT

The study was aimed at molecular investigation of multiplicity of infections and drug resistance to sulphadoxine-pyrimethamine (SP) in *Plasmodium falciparum* malaria. This molecular epidemiological study involved 141 blood samples from patients aged less than five years from malaria-endemic Mlimba division of Kilombero District, Morogoro, south eastern Tanzania. Blood samples were collected on filter papers (3MM Whatmann) and parasite DNA was extracted by Chelex technique. Molecular analysis on the merozoite surface protein 2 (MSP2), dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) was based on polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) of PCR products (PCR-RFLP). These techniques made it possible to determine the multiplicity of infections and SP resistance-associated point mutations anticipated at sorting out recrudescence from new infections. The commonly reported point mutations occurring at codons 51, 59, 108 and 164 in the DHFR and codons 437, 540 and 581 in the DHPS domains were investigated. The results showed the multiplicity of infection array of single to six infections per patient with an average multiplicity of 2.58 infections per patient. Fifty-one patients possessed single alleles of either allelic families of the MSP2 gene in PCR-RFLP successful samples. Double, triple and multiple infections were detected in 37.7%, 11.9% and 5.9% of patients, respectively. Regarding drug resistance molecular markers, 66.9% carried mutations at codon 108, 62.7% at codon 51 and 48.8% at codon 59 of DHFR domain. Fifty-six (43.7%) of samples carried mutations at codon 437, 39.2% at codon 540 and 0.8% at codon 581 on the DHPS domain. Proportions of mixed variants in the DHFR domain ranged from 0 – 21.5% and 0.8 – 6.3% in the DHPS domain. About 44 (36.4%) of isolates

harboured triple mutant DHFR genotypes, whereas quintuple mutation was observed in 24 (19.8%) of isolates. Ten (8.3%) isolates possessed at least double DHFR and double DHPS mutants. This study found a high proportion of SP resistance-associated point mutations in Mlimba two years after deployment of SP as a first-line antimalarial drug in Tanzania. However, the adequate clinical response (81.1%) observed clinically reflects the role of semi-immunity component in the study population. This implies that used molecular markers for monitoring drug resistance, be done simultaneously with studies on confounding factors pertaining to development of resistance against SP in falciparum malaria. The extensive use of antifolates other than SP for treatment of infections other than malaria is a probable candidate for potentiating selection of mutations ascribed to SP resistance. The SP resistance potential detected in this study, caution on its useful therapeutic life as an interim first-line drug against malaria in Tanzania.

DECLARATION

I, Erasto Vitus Mbugi do hereby declare to the Senate of Sokoine University of Agriculture that, this dissertation is my own original work and that it has neither been submitted nor is concurrently submitted for a degree award at any other University.

Signature.....

Date.....18/11/2004.....

COPYRIGHT

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my almighty God who made me alive and healthy for the whole period of my study and presently. Indebted appreciation be to Dr S.T. Balthazary of Sokoine University of Agriculture (SUA) and Dr. H. Mshinda of Ifakara Health Research and Development Centre (IHRDC) whose initiatives implanted me to carry my research work successfully at IHRDC Molecular Epidemiology Laboratory. My idiosyncratic thanks are due to my Principal supervisor, Prof. B.M. Mutayoba who felt pompous with delighted outcome of this work. His parental guidance, close supervision, available and reliable assistance from coursework towards triumph of this research work makes me feel privileged having been supervised by him. I also wish to address my heartfelt thanks to Dr. S.T. Balthazary and Dr. H. Mshinda for their co-supervisory work and encouragement during my coursework and research work.

Great thanks to the Head, Department of Veterinary Physiology, Biochemistry, Pharmacology and Toxicology, and all staff members in the department for their academic and social assistance and co-operation during my studies. Their moral support they delivered to me has rendered this work sound. Exclusive appreciation is to Dr. P. Mlay, whose teaching, advice and guidance during coursework lead me to build capacity in research proposal development and improve my report writing skills. This portion of acknowledgement won't be complete if I forget the tireless unlimited assistance of Dr. Magwisha in various computer graphics and other computer writing skills, which made me push faster in writing this dissertation. I am also pleased to address the high contribution of Prof. P.S. Gwakisa in boosting my

knowledge in molecular biology and immunology, which was the key of this research work. I further thank my classmate Justice John Muumba for his incomparable majesty he showed when we were together.

Special acknowledgement is due to Directorate of IHRDC, Dr Mshinda in particular for providing the samples used in this study and allowing and providing all facilities for carrying this research at IHRDC. I am also obliged to Multilateral Initiative on Malaria (MIM) Project, which funded the sample collection and allowed using their samples for this particular study. The co-operation delivered by members of Molecular Epidemiology Laboratory and all other IHRDC staff members is unprecedentedly high and excellent. To mention few without order of preference, I wish to appreciate the contribution of Allen Malisa, Kefas Mugittu, Selina Churu, Valeriana Mayagaya, Honorath Urasa, John Wigayi, Ezra Mrema, Joseph Paschal Mugasa, Abdallah Mkopi and my fellow student Winfrida Kidima. The moral and scientific support they brought made me pursue my research work with maximum comfort and confidence.

I also show gratitude to Muhimbili University College of Health Sciences (MUCHS) through its Malaria Vaccine Initiative Project and DAAD for sponsoring this study. Distinctive admiration is due to Dr. T.B. Nyambo and other colleagues at Biochemistry Department (MUCHS) for motivation and facilitation of my studies at this level.

and sacrifices for my education. I feel honored to have such parents who knew the importance of getting me to school. My mother in particular, despite having no formal education, she managed to teach me how to read and write, right at home. The co-operation I received from my fellow postgraduate students is highly appreciated.

DEDICATION

This work is specially dedicated to my mother, Esterina Chogimembe Kisinda and my late father, Vitus Lyambangi Gonelimwani Temigunga Mbugi for sending me to school and primarily facilitating my academic performance.

TABLE OF CONTENTS

ABSTRACT ii

DECLARATION iv

COPYRIGHT v

ACKNOWLEDGEMENT vi

DEDICATION ix

TABLE OF CONTENTS x

LIST OF TABLES xiii

LIST OF FIGURES xv

LIST OF APPENDICES xvii

ABBREVIATIONS AND SYMBOLS xviii

CHAPTER ONE 1

1.0 INTRODUCTION 1

CHAPTER TWO 8

2.0 LITERATURE REVIEW 8

2.1 Background information 8

2.2 The life cycle 8

2.3 Merozoite surface protein 2 (MSP2) as molecular markers of multiplicity of infections 12

2.4 Antimalarial Resistance 16

2.5.0 Molecular techniques for studying multiplicity of infections and SP resistance 28

2.5.1 Polymerase Chain Reaction (PCR) 28

2.5.2 Restriction Fragment Length Polymorphisms (RFLPs) 29

CHAPTER THREE **30**

3.0 MATERIALS AND METHOD **30**

3.1 Study Area **30**

3.2 Sample size **30**

3.3 Ethical Considerations **31**

3.4 Written informed consent **31**

3.5 Sample Collection **32**

3.6 Sample analysis **32**

3.7 DNA Extraction **33**

3.8.0 PCR-RFLP Analysis **36**

3.8.1 Amplification of parasite's DNA by PCR **36**

3.8.1.1 Amplification of the MSP2 gene **36**

3.8.1.2 Amplification of the drug target genes **37**

3.8.2 Restriction enzyme digestion **38**

3.9.0 Electrophoresis **39**

3.9.1 Agarose gel electrophoresis **39**

3.9.2 Polyacrylamide gel electrophoresis **39**

3.9.3 Documentation and storage of information **40**

3.9.4 Statistical Analysis **40**

CHAPTER FOUR **42**

4.0 RESULTS **42**

4.1 Clinical results **42**

4.2.0 PCR amplification of molecular markers for multiplicity of infections (MSP2 genes) and drug resistance (DHFR and DHPS genes) **44**

4.2.1 PCR amplification of the MSP2 gene 44

4.2.2 PCR amplification of the DHFR and DHPS genes 46

4.2.3.0 RFLP analysis 52

4.2.3.1 Multiplicity of *P. falciparum* infections 52

4.2.3.2 Drug resistance in *P. falciparum* infections 60

CHAPTER FIVE 77

5.0 DISCUSSION 77

5.1 Clinical data 77

5.2 Multiplicity of infection 78

5.3 Drug resistance target genes 85

CHAPTER SIX 96

6.0 CONCLUSIONS AND RECOMMENDATIONS 96

6.1 Conclusions 96

6.2 Recommendations 98

7.0 REFERENCES 99

8.0 APPENDICES 119

LIST OF TABLES

Table 1: Polymorphic genes investigated, mutation sites, primers and primer sequences, PCR reaction conditions, restriction enzymes and control DNA used in this study.....35

Table 2: The trend of clinical response to the study drug, treatment failures, exclusions and loss during follow up (Day 3).....43

Table 3: The trend of clinical response to the study drug, treatment failures, exclusions and loss during follow up (Day 7).....43

Table 4: The trend of clinical response to the study drug, treatment failures, exclusions and loss during follow up (Day 14).....44

Table 5: Frequency of different MSP2 alleles of *P. falciparum* found in this study in 101 PCR-RFLP successful samples of children from Mlimba.....56

Table 6: Overall frequency of multiplicity of infections of the MSP2 genotypes of the *P. falciparum* 3D7 and FC27 allelic families from Mlimba 57

Table 7: MSP2 PCR-RFLP product detection from fifteen follow-up samples analysed post-medication in this study.....59

Table 8: Proportions of point mutations on DHFR and DHPS gene related to parasite resistance to antifolates.....69

Table 9: Overall proportions of known DHFR and DHPS mutations of investigated in this study.....71

Table 10: Various DHFR/DHPS combinations obtained from restriction analysis to determine point mutations in these genes responsible for SP resistance...72

Table 11: Proportions of mixed DHFR/DHPS genotypes detected in this study.....74

Table 12: Trend of PCR-RFLP product as detected for drug resistance markers from fifteen follow-up samples analysed post-medication in this study.....76

LIST OF FIGURES

Figure 1: The general Life Cycle of *Plasmodium species* causing human malaria.....9

Figure 2: Map of Tanzania showing geographical location of study sites, SP resistance, and prevalence of *Plasmodium falciparum* DHFR and DHPS genotypes.....19

Figure 3: The proposed *de novo* (solid arrows) and salvage pathways (dashed arrows) for tetrahydrofolate in *P. falciparum*.....22

Figure 4: Proportion of point mutations related to resistance to group I antifolates in different regions of Africa (percentage showing proportion of mutation-positive samples from every geographical region).....26

Figure 5: Proportion of point mutations related to resistance to group II antifolates in different regions of Africa (percentages showing proportion of mutation-positive samples from every geographical region).....27

Figure 6: Representative agarose gel showing PCR products from amplification of MSP2 gene using S2/S3 and S1/S4 primers.....45

Figure 7: MSP2 gene PCR products run on 2% agarose gel.....46

Figure 8: Representative DHFR gene PCR amplified fragments (using F + M4 primers), separated on 2% agarose.....48

Figure 9: A representative 2% agarose gel showing PCR amplicons resulted from amplification of DHFR gene with F + M3 primers.....49

Figure 10: A 2% agarose gel showing representative PCR amplification of DHPS gene using K + K/ primers.....50

Figure 11: Agarose gel showing representative PCR amplification of the DHPS gene using L +L/ primers.....51

Figure 12: Polyacryamide gel (10%) showing MSP2 RFLP products.....53

Figure 13: A 10% representative PAA gel for the MSP2 PCR-RFLP showing different restriction patterns in multiple infections.....54

Figure 14: Venn diagram showing distribution of MSP2 allelic families among isolates.....56

Figure 15: RFLP with *Alu*I and *Xmn*I discriminating the wild type, mutant and mixed genotypes.....61

Figure 16: *Alu*I and *Xmn*I representative restriction digests separated on 10% PAA gel.....62

Figure 17: *TSP 509*I and *Dra*I DHFR representative restriction digests for codons 51 and 164, respectively.....63

Figure 18: *BstU*I and *Ava*II restriction digests separated on 10% PAA gel.....66

Figure 19: *Fok*I restriction digestion showing wild type and mutant genotypes on 10% PAA gel.....67

LIST OF APPENDICES

Appendix 1: WHO-set criteria for the study and for participating patients used in the study as adopted from MIM/TDR Guidelines (2001).....119

Appendix 2: Details of diverse number of MSP2 genotypes of *P. falciparum* 3D7 allelic family from Mlimba.....126

Appendix 3: Details of the MSP2 genotypes of the *Plasmodium falciparum* FC27 allelic family from Mlimba.....127

Appendix 4: Summary of multiplicity of infections determined in this study.....128

ABBREVIATIONS AND SYMBOLS

%	Percentage
<	Less than
>	Greater than
µg/ml	microgram per millilitre
µl	Microlitre
µM	Micromoles
^{0}C	Degrees centigrade
A437G	Substitution of glycine for alanine at codon 437
A581G	Substitution of glycine for alanine at codon 581
ACR	Adequate Clinical Response
ADRN	Antimalarial Drug Resistance Network
Ala	Alanine
APS	Ammonium per sulphate
Arg	Arginine
Asn	Asparagine
Bp	Base pairs
BSA	Bovine Serum Albumin
C59R	Substitution of arginine for cysteine at codon 59
CDC	Center for Disease Control and Prevention, USA
CQ	Chloroquine
D0	Day zero
D14	Day fourteen
D3	Day three

D7	Day seven
DHF	7, 8-dihydrofolate
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate synthase
DIC	Disseminated intravascular coagulopathy
DNA	Deoxyribonucleic Acid
DNTPs	Deoxyribonucleotide Triphosphates
EDTA	Ethylenediamine Tetraacetic Acid
ETF	Early Treatment Failure
Glu	Glutamate
GLURP	Glutamate-rich proteins
Gly	Glycine
GPI	Glycosylphophatidylinositol
HCl	Hydrochloric Acid
I	Isoleucine
i.e.	That is
I164L	Substitution of leucine for isoleucine at codon 164
IHRDC	Ifakara Health Research and Development Centre
Ile	Isoleucine
K540D	Substitution of glutamate for lysine at codon 540
Kb	Kilo base
KCl	Potassium Chloride
KDa	Kilodalton
Km	Kilometre(s)

L	Leucine
LapDap	Chlorproguanil-dapsone
Le	Leucine
LTF	Late Treatment Failure
Lys	Lysine
mg/dL	milligram per decilitre
$MgCl_2$	Magnessium Chloride
MIM	Multilateral Initiative on Malaria
min	Minutes
ml	Millilitre
Mm	Millimole
MOH	Ministry of Health, Tanzania
MOI	Multiplicity of infection
MSP1	Merozoite Surface Protein 1
MSP2	Merozoite Surface Protein 2
MUCHS	Muhimbili University College of Health Sciences
N51I	Substitution of isoleucine for asparagine at codon 51
NIMR	National Institute for Medical Research, Tanzania
PAA	Polyacryalamide
PABA	para-aminobenzoic acid
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PRF	Patient Record File
RBC	Red blood Cells (erythrocytes)

RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
S108N	Substitution of asparagine for serine at codon 108
SDS	Sodium Dodecyl Sulphate
Sec	Second(s)
SMP	Sulphamethopyrazine-pyrimethamine
SP	Sulphadoxine-pyrimethamine
STI	Swiss Tropical Institute
SUA	Sokoine University of Agriculture
Taq	Thermus aquaticus
TBE	Tris, Borate and EDTA
TDR	WHO Special Programme for Research & Training in Tropical Diseases
TEMED	N, N, N', N', -tetramethylethylenediamine
THF	5, 6, 7, 8-tetrahydrofolate
U	Units
U/µl	Units per microlitre
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volts
v/v	volume by volume
w/v	Weight by volume
WHO	United Nations World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

Falciparum malaria is predominantly the cause of more than 120 million clinical cases and over one million deaths occurring in the world each year (WHO, 2003). Eighty per cent of the cases occurs in tropical Africa, where malaria accounts for 10% to 30% of all hospital admissions and is responsible for 15% to 25% of all deaths of children under the age of five (WHO, 2003). In addition, around 800,000 children under the age of five die from malaria every year, making this disease one of the major causes of infant and juvenile mortality. In pregnant women, the disease is responsible for a substantial number of miscarriages and low birth weight babies. Inadequate health services and poor socio-economic conditions aggravate the problems of controlling the disease in malaria-endemic countries. Travellers from malaria-free areas entering malaria endemic areas are especially severely afflicted with the disease because of their low or no immunity to the disease (WHO, 2003). On the other hand, migratory populations are considered to play a great role in malaria transmission due to their movement hence introducing the disease to previously disease-free areas (Philips, 2001). Malaria therefore has social consequences and is a heavy burden on economic development and hence control of the disease is a positive precursor to development. Failure to control the disease undoubtedly will have negative effects on country's developments in tourism, agriculture and other sectors. In Tanzania for example, 33% of the hospital outpatient is due to malaria thus being a leading cause of outpatient attendance (Ministry of Health, Statistics Abstract, 1998; Winstanley *et al.*, 2002).

Malaria is caused by protozoan parasites of the genus *Plasmodium* of which *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* are most important. However, *Plasmodium falciparum* is the most widespread and dangerous of the four and if left untreated it can lead to fatal cerebral malaria.

Malaria parasites are transmitted by the female Anopheline mosquitoes and about 380 species of these mosquitoes are known. However, only about 60 are able to transmit the parasite, the commonest species being *Anopheles gambiae* (WHO, 2003).

Malaria has been known since time immemorial, and a variety of drugs have been used to treat the disease, since ancient times (WHO, 2003). Systematic control of malaria started after the discovery of the malaria parasite by Laveran in 1889 (WHO, 2003), and the demonstration by Ross in 1897 that the mosquito was the vector of malaria. These discoveries quickly led to development of control strategies and synthesis of effective and inexpensive drugs of the chloroquine group (Cowman, 1998).

Currently an enormous number of prophylactic and therapeutic drugs have been developed to combat the disease, in addition to quinine and chloroquine. These compounds have been sorted into four main groups (Danis and Bricaire, 2003), probably basing on their different modes of action on the target parasite: (a) 4-aminoquinolines (chloroquine, amodiaquine and piperaquine) (b) Amino-alcohols (quinine, mefloquine, halofantrine and lumefantrine) (c) Artemisinin derivatives

(artesunate, artemether and dihydroartemisinin). The drugs in these three categories block the metabolism of haemoglobin digested by the parasite in its vacuoles, but at different levels of parasite development (d) Antifolates or folate inhibitors (sulphonamides and sulphones (sulphadoxine, dapsone etc.) which block the folate biosynthetic pathway through inhibition of the enzyme dihydropteroate synthase (DHPS). This group also incorporate the pyrimidine derivatives (pyrimethamine, proguanil or cycloproguanil) and atovaquone targeting the same metabolic chain at higher level along the pathway, essentially by blocking the enzyme dihydrofolate reductase (DHFR).

Unfortunately, the ability of the malaria parasite to develop resistance to these new and old drugs has overwhelmed the efforts to get rid off the disease. Development of resistance to formally effective antimalarial drugs stimulated scientists to synthesise drug combinations with different modes of action to combat the disease (Cowman, 1998). Such combinations included the use of sulphonamides potential with proguanil and pyrimethamine (Cowman, 1998). Currently, a combination, sulphadoxine-pyrimethamine (Fansidar, SP) is effective and has replaced chloroquine in most malaria-endemic areas including Africa (Cowman, 1998; WHO, 2003).

Sulphonamides and pyrimidine-derived drugs such as pyrimethamine function by blocking the folate biosynthetic pathway, which is important obligatory pathway for DNA synthesis by the plasmodium. The combination of sulphadoxine and pyrimethamine (SP) has a potent synergistic activity as folate biosynthesis inhibitors. These drugs target on two different enzymes in the same biosynthetic pathway. The

target enzymes for pyrimethamine and sulphadoxine in the parasite are dihydrofolate reductase (DHFR) and the dihydropteroate synthase (DHPS), respectively (Cowman, 1998). The enzyme DHFR catalyses the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) by the cofactor NADPH and DHPS is a critical enzyme in DHF biosynthesis in the same biosynthetic pathway of *Plasmodium falciparum*. Pyrimethamine is a competitive inhibitor of DHFR and sulphadoxine is a competitive inhibitor of para-aminobenzoic acid (PABA) in the synthesis of dihydropteroic acid leading to the synthesis of THF due to structural analogue. The two synergistic biosynthetic steps are essentially important heading towards parasite DNA synthesis. The use of SP drug combination causes a lethal disruption of this folate pathway, required for the synthesis of deoxythymidine monophosphate (dTMP), histidine and methionine. In this way, the parasite DNA synthesis is blocked and parasite death is an outcome.

However, studies have shown that the malaria parasites have also developed resistance to SP, an antifolate combination that proved effective against the parasite for quite sometime (Basco *et al.*, 1998; Warsame *et al.*, 1999; Omar *et al.*, 2001). In addition, low-dose treatment with SP has also been found to have stepwise selection for drug-resistant *falciparum* strains (Kun *et al.*, 1999). This, in most cases has resulted from poor knowledge on the part of the patient on the importance of completing the course of treatment thus keeping the rest of drug for next time in case one falls sick from malaria (Danis and Bricaire, 2003). The resistance to SP by the malaria parasites seems to be largely correlated with mutations in the DHFR and DHPS genes. This rapid spread of resistance may compromise the use of SP, which is

currently, an interim first-line drug for treatment of uncomplicated malaria in Tanzania.

A number of mutations in the DHFR and DHPS enzymes in *P. falciparum* are said to be associated with resistance to SP (Plowe *et al.*, 1997; Cowman, 1998; Ranford-Cartwright *et al.*, 2002). SP resistant strains also occur in patients after treatment with the relevant drug (Wang *et al.*, 1997b; Curtis *et al.*, 1998). Studies by Kun, *et al.* (1999) have shown considerable recrudescent parasites detected following treatment by pyrimethamine, sulphadoxine or both. The resistance to SP by *P. falciparum* strains has been observed to spread very fast partly due to the long half-life of the drug combination (slowly acting), which makes the drug to remain in the body for a long time at sub-optimal amounts. Reports by Winstanley *et al.* (2002) and Plowe (2003) indicated that despite the pyrimidine-derived antimalarial combinations (SP and chlorproguanil-dapsone, LapDap) having similar effects on the inhibition of parasite's DHFR enzyme, fewer recrudescences follow LapDap than is the case for SP. The better efficacy and short half-life of LapDap seem to result into less selection for antifolate–resistant parasites compared with SP. Supportive report by Berglez *et al.* (2004) has recently pointed out the long half-life of sulphadoxine to contribute to the rapid emergence of drug resistance. The WHO now plans for replacement of sulphadoxine with dapsone but the immediate use of the drug is still questionable as it is thought to have marked adverse effects in people with glucose-6-phosphatase deficiency. Yet LapDap is reported to cause anaemia in field trials (Danis and Bricaire, 2003). Nevertheless, the reported partial cross-resistance between cyloguanil and pyrimethamine (Basco and Ringwald, 2000) and inadequate efficacy of

chloroquine-proguanil combination against malaria parasite (Basco, 2002) raises another confront on the best future antimalarial drug alternative. In addition, unabating political crises in many tropical countries have lead to people buying and using antimalarial drugs without proper prescription thus leading to under-dosage with consequent selection of mutations responsible for SP resistance (WHO, 2003). Ignorance partly plays a role in selection of mutations leading to SP resistance due to uncontrolled and inappropriate use of the drug. In this case, a properly prescribed drug may be misused through patients sharing a single dose in sub-dosage amounts (personal observation). Therefore thinking of malaria being a disease prevailing mainly in poor sub-Saharan countries, production of cheap, simple, reliable, effective and safe antimalarial drugs seem to be of great value. In this context, the cost and safety issues are among challenges facing combination antimalarial therapy in Africa (Plowe, 2003). This reflects that despite production of effective antimalarial drug, it should be safe to users while being available and reliable at prices affordable by the majority.

Molecular markers are potentially powerful public health tools for surveillance of drug resistance. Using molecular diagnostic methods currently available (Plowe *et al.*, 1995; Jelinek *et al.*, 1997), resistant parasites can be detected. These molecular tools are based on the detection by Polymerase Chain Reaction (PCR) of point mutations in the parasite genes followed by restriction digestion of amplicons by Restriction Fragment Length Polymorphisms (RFLPs). These methods enable monitoring of frequencies of the mutations in the local population of parasites (Ranford-Cartwright *et al.*, 2002). In addition, the combined techniques PCR-RFLP also allow predictions

on the spread of drug resistance (Plowe *et al.*, 1995; Ranford-Cartwright *et al.*, 2002). Epidemiological studies using molecular typing have therefore been particularly productive in elucidating the diversity and the significance of multiplicity of infection of the malaria parasite. These molecular techniques have been validated and are used in monitoring resistance to antimalarials (Jelinek *et al.*, 1997; STI Report, 2000).

The general objective of this study was to investigate the multiplicity of infections and drug resistance to sulphadoxine-pyrimethamine (SP) in *Plasmodium falciparum* malaria using PCR-RFLP techniques.

The specific objectives were:

(i) To establish the parasite diversity and enumerate multiplicity of infections in sampled population in Mlimba Division by using MSP2 genes.

(ii) To establish the level of recrudescence and new infection in SP treated individuals using MSP2 genes.

(iii) To assess the level of genetic resistance to SP by the PCR-RFLP assay basing on the DHFR/DHPS gene mutations as drug targets.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background information

Malaria has been and still is the cause of high human morbidity and mortality in malaria endemic areas in the world. Although the disease has been eradicated in most temperate zones, it continues to be endemic throughout much of the tropics and subtropics (WHO, 2003). Forty percent (40%) of the world's population lives in malaria-endemic areas with 90% of deaths occurring in sub-Saharan Africa (WHO, 2003). In these areas epidemics have devastated large populations. In addition, malaria poses a serious barrier to economic progress in many developing countries (WHO, 2003).

A number of protozoan parasites of the genus *Plasmodium* are known to cause malaria. Four species are particularly important etiological agents for human malaria. These include *Plasmodium falciparum*, *Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae*. However, *P. falciparum* is the most widespread and deadly.

2.2 The life cycle

The malaria parasite like other members of apicomplexa have a complex life cycle pigeonholed by three distinct processes: sporogony, merogony and gametogony (Fig 1). *P. falciparum* life cycle involves an insect vector (mosquito) and a vertebrate host (human). Humans acquire infection when sporozoites are injected with the saliva

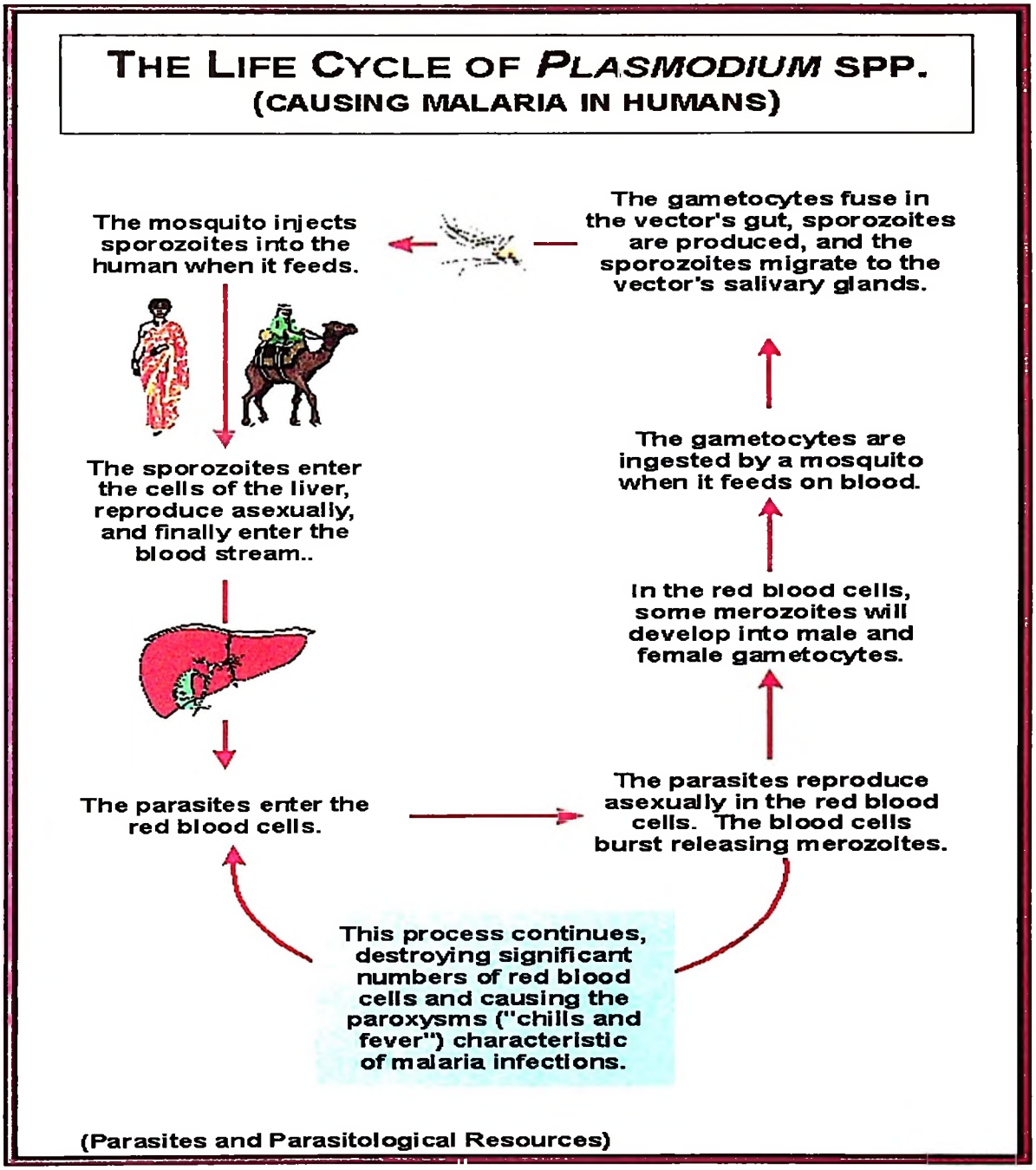

Figure 1: The general Life Cycle of *Plasmodium species* causing human malaria

Source: http://www.biosci.ohio-state.edu/~parasite/lifecycles/plasmodium_lifecycle.html

during mosquito feeding on host's blood. The sporozoites enter the circulatory system and within 30-60 minutes invade the liver cells. Host cell entry, is facilitated by the apical organelles. After invading the hepatocyte, the parasite undergoes an asexual replication. This replicative stage is often called exoerythrocytic (or pre-erythrocytic) schizogony. During schizogony the parasite undergoes multiple rounds of nuclear division without cytoplasmic division followed by a budding, or segmentation, to form progeny. The progeny, called merozoites, are released into the circulatory system following rupture of the infected host hepatocytes and immediately invade erythrocytes. The merozoites recognise specific proteins on the surface of the erythrocyte and actively invade the cell. Inside erythrocytes a series of asexual events (3 – 5 rounds) takes place involving erythrocytic schizogony with differentiation of late schizonts to individual merozoites. The host erythrocyte ruptures and releases the merozoites. These merozoites invade new erythrocytes and initiate another round of schizogony. The blood-stage parasites within a host usually undergo a synchronous schizogony. The simultaneous rupture of the infected erythrocytes and the concomitant release of antigens and waste products accounts for the intermittent fever paroxysms associated with malaria. Blood stage schizogony in *P. falciparum* differs from the other human malarial parasites in that trophozoite- and schizont-infected erythrocytes adhere to capillary endothelial cells and are not found in the peripheral circulation. This sequestration is associated with severe cerebral malaria (Wiser, 2004) and most recrudescence observed in longitudinal epidemiological studies (Contamin *et al.*, 1995).

Simultaneous to the schizogony some of the parasites take an alternative route undergoing a sexual cycle and terminally differentiating into either micro- or macrogametocytes. Ingestion of these micro- and macrogametocytes by the mosquito induces gametogenesis with differentiation to microgametes (male) and macrogametes (female). In the vector host the highly mobile microgametes fuse with macrogametes to form a zygote. The zygote develops into ookinete, which is invasive stage in the mosquito and will traverse the gut epithelium to the extracellular space where it differentiates to oocyst. The oocysts undergo an asexual replication (sporogony), which culminates in the production of several thousand sporozoites under favourable conditions. Upon maturation the oocyst ruptures and releases the sporozoites, which cross the basal lamina into the hemocoel (body cavity) of the mosquito. These motile sporozoites specifically recognise and invade the salivary glands traversing epithelial cells and coming to lie within its lumen. The sporozoites will be induced into the vertebrate host as the mosquito takes a blood meal, and thus reinitiating the infection in the vertebrate host.

During schizogony in the human host, the parasite antigenic surface proteins, which aid the parasite to invade host cells, develop. The most common being the merozoite surface protein 1 (MSP1) and merozoite surface protein 2 (MSP2). The MSP2 support the parasite to anchor on the surface of erythrocytes through specific receptors (e.g. glycophorins, sialic acid). The gene involved in the synthesis of MSP2 (the MSP2 gene) is highly polymorphic and has been typed by PCR-RFLP techniques (Felger *et al.*, 1999; Irion, 2000) and found to be useful in characterising the parasite and enumerating variety of parasite clones concurrently infecting the

patient at one particular time. Studies on MSP2 have made it possible to discriminate recrudescent and new infections in drug trials (Irion *et al.*, 1998) thus the base for its exploitation in this present study.

2.3 Merozoite surface protein 2 (MSP2) as molecular markers of multiplicity of infections

Polymorphism and antigenic variation are common survival strategies of the malaria parasites in human populations. Polymorphism is defined as the expression of distinct alleles of a gene at a single gene locus in different clones of an organism, parasite, whereas variation shows the ability of a clonal population to switch the antigenic phenotype with unchanged genotype. Allelic polymorphism is usually a between-host survival strategy, providing an individual pathogen with maximum fitness for successful infection of its host (Reeder *et al.*, 1996).

Individuals living in areas where *P. falciparum* is endemic experience numerous episodes of infection. These episodes may or may not be symptomatic, with the outcome depending on a combination of parasite factors (the structure of the parasite population and the survival strategies of the parasite) and host factors reflected by the immune response, and the implications of interventions (Hoffman *et al.*, 2001). One parasite factor is believed to be the particular alleles of several parasite proteins to which the host is capable of mounting protective immune responses (Weisman *et al.*, 2001). Among most important surface proteins expressed by the blood-stage parasite to which host's immune response is built are merozoite surface protein 1 and 2 (MSP1 and MSP2) and glutamate–rich proteins (GLURP). These parasite surface

proteins are polymorphic and have antigenic role to the parasite survival (Aubouy *et al.*, 2003b).

Polymorphic antigens have been described in several parasite life cycle stages but are particularly a feature of the antigens associated with the surface of the asexual blood-stage merozoites, MSP2 (Scythe *et al.*, 1990; Felger *et al.*, 1999). The MSP2 is a 45- to 52-kDa integral membrane glycoprotein encoded by a single-copy gene, and is anchored on the surface of the merozoite by a glycosylphosphatidylinositol (GPI) moiety. MSP2 sequences usually are assigned to one of two families, FC27 and IC-1/3D7, on the basis of the non-repetitive sequences (Fenton *et al.*, 1991; Smythe *et al.*, 1991; Snewin, 1991). Sequencing of MSP2-alleles and PCR-RFLP from a large number of parasite isolates from different geographical locations has shown a virtually dimorphic structure of the molecule (Felger *et al.*, 1999).

The finding of structural identity between MSP2 alleles isolated from human hosts geographically and chronologically separated (Snounou *et al.*, 1999; Weisman *et al.*, 2001) is consistent with the idea that the evolution of MSP2 variants may be limited by functional compulsion. In addition, Weisman *et al.* (2001) reported to have identified the first homologous gene for a *Plasmodium species* (*P. reichenowi*) other than *P. falciparum* showing almost identical conserved regions. The fact that the degree of antibody reactivity to MSP2 is sequence dependent (Ranford-Cartwright *et al.*, 1996) is indicative of antigenic diversity. This is further complemented by the differences in immune response in that, antibodies that are inhibitory to parasites expressing a particular form of MSP2 do not inhibit parasites expressing a different

form (Saul *et al.*, 1989). Field studies on parasite genomic DNA extracted from infected blood suggest that there is a large repertoire of circulating strains (Eisen *et al.*, 1998; Konate *et al.*, 1999). Much of these data come from various molecular analytical methods such as restriction fragment length polymorphisms (RFLPs) analysis of PCR products and Southern hybridization using MSP2-specific probes, and rely on size differences between repeat regions.

Characterisation of the polymorphic MSP2 antigen has also been used to establish whether a parasitaemia observed after treatment is caused by a recrudescence of resistant parasites or by a new infection (Babiker *et al.*, 1994; Al-Yaman *et al.*, 1997; Felger *et al.*, 1997). This could be an important information when carrying out ***in vivo*** tests, particularly in areas with a considerable amount of transmission where, after a certain time, it is impossible to distinguish between recrudescence and new infection. This antigenic diversity of the parasites renders the highly polymorphic MSP2 gene suitable as a marker for genotyping of *P. falciparum* and enumerating multiple concurrent infections in blood samples and distinguishing individual alleles (Scythe *et al.*, 1990; Felger *et al.*, 1999; Aubouy *et al.*, 2003b). Amplification of MSP2 is achieved by Polymerase Chain Reaction (PCR) with subsequent restriction digests of PCR products by Restriction Fragment Length Polymorphism (RFLP). Genotyping of field isolates is useful in detecting not only the multiplicity of mixed clone infections, but also aids in studying infection dynamics and finding markers for virulence and drug resistance (Felger *et al.*, 1999, Owusu-Agyei *et al.*, 2002). Multiplicity of infections is reported to affect both the prevalence of parasite genetic markers for

antimalarial drug resistance and the risk of clinical disease (Owusu-Agyei *et al.*, 2002).

Restriction digestion of nested PCR products of the MSP2 gene produces two allele-specific fragment patterns. The fragments are obtained from *Hinf*I restriction sites on the dimorphic region of the gene located in the variable non-repetitive region. These *Hinf*I restriction sites are shared by most alleles of the same family. Genotyping of single clone infections is done by checking the *Hinf*I restriction fragment pattern for the presence of the family-specific conserved fragments followed by determination of the allele-specific fragment (Felger *et al.*, 1999). Two *Hinf*I digests belonging to FC27-type and two belonging to 3D7-type alleles result from single restriction digestion of nested PCR products. Fragments of 115 and 137 base pairs (115 and 137 bp) are specifically discriminative conserved fragments for the FC27-type alleles. Two fragments of 70 and 108 bp are specifically of 3D7-type alleles. Discrimination of these fragment sizes can easily be achieved on the 10% polyacryamide gel. Precise comparison of consecutive DNA samples is achieved by running side by side the consecutive DNA restriction digests in the same gel.

According to Felger *et al.* (1999) and Irion (2000), restriction analysis of the MSP2 allelic families need be started with screening of the 137 and 115 base pair (137 and 115-bp) fragments indicating the presence of at least one FC27-type allele. That is done along with the screening of 70-bp and 108-bp fragments indicative of the presence of at least one or more 3D7-type alleles. Secondly, identification of the 96-bp fragment follows which indicates the presence of at least one FC27-type allele

with more than one 96-bp repeat. Then determination of fragments of size between 150 and 300-bp which in most cases are of FC27-type allele representing the large number of internal *Hinf*I fragment of FC27-type alleles with several 36-bp repeats. The central *Hinf*I fragments of these alleles have fixed sizes (Irion *et al.*, 1997). The remaining high molecular fragments in general are of 3D7-type allele, and very variable in size. For instance, fragments of sizes 115, 137 and say 162 bp indicative of FC27-type allele and 70, 108 and 400 bp belonging to 3D7-type allele in that order, are therefore expected to be obtained. In both cases the first two fragments are conserved and the latter highly variable. Felger *et al.* (1999) identified 9 of the known 41 MSP2 genotypes of the *P. falciparum* 3D7 allelic family and 41 of FC27 allelic family in Tanzania. However, Irion *et al.* (1997) reported occurrence of new fragment sizes in *Hinf*I digestion to be uncommon due to recombination of the two allelic families in the parasite population. In addition to *Hinf*I, restriction digestion using *Dde*I, *Rsa*I and *Scr*FI restriction enzymes can be done where ambiguities arises and especially when infections from different blood samples need to be compared between each other (Felger, *et al.* 1999; Irion, 2000). Extra digests considerably increase the discriminatory power of the genotyping scheme and allow the identification of individual alleles.

2.4 Antimalarial Resistance

For many years chloroquine (CQ) has been the official first-line drug for the treatment of uncomplicated malaria in nearly all African countries as it is cheap, effective and safe. In Tanzania chloroquine was the only antimalarial drug available in most health centres and dispensaries together with second and third line therapies

SP or sulfamethopyrazine-pyrimethamine (SMP) and quinine (Ministry of Health, Tanzania, 1998). CQ resistance spread slowly at first, but from the mid-1980s the rate of growth accelerated rapidly in most malaria-endemic areas. Currently the CQ resistance is common in practically all malaria-endemic countries in sub-Saharan Africa, including Tanzania (WHO, 1996). Lookout site surveys by the Ministry of Health, Tanzania in 1999 found CQ treatment failure in between 28% and 72% of patients. The rapid spread of CQ resistance resulted into removal of CQ as the official first-line drug for treatment of malaria in Tanzania and SP was approved instead by August 2001. The drug was approved despite the failure rates of 6% to 34% in clinical trials (Ministry of Health, Tanzania, 1999) due to its relative cheapness as compared to other available antimalarial drugs basing on the fact that malaria is mostly a disease of poor communities of tropical and subtropical countries. To date a change to the use of SP as the first-line drug has already been implemented in other African countries such as Malawi, Kenya, Botswana and South Africa. However, the reported rapid spread of resistance against SP in northern Tanzania (Mutabingwa *et al.*, 2001) and elsewhere in Africa (Jelinek *et al.*, 2002; Kublin *et al.*, 2002; Bwijo *et al.*, 2003) creates a debate on its persistent use as first-line drug versus malaria parasite in the region. The distribution of point mutations on the DHFR and DHPS associated with SP resistance in Tanzania has been recently reported (Fig 2) by Mugittu *et al.* (2004).

Resistance of *P. falciparum* to antimalarial drugs has been reported in other countries where transmission of *P. falciparum* occurs including, Kenya, Malawi and Mali (Diourte *et al.* 1999). A number of point mutations in the dihydrofolate reductase

(DHFR) and dihydropteroate synthase (DHPS) genes of *P. falciparum* have been shown to have an association with resistance to SP. This rapid spread of resistance to SP threatens the utility of the drug that in countries like Tanzania has just been introduced as the first-line chemotherapeutic against the parasite (Diourte *et al.* 1999). To date effective antimalarial chemotherapy using known antimalarial drugs is increasingly becoming difficult due to the spread of multiple drug-resistant strains of *P. falciparum* (Mookherjee *et al.*, 1999). Thus in anticipation of the future deployment of new antifolates, and the continuing use of current antifolates, assessing the distribution of *P. falciparum* DHFR and DHPS genotypes currently prevalent in the field is necessary.

Dihydrofolate reductase (DHFR) enzyme catalyses the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, which is an essential step in the production of methyl groups for nucleic acid biosynthesis. Blocking the folate pathway at DHFR level prevents the biosynthesis of deoxythymidine, methionine, and histidine, which are essential for parasite's DNA synthesis and multiplication (Cowman, 1998). This leads to failure of nuclear division at the time of schizont formation in erythrocytes and liver.

Pyrimethamine and other pyrimidine-derived antimalarial drugs, competitively inhibit the enzyme DHFR in *P. falciparum*. On the other hand, sulphadoxine and other sulphonamides inhibit the utilization of para-aminobenzoic acid (PABA) in the synthesis of dihydropteroic acid, which is also an essential step in a series of events (Figure 3) towards the synthesis of parasite DNA (Mathews *et al.*, 2000; King, 2003).

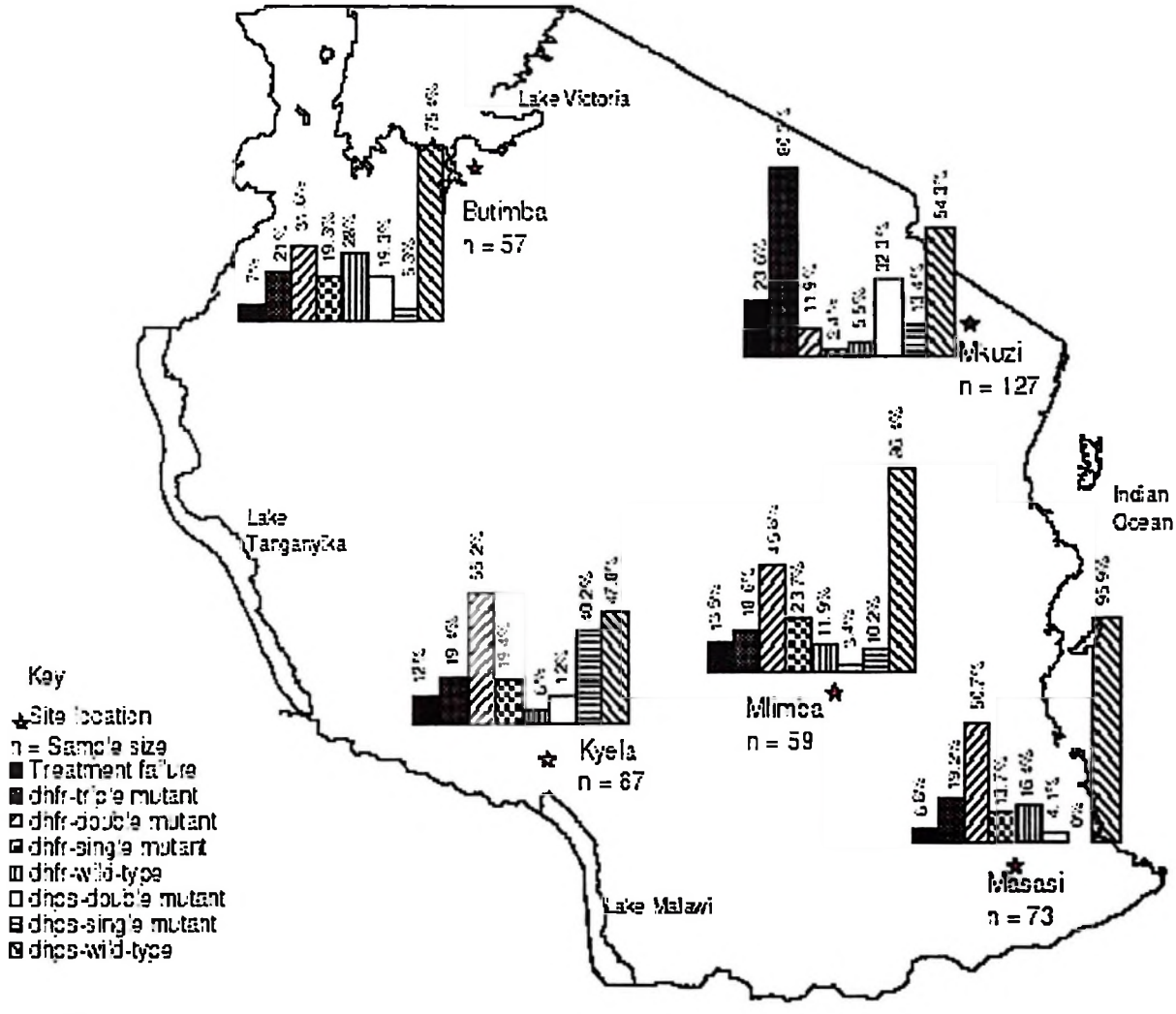

Figure 2

Map of Tanzania showing geographical location of study sites, SP resistance, and prevalence of *P. falciparum* DHFR and DHPS genoptypes. The triple-DHFR (51^{Ile}, 59^{Arg} 108^{Asn}) and double-DHPS (437^{Gly} and 540^{Glu}) mutant genotypes are highly prevalent in Mkuzi, an area with highest level of SP resistance. The rest of the sites (Butimba, Kyela Masasi and Mlimba) have moderate levels of SP resistance and prevalence of this mutant genotype but high prevalence of wild-type genotype.

Source: Mugittu *et al.* (2004)

In addition, sulphonamides are metabolised into sulpha-containing folate analogues that have been shown to compete with dihydrofolate (DHF) and inhibit growth in yeast model (Patel *et al.*, 2003). The combination of pyrimethamine and sulphadoxine thus offers two-step synergistic blockade of plasmodial division. Biochemical analysis, studies of parasites in culture, and fieldwork (Peterson *et al.*, 1990; de Pecoulas *et al.*, 1996; Plowe *et al.*, 1996; Hankins *et al.*, 2001; Nagesha *et al.*, 2001) have shown that resistance to pyrimethamine is strongly associated with a point mutation in the *P. falciparum* DHFR gene. This point mutation encodes a substitution of asparagine for serine at codon 108 thus reducing the binding of pyrimethamine to the active site of the enzyme. Additional point mutations at positions 51, 59 and 164 resulting into substitution of asparagine to isoleucine, cysteine to arginine and isoleucine to leucine respectively, augment the resistance (Mookherjee *et al.*, 1999; Diourte *et al.* 1999; Hankins *et al.*, 2001; Nzila *et al.*, 2002). These additional mutations (Basco *et al.*, 1995; Reeder *et al.*, 1996; Triglia *et al.*, 1997; Rallon *et al.*, 1999) together with mutations in the enzyme dihydropteroate synthase (DHPS) that mediate resistance to sulphonamide-based drugs such as sulphadoxine are said to confer resistance at higher levels of the drug combination.

Mutations occur at various sites on the DHPS gene causing resistance to increasing concentrations of sulphonamides *in vitro* (Wang *et al.*, 1997b). The point mutation at codon 437 of this enzyme gene, however, seems to be especially important for the resistance to sulphadoxine (Curtis *et al.*, 1998; Kublin *et al.*, 1998). This mutational exchange of an alanine (Ala) to a glycine (Gly) alters the affinity of the drug to its potential target (Trigilia *et al.*, 1997). Additional mutations at positions 540 (lysine to

glutamate) and 581 (alanine to glycine) have found to mediate resistance to higher dosages of the drug. Studies on transfection experiments with yeast have shown DHFR mutated genes from *P. falciparum* to induce pyrimethamine resistance to sensitive yeast (Wooden *et al.*, 1997; Cortese and Plowe, 1998). Similarly, when sulphadoxine sensitive *P. falciparum* strains are transfected with mutated DHPS genes, the resulting transfectants have shown to be resistant against sulphadoxine (Triglia *et al.*, 1998).

Wernsdorfer and Noedl (2003) has shown that for marked resistance to SP combination, *P. falciparum* isolates need to exhibit mutations in at least three DHFR and two DHPS codons, where DHFR 108^{Asn} and DHPS 437^{Gly} are practically always present. In similar viewpoint, the quintuple combination of DHFR 51^{Ile}, 59^{Arg} and 108^{Asn} and DHPS 437^{Gly} and 540^{Glu} is considered a strong indicator of resistance to SP (Nzila *et al.*, 2000). Bwijo *et al.* (2003) reported a prevalence of 78% quintuple mutant considered as the most specific molecular marker for SP treatment failure of *P. falciparum* malaria in Malawi.

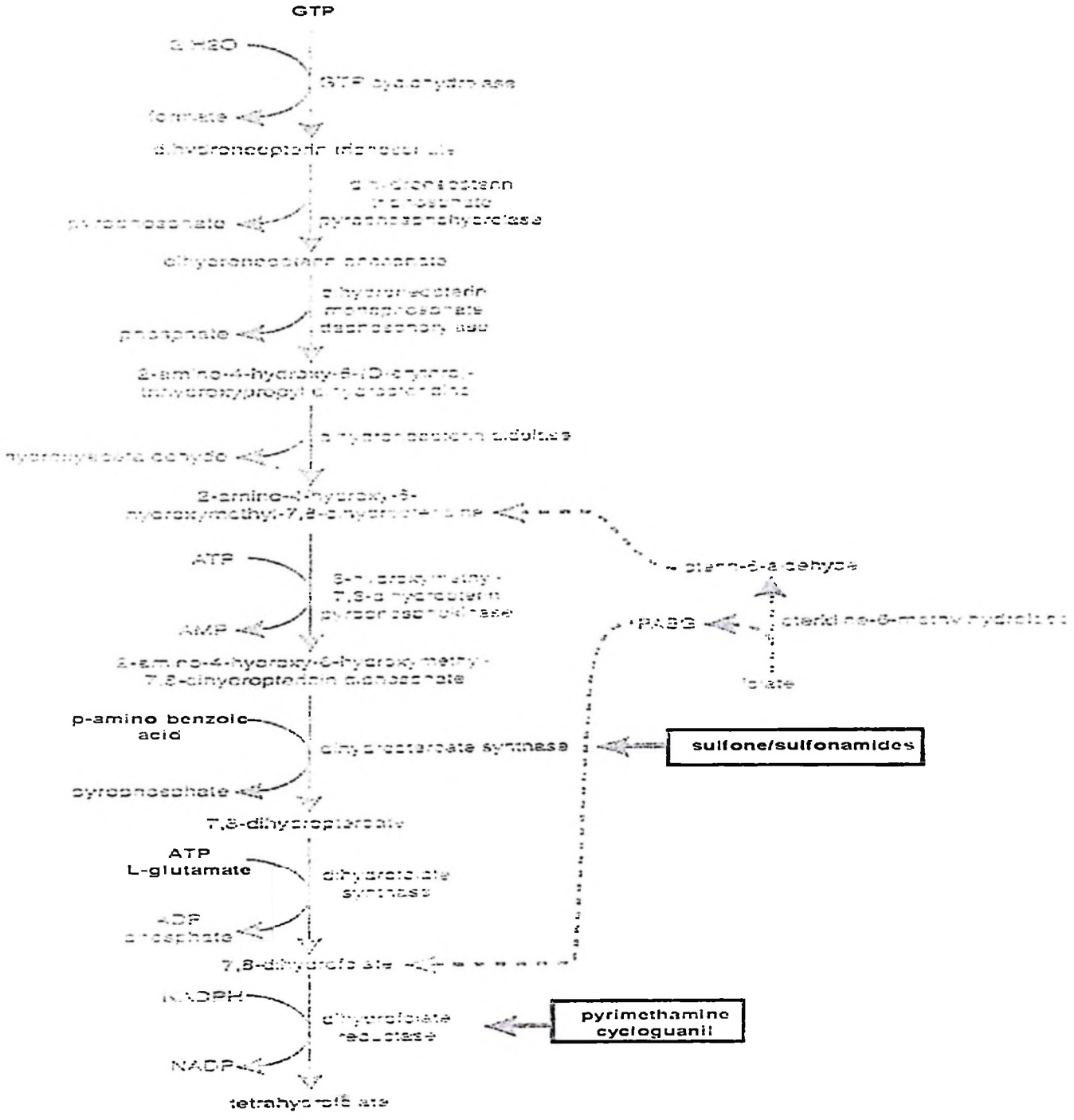

Figure 3:

The proposed *de novo* (solid arrows) and salvage pathways (dashed arrows) for tetrahydrofolate in *P. falciparum*. The site of action of the antimalarial drugs is shown for the sulphones and sulphonamides (DHPS) and also for pyrimethamine and cycloguanil (DHFR).

Source: Cowman (1998)

The presence of quintuple mutation aforementioned is accurately predicted by simultaneous presence of DHFR 59^{Arg} and DHPS 540^{Glu} (Kublin *et al.*, 2002). Further studies (Méndez *et al.*, 2002) has shown that despite presence of 108^{Asn} alone being insufficient to compromise the curative efficacy of SP, it delays parasite clearance and thereby increases gametocyte production and most often, when both 51^{Ile} and 108^{Asn} of DHFR are concurrently present at one particular moment. Mutation occurring at DHFR gene codon 164 resulting in substitution of leucine for isoleucine (I164L) has been observed to be widespread in Southeast Asia (Wang *et al.*, 1997a). This mutation when occurs together with the three other mutations (51^{Ile}, 59^{Arg} and 108^{Asn}) on the DHFR gene, a quadruple mutant strain results that can no longer be treated effectively with SP (Hankins *et al.*, 2001; Aubouy *et al.*, 2003). This is regardless whether mutations on the DHPS gene occur or not. Plowe (2003) reported the co-existence of quadruple mutation in the DHFR gene to confer the high-level resistance to both pyrimethamine and chlorcycloproguanil both of which being effective inhibitor of the parasite's DHFR enzyme. Although SP has been used widely in Africa, the I164L mutation has not yet been reported using standard protocols for the detection of this point mutation in DHFR (PCR-RFLP and sequencing). However, Hastings *et al.* (2002) reported the presence of the I164L mutation in three SP resistant isolates collected from Muheza, Tanzania using the yeast complementation approach which is based on the expression of plasmodium DHFR genes in yeast cells followed by selection of cells expressing highly resistant alleles. Muheza is an area with high resistance to SP (Mutabingwa *et al.*, 2001). The occurrence of this mutation in Africa threatens the useful therapeutic life of SP and renders the new antifolate combination such as chlorproguanil-dapsone (LapDap)

ineffective (Watkins *et al.*, 1997). Studying these mutations and the drug combinations currently used as effective antimalarial drugs in various countries including Tanzania, will enable the finding out of the appropriate drug combination against parasite which are resistant to currently used drugs. Molecular markers available for detection of parasite resistance are indispensable with reasonable reliability (Kublin *et al.*, 2002) towards new drug discovery. Nevertheless treatment outcomes of the new drug may depend on other factors such as host immunity, micronutrient levels and bioavailability and pharmacokinetics of the drug in question. These confounding factors need be considered in these drug studies.

Recent studies by Bousema *et al.* (2003) in Western Kenya have shown a successful treatment with SP against malaria parasite to induce gametocytes. In addition, the gametocyte density was found to be higher upon SP treatment failure. This brings a challenge of concern, as the increased gametocyte prevalence and density after SP treatment failure may increase the spread of SP-resistant strains in the population. A concern is also raised on the use of the antifolate drugs such as trimethoprim and sulphamethoxazole for treatment of other infectious diseases in selection of malaria parasites with mutations in the DHFR and DHPS genes. Kofoed *et al.* (2004) reported that although not linked in the individual child, both bacterial resistance and mutations in the parasitic genes (triple mutations at codons 51, 59 and 108 of DHFR and at 437 of DHPS genes) are common following *E. coli* treatment with trimethoprim/ sulphamethoxazole. Bwijo *et al.* (2003) also reported the selection of DHPS mutations following treatment of infectious diseases other than malaria with sulphonamides in individuals. Therefore, restricting the use of sulphadoxine-

pyrimethamine for the treatment of chloroquine resistant malaria might not be sufficient to prevent the development of resistance in the parasites as long as other antifolate drugs are used extensively. The prevalence of mutations on the DHFR and DHPS genes has been mapped indicating the levels of potential drug resistance in endemic areas world-wide (Nzila *et al.*, 2000; Jelinek *et al.*, 2002; Schelenberg, 2002). Figures 3 and 4 below show the distribution of all known point mutations on the DHFR and DHPS genes in Africa, classified as group I (DHFR inhibitors) and group II (DHPS inhibitors) antifolate resistance related mutations, respectively.

In this study, only four point mutations on the DHFR gene (51^{Ile}, 59^{Arg}, 108^{Asn} and 164^{L}) and three point mutations on the DHPS gene (437^{Gly}, 540^{Glu} and 581^{Gly}) which are considered important cause of parasite resistance against SP in Africa were dealt with.

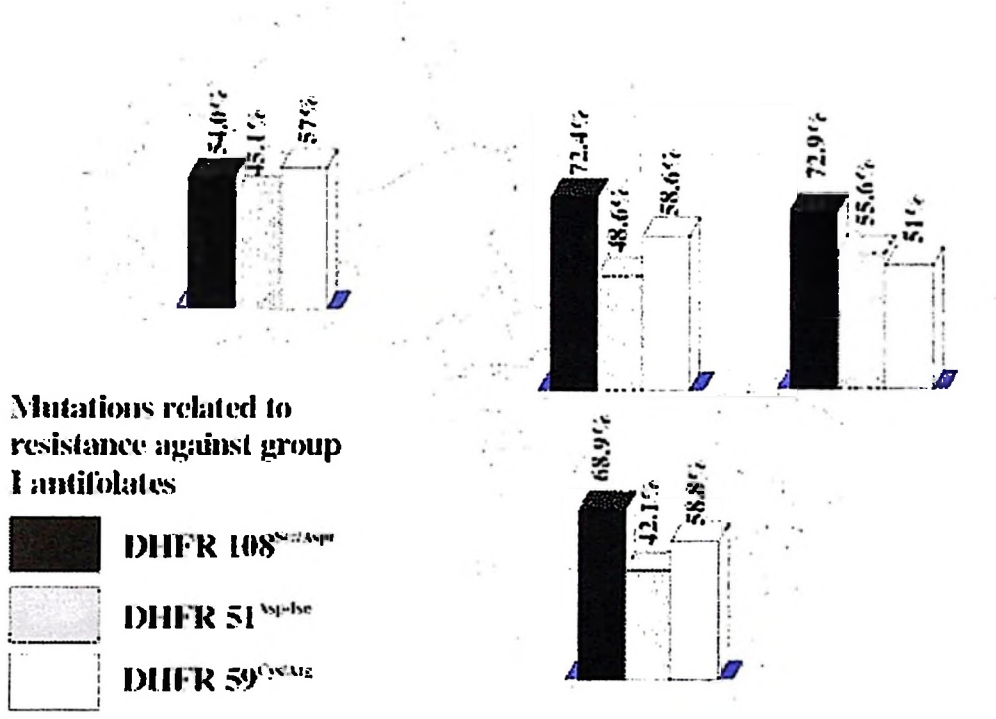

Figure 4:

Proportion of point mutations related to resistance to group I antifolates in different regions of Africa (percentage showing proportion of mutation-positive samples from every geographical region)

Source: Jelinek, *et al.* (2002)

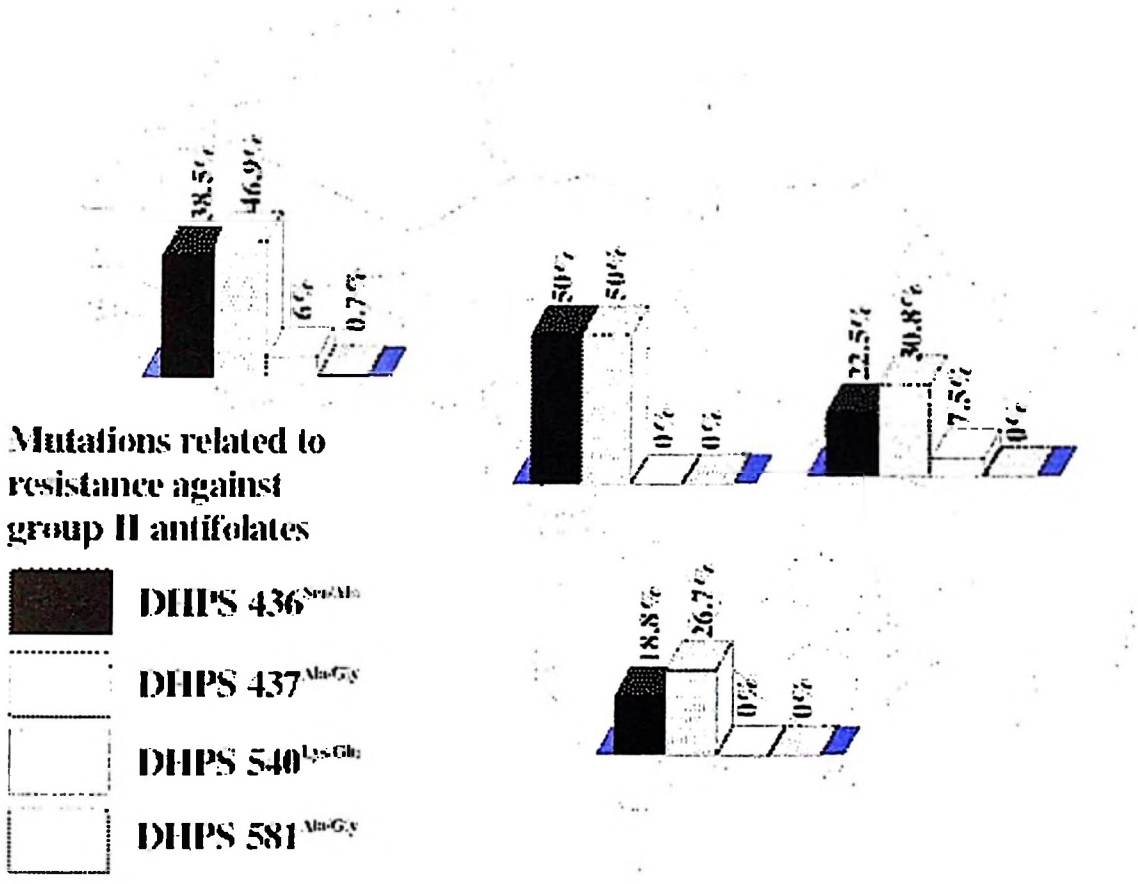

Figure 5:

Proportion of point mutations related to resistance to group II antifolates in different regions of Africa (percentage showing proportion of mutation-positive samples from every geographical region)

Source: Jelinek, *et al.* (2002)

2.5.0 Molecular techniques for studying multiplicity of infections and SP resistance

2.5.1 Polymerase Chain Reaction (PCR)

A variety of methods have been used in molecular characterization of mutations in DHFR and DHPS genes. These include gene sequencing (Basco *et al.*, 2000), allele specific amplification, (Gyang *et al.*, 1992; Wang *et al.*, 1995), single sequence oligonucleotide probing (Roper *et al.*, 2000) and restriction analysis of PCR products (Duraisingh *et al.*, 1998).

Polymerase Chain Reaction has a profound impact upon molecular biology due to its practical applications in gene manipulation through enormous amplification secondary to the aim of altering the amplified sequence. Development of the PCR has provided new ways of studying the malaria parasite, its vector and its host (Greenwood, 2002). Recent studies by Nsobya *et al.* (2004) have shown 47% prevalence of malaria in asymptomatic children detected by PCR as compared to 17% which was obtained by microscopy. The technique has been used to amplify the MSP2, DHFR and DHPS genes for evaluation of multiplicity of infections and drug-resistance conferring mutations in *Plasmodium falciparum* (Kun *et al.*, 1999). Good molecular prints for drug resistance and multiplicity of infection genes are available (Table 1). High sensitivity necessary for detecting very low parasite densities is achieved by performing two consecutive rounds of PCR, a primary and the nested PCR. In the two PCR rounds two different sets of primers are used, the nested primers are normally located within the primary PCR product generated by the outer

primers. In both cases the thermally stable enzyme, Taq polymerase catalysis, increases the efficay of the reactions (Krawetz, *et al.*, 1989, Primrose *et al.*, 2001).

2.5.2 Restriction Fragment Length Polymorphisms (RFLPs)

This technique uses restriction enzymes to cut genomic DNA at precise restriction sites producing a collection of DNA fragments of precisely defined length or size. Electrophoresis separates these fragments, with smaller fragments migrating farther than the larger fragments forming a fingerprint map. Using a DNA size ladder, DNA fragment sizes of interests can be compared (Felger *et al.*, 1999). Specific restriction enzymes (Table 1, next chapter) are used, and were also employed in the present study.

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Study Area

The study was conducted at Ifakara Health Research and Development Center (IHRDC) situated at Ifakara Town in Kilombero District, Morogoro, Tanzania. The IHRDC is currently conducting various research projects on malaria on routine basis using both conventional and molecular-based techniques. The center kindly accepted and offered assistance for carrying out this study. Samples used in this study were previously collected by the centre during the period of January to August 2002 from Mlimba, an area about 150 km from IHRDC. The area is among nine sentinel sites for National Malaria Control Program since 1997. Its human population dynamics is being closely monitored on a monthly basis by the Ifakara Centre Demographic Surveillance System (IC-DSS) since 1996. Essentially, Mlimba is an area along Kilombero River where falciparum malaria is endemic with perennial transmission. Recruitment of patients and sample collection was done by the research team at Mlimba Health Centre, which has well-established health facilities.

3.2 Sample size

Between January and August 2002, about 172 patients consisting of males and females with acute uncomplicated falciparum malaria and aged less than 5 years were initially recruited by the IHRDC staff in this study. This sample size provided enough samples to determine any associations between parasite gene mutations and drug resistance, with an odds ratio of 2.5 or more at the 95% confidence level and power of 80% (EPI Info, CDC, Atlanta). All positive cases were treated with a single dose

of SP scaled by body weight and was given as a tablet containing 500mg of sulphadoxine and 25mg of pyrimethamine (Fansidar®). However some of the patients either were excluded from the study due to failure to comply with criteria for participating in the study as described in the protocol (Appendix 1) or were lost during follow up in the due course of the study. Therefore 141 (82% of all recruited patients) blood samples were available and were used in this study. The samples were collected using the WHO-set criteria (Appendix 1) described in the MIM/TDR guidelines for protocols involving molecular markers for drug resistance and multiplicity of infections (2003).

3.3 Ethical Considerations

The study involved human subjects thus ethical consideration was a prerequisite prior to conducting this research. The clearance certificate for conducting the research was obtained from National Institute of Medical Research (NIMR) ethical committee and Institutional Ethical Clearance was obtained from IHRDC ethical committee. The clinical assessment of antimalarial drug resistance was conducted in accordance with the principles laid down by the World Health Assembly of 1975 on Ethics in Human Experimentation and the Helsinki Declaration (WHO, 1991). The study adhered to the standards established for Good Clinical Practices (GCP) and conformed to the MIM/TDR Standard Operating Procedures (SOP) 2003.

3.4 Written informed consent

Before recruiting patients into the study the IHRDC research team had to obtain an informed consent from participants. A written or witnessed verbal informed consent

was obtained from each prospective patient's parent or legal guardian at the start of the study. Each subject's parent or guardian was informed of the objectives, methods, anticipated benefits and potential hazards of the study. The subject's parent or guardian was informed that he/she is at liberty to abstain from participation in the study and that he/she is free to withdraw the consent of participation at any time without loosing any benefits which the parent/guardian or child are entitled.

3.5 Sample Collection

Samples were collected by the IHRDC through MIM Project. Collection of samples for parasite genotyping was done on filter paper (3MM Whatman). All samples were clearly labeled and identified. The samples on filter papers were put in a dry clean container with desiccant for a minimum of 3 hours to dry. All these procedures were done with gloved hands to avoid any contamination. The research team made follow-ups at day 3 and thereafter weekly for 4 weeks after treatment (Day 0). In addition, further follow-ups were done at any other day if the child was unwell. During all these visits, finger-prick blood was obtained for thick smears on clear microscopic slides and also transferred on filter papers for molecular genotyping. The dried finger-prick blood samples on filter papers were stored in self-sealing plastic bags at room temperature (25^0C) at IHRDC molecular parasitology laboratory to when molecular work was done.

3.6 Sample analysis

Sample analysis was based on the standardised PCR-RFLP techniques following DNA extraction. In this study, PCR-RFLP techniques were used in identifying *P.*

falciparum infection dynamics and investigating mutations in *P. falciparum* populations in Mlimba. The technique was also used to determine whether the infection following SP recovery after medication was a new infection or recrudescent one. Using nested PCR approach (Ranford-Cartwright *et al.*, 1997), the regions of DHFR and DHPS gene containing antifolate resistance-associated point mutations were amplified for RFLP analysis.

3.7 DNA Extraction

The Chelex Method (Plowe *et al.*, 1995) was used for DNA extraction. Essentially a blotted filter paper of each blood sample was cut in approximately 4mm or 5mm depending on the amount of blood stained, and then placed in a 1.5ml Eppendorf tube (BRAND Eppendorf, Gilson, German). To the tube 50µl of 10% saponin and 1ml of 1x phosphate-buffered saline (1x PBS, pH 7.4) were added, inverted several times and stored at 4°C overnight as per protocol. After overnight incubation at 4°C, the tubes were then centrifuged at 14,000 rpm for 1min, and all the supernatant discarded. To each tube, 1ml of 1x PBS (pH 7.4) was added, inverted several times and incubated at 4°C for 80 min (15 min to 2 hrs). The tubes were centrifuged at 14,000 rpm for 2 min and fluid was aspirated to dry the filter paper. A new set of 0.5ml Eppendorff tubes was prepared to which 100µl of sterile water (Sigma®) followed by 50µl of 20% Chelex solution were added. Before DNA extraction, the Chelex solution in the latter set of microfuge tubes was heated to 100°C and the filter papers transferred into heated Chelex solution. The parasite DNA was extracted by incubating the tubes in 100°C heating block for 20 minutes, vigorously vortexing for 30 seconds initially, then few seconds every 3 minutes. After incubation, the tubes

were centrifuged at 14,000 rpm for 2 min. Two sets of tubes were labelled for transferring DNA, the second set for final storage of DNA. This was done by transferring as much solution as possible from the spun tubes to the first set of microfuge tubes, spun again for 10 minutes and then the final white to yellowish supernatant transferred to the final set of labelled tubes. The DNA was stored at -20°C for further analysis.

The extracted DNA was amplified using respective primers (Table 1). Using respective site-specific restriction enzymes (Table 1), the PCR amplicons were digested aiming at obtaining restriction fragments of interest.

Table 1: Polymorphic genes investigated, mutation sites, primers and primer sequences, PCR reaction conditions, restriction enzymes and control DNA used in this study.

Gene	Mutation	Primers	Primer Sequences	PCR conditions	Enzyme digest	Control DNA
MSP2 Primary		S3 S2	5' GAAGGTAATTAAAACATTGTC 3' 5' GAGGGATGTTGCTGCTCCACAG 3'	94°C-3min, 94 °C-30sec, 42 °C-60sec. 65 °C-2min, x30, 72 °C-3min, 4 °C-hold		HB3, 3D7 W2
MSP2 Nested		S1 S4	5' GAGTATAAGGAGAAGTATG 3' 5' CTAGAACCATGCATATGTCC 3'	94 °C-30sec, 50 °C-60sec, 72 °C-2min, x30, 72 °C-3min, 4 °C-hold	*Hinf*I	HB3, 3D7 W2
DHFR Primary		M1 M5	5' TTTATGATGGAACAAGTCTGC3' 5' AGTATATACATCGCTAACAGA3'	94°C-3min, 94 °C-1min, 50 °C-2min, 72 °C-2min, x40, 72 °C-10min, 4 °C-hold		
DHFR Nested	N51I	M3 F/	5'TTTATGATGGAACAAGTCTGCGACGTT3' 5'AAATTCTTGATAAACAACGGAACCTttTA3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72 °C-2min, x35, 72 °C-10min, 4 °C-hold	*Tsp509*I	(+) K1
	C59R	F M4	5'GAAATGTAATTCCCTAGATATGGAATATT3' 5'TTAATTTCCCAAGTAAAACTATTAGAGCTTC3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72 °C-2min, x35, 72 °C-10min, 4 °C-hold	*Xmn*I	(+) T9/96
	S108N	F M4	5'GAAATGTAATTCCCTAGATATGGAATATT3' 5'TTAATTTCCCAAGTAAAACTATTAGAGCTTC3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72 °C-2min, x35, 72 °C-10min, 4 °C-hold	*Alu*I	(+) HB3 (+) T9/96 (+) FCR3
	I164L	M3 F/	5'TTTATGATGGAACAAGTCTGCGACGTT3' 5'AAATTCTTGATAAACAACGGAACCTTTTA3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72 °C-2min, x35, 72 °C-10min, 4 °C-hold	*Dra*I	(+) V1/S (+) HB3 (+) DD2
DHPS Primary		R2 R/	5' AACCTAAACGTGCTGTTCAA3' 5' AATTGTGTGATTTGTCCACAA3'	94°C-3min, 94 °C-1min, 50 °C-2min, 72 °C-2min, x40, 72 °C-10min, 4 °C-hold		
DHPS Nested	A437G	K K/	5'TGCTAGTGTTATAGATATAGGATGAGcATC3' 5'CTATAACGAGGTATTGCATTTAATGCAAGAA3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72 °C-2min, x35, 72 °C-10min, 4 °C-hold	*Ava*II	(+) K1 (+) FCR3
	K540D	K K/	5'TGCTAGTGTTATAGATATAGGATGAGCATC3' 5'CTATAACGAGGTATTGCATTTAATGCAAGAA3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72 °C-2min, x35, 72 °C-10min, 4 °C-hold	*Fok*I	(+) TN-1 (+) VIS (+) W2
	A581G	L L/	5'ATAGGATACTATTTGATATTGGACCAGGATTCG3' 5'TATTACAACATTTTGATCATTCGCGCAACCGG3'	94°C-2min, 94 °C-1min, 45 °C-1min, 72 °C-2min, x35, 72 °C-10min, 4 °C-hold	*Bst*UI	(+) K1 (+) DD2

Source: Lowe *et al.* (1990), Foley *et al.* (1992), Felger *et al.* (1993), Duraisingh *et al.* (1998)

3.8.0 PCR-RFLP Analysis

Assays for MSP2 by PCR-RFLP, was used as standard assays to distinguish new infection from recrudescent parasites. Restriction fragment sizes were estimated by comparison with a DNA size marker (1kb ladder, Gibco BRL Life Technologies). To distinguish a resistant parasite from a new one, samples from longitudinal studies were compared with each other by running side by side on the same gel (Felger *et al.*, 1999; Irion *et al.*, 2000). In addition, the PCR followed by RFLP (PCR-RFLP) protocol was adopted in drug resistance study whereby parasites were examined for antifolate-resistance associated point mutations in the DHFR (chromosome 4) (Duraisingh *et al.* 1998) and DHPS (chromosome 8) genes using a nested PCR-RFLP method as described by Kublin *et al.* (2002).

3.8.1 Amplification of parasite's DNA by PCR

The parasite DNA was amplified in two steps, the primary PCR and the nested PCR the former amplifying the target parasite DNA (outer primers) to initiate the exponential expansion of PCR products and the latter specifically amplifying the shorter parasite DNA fragment where the point mutation occurs (nested primers).

3.8.1.1 Amplification of the MSP2 gene

As pinpointed earlier in this chapter, the parasite DNA was extracted from filter paper using Chelex technique. Genotyping of parasite MSP2 genes involved two primer pairs, S2/S3 as forward and reverse primers in primary reaction and S1/S4 in secondary reaction. The block 3 polymorphic region of MSP2 was amplified by nested PCR. Initial PCR primers corresponded to conserved sequences flanking the

MSP2 gene polymorphic region (Zwetyenga *et al.*, 1998). The nested PCR primers (S1/S4) were then used to amplify the 3D7 and FC27 allelic families of MSP2. For controls, purified genomic DNA from HB3, 3D7, 7G8 and V1S laboratory strains were isolated on similar standard method and used accordingly.

3.8.1.2 Amplification of the drug target genes

In the multiplex parasite DNA amplification of the drug target genes, two primer pairs M1/M5 and R2 + R/ were used as forward and reverse primers in primary (nest I) PCR reaction for the DHFR and DHPS domains, respectively. In secondary (nest II) PCR reaction M3 + F/ and F + M4 were used as forward and reverse primers to amplify the four regions on the DHFR gene where the point mutation was anticipated to occur (Duraisingh *et al.* 1998). On the other hand, K +K/ and L + L/ primers were used to amplify points on the DHPS gene where resistance-associated mutation are said to occur (Kublin *et al.*, 2002). The details of primer sequences, annealing temperatures and controls are shown in Table 1 as described elsewhere (Duraisingh *et al.*, 1998). In both primary and secondary DNA amplification, reaction volumes ranged from 20µl to 30µl. The final concentration of each reagent was 1x PCR reaction buffer (10x PCR buffer – $MgCl_2$, Invitrogen), 1.5 mM $MgCl_2$, 125µM dNTP (Promega, Madison, WI, USA), 250nM primers (QIAGEN, Operon) and 0.02 U/µl Taq Polymerase (Invitrogen). Molecular biology grade PCR water (Sigma) was used as a diluent. The master mix was prepared in a 1.5ml reaction tube and aliquots made in PCR tubes (0.2ml size). To each PCR tube, 5µl of DNA was added in primary reaction and 2µl was re-amplified in the nested PCR reaction. The known purified genomic DNA from HB3, 3D7, W2, K1, T9/96, FCR3 and V1/S laboratory parasite

clones (strains) were used as positive controls and NT (No template, just PCR water) was used as negative control for detection of any possible contamination on DNA samples and for comparison purposes. PCR was performed in a Programmable Thermo Controller, (PTC-100 (TM) MJ Research, Inc., Watertown, MA, USA). Samples with no detectable PCR products were re-examined at least twice starting from the DNA preparation before were declared negative.

3.8.2 Restriction enzyme digestion

The PCR amplicons of the parasite DNA were subjected to site-specific restriction enzyme digestion. Eight different restriction enzymes were used in this study (Table 1). For digestion of MSP2 nested PCR amplicons, *Hinf*I (New England Biolabs) was used. The nested drug target genes PCR amplicons were digested by *TSP509*I, *Xmn*I, *Alu*I (DHFR domain) and *Ava*II, *Fok*I, *Bst*UI (DHPS domain) enzymes, respectively. Restriction digestion was performed in 1.5ml tubes depending on manufacturer's recommendations. Essentially 25µl reaction volume was used containing 1x restriction buffer, varying volume of restriction enzymes and 8µl of PCR product. Each tube containing the enzyme restriction reaction mixture was overlaid with mineral oil and then capped to prevent evaporation. The restriction digestions were carried out overnight at respective temperatures as indicated by the suppliers (New England Biolabs). Essentially, restriction digestions involving the enzymes *Hinf*I, *Xmn*I, *Alu*I, *Ava*II and *Fok*I were incubated at 37^0C overnight. Where *TSP509*I and *Bst*UI were used, the reaction mixture was incubated at 65^0C and 60^0C, respectively. Restriction digestions involving *Xmn*I, in addition required 1x BSA (bovine serum albumin). To stop the reaction, 5µl of 6x loading buffer (Sigma chemicals Co. ST

Luis USA) was added and the reaction mixtures were subsequently preserved at 4^0C before polyacrylamide gel electrophoresis.

3.9.0 Electrophoresis

3.9.1 Agarose gel electrophoresis

Two percent (w/v) agarose gel was prepared by dissolving 2g of ultra PURE agarose powder (Gibco BRL Life Technologies) in 100ml of 1x Tris Borate EDTA (1x TBE) solution. This was a desired gel concentration recommended for electrophoresis of parasite's DNA PCR amplicons (Duraisingh *et al.*, 1998). Five microlitres of 0.5µg/ml ethidium bromide was added as a staining reagent. The mixture was melted at 100^0C and then cooled to 50^0C. The cooled solution was then poured in an electrophoresis casting glass plates and allowed to polymerize for 45 minutes. The PCR amplicons (5µl) were mixed with a 5x loading dye (1/5 dye : DNA ratio) and 3µl of which was loaded onto the gel. A standard molecular marker (1kb DNA marker) was used as a ladder. Electrophoresis of nested PCR products was carried out using 2% agarose gel in a horizontal gel electrophoresis apparatus in 1x TBE buffer. Effective electrophoresis was achieved by applying a direct current of electricity at constant voltage of 10v/cm gel for 45 to 60 minutes. This allowed satisfactory movement and separation of DNA to a desired length in the gel.

3.9.2 Polyacrylamide gel electrophoresis

In performing polyacryamide gel electrophoresis, the procedure described by Sambrook *et al.* (1989) was followed. Electrophoresis of the restriction digests was carried out on 10% polyacryamide gel (PAA) prepared at least a day before and

stored at 4^0C for good resolution (Felger *et al.*, 1999). To prepare a desired percentage gel, appropriate volumes of 40% Acrylamide/Bisacrylamide solution (Q. Biogene), distilled H_2O, 5x TBE (5 M Tris-HCl; 10mM EDTA; 4 M Boric acid) pH 8, 20% (w/v), Ammonium persulphate (APS) and 0.5% (w/v) N, N, N', N'-tetramethylethylenediamine (TEMED) (Gibco BRL Life Technologies) were mixed. The prepared solution was cast onto glass plates to polymerise with relevant comb fixed to produce slots for loading DNA restriction digests. About 32µl of restriction digestion product containing 5µl of loading dye was loaded on the gel for electrophoresis. Loading was done hand in hand with the 1kb DNA Marker (Gibco BRL Life Technologies) as reference for sizing the DNA restriction fragments. Electrophoresis of PAA gel was done in a vertical electrophoresis apparatus (Gibco BRL Life Technologies, Gaithersburg, MD-USA) filled with 1x TBE buffer by applying direct current electricity at constant voltage of 11.25v/cm gel for 2.30 hours. The gel was then stained with 0.5µg/ml ethidium bromide by thorough mixing of 30µl ethidium bromide with 500 ml 1x TBE buffer in a plastic, staining tank.

3.9.3 Documentation and storage of information

Following electrophoresis, the DNA bands were visualised under Ultra Violet trans illuminator light and documentation of the results was done using both Polaroid camera photographing and electronic records using digital cameras.

3.9.4 Statistical Analysis

Data were entered and analyzed using the EPI Info Version 6.04 epidemiological software (Centers for Disease Control and Prevention, Atlanta, GA, USA). This made

possible to estimate the frequency of point mutations on the DHFR and DHPS genes responsible for parasite resistance against SP thus determining the prevalence of these mutations. The prevalence of each family in the MSP2 gene was calculated as the percentage of baseline (D0) samples containing alleles of that particular family (Aubouy *et al.*, 2003b). The distribution of specific families was estimated as the percentage of fragments assigned to one family within the overall number of fragments. The multiplicity of infections and parasite diversity, was found by counting and comparing the number of different alleles within and between the two allelic families basing on the different restriction fragment sizes (Felger *et al.*, 1999). In this context, multiplicity of infection (MOI) was defined as the number of genotypes per infection and was calculated as the highest number of genotypes at the MSP2 locus of the parasite DNA and was the basis for establishment of parasite diversity (Aubouy *et al.*, 2003b).

CHAPTER FOUR

4.0 RESULTS

4.1 Clinical results

Clinical results formed the basis and were used to select samples for the molecular work carried out in this particular study. The clinical data sets were provided by IHRDC and showed significant differences observed during follow up in the study. Essentially, 172 patients were initially recruited between January-August 2002 in the study, but adequate clinical response was observed in 65.1% (112/172) of the recruited patients at day 3 during follow up (Table 2). Early treatment failure was observed in 30.8% (53/172) of the patients. One (0.6%) patient developed severe malaria and was excluded from the study while 6 (3.5%) of patients were lost from the study during follow up. At day 7, 13 (7.7%) patients were lost from the study. Late treatment failure was encountered in 10 (5.9%) of the patients (Table 3). Adequate clinical response was observed in 146 (84.6%) of all patients. A total of 142 patients completed the study. Of these patients, adequate clinical response was observed in 128 (81.1%) patients and 14 (8.9%) was either having recrudescent parasite or new infection (Table 4). No sample was available for one patient for PCR-RFLP evaluation basing on the study objectives. This left a total of 141 samples, which were used in this study. Therefore data presented in this chapter is bases on the 141 samples to which molecular analysis was done. The trends of clinical response to SP treatment by the study drug are summarized in Table 2 – 4.

Table 2: The trend of clinical response to the study drug, treatment failures, exclusions and loss during follow up

Day 3			
Observation	Frequency	Percentage (%)	Cumulative (%)
ACR	112	65.1	65.1
ETF	53	30.8	95.9
EX	1	0.6	96.5
LOSS F/UP	6	3.5	100
Total	172	100	

Key: ACR = Adequate clinical response, ETF = Early treatment failure, EX = Excluded from the study, LOSS F/UP = Lost during follow up

Table 3: The trend of clinical response to the study drug, treatment failures, exclusions and loss during follow up

Day 7			
Observation	Frequency	Percentage (%)	Cumulative (%)
ACR	146	86.4	86.4
LOSS F/UP	13	7.7	94.1
LTF	10	5.9	100
Total	169	100	

LTF = Late treatment failure

Table 4: The trend of clinical response to the study drug, treatment failures, exclusions and loss during follow up

	Day 14		
Observation	Frequency	Percentage (%)	Cumulative (%)
ACR	128	81.1	81.1
LOSS F/UP	16	10.1	91.1
NI	14	8.9	100
Total	158	100	

NI = New infection

4.2.0 PCR amplification of molecular markers for multiplicity of infections (MSP2 genes) and drug resistance (DHFR and DHPS genes)

4.2.1 PCR amplification of the MSP2 gene

A total of 141 samples were analyzed for multiplicity of infections in this study. PCR amplification of the parasite MSP2 gene was done using S2/S3 and S1/S4 as primary and nested primers, respectively. Amplification was repeated at least twice before the sample was declared negative, frequently starting from DNA preparation (Felger *et al.*, 1999). Successful PCR amplification was achieved in 125 (88.7%) out of 141 samples while 16 (11.3%) samples were PCR negative. PCR was essentially done to confirm presence of appropriate DNA fragments before subjecting to RFLP analysis in attempt to establish the concurrent infections per blood sample (multiplicity of infections).

Figure 5 and 6 depicts the PCR amplification of the MSP2 gene using S1/S4 primers of some representative samples. The band sizes of PCR amplicons ranges between 378 and 740 bp.

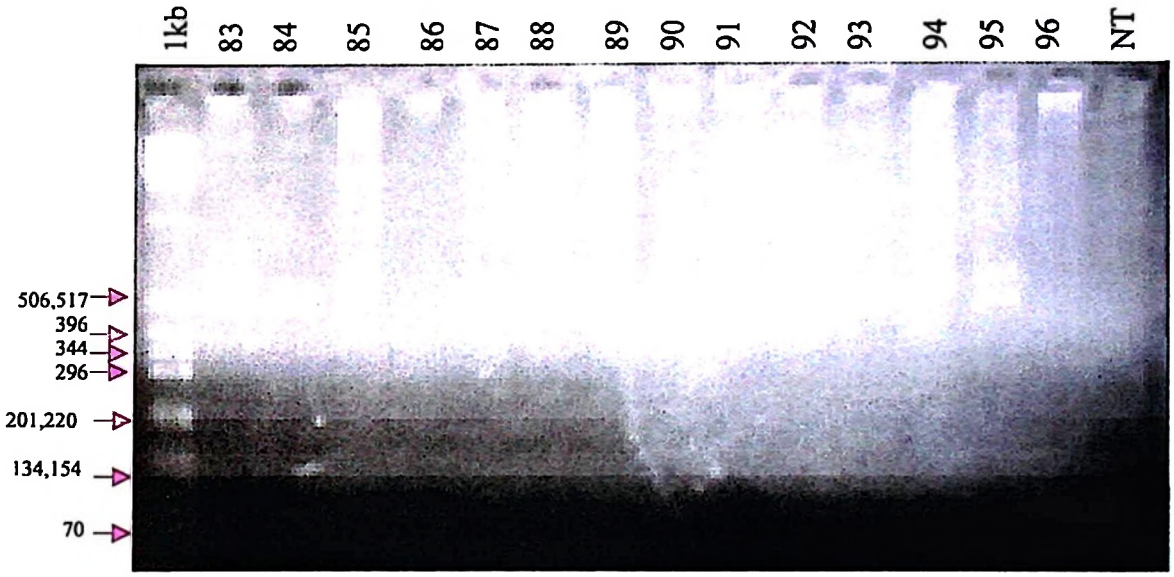

Figure 6

MSP2 gene PCR products run on 2% agarose gel. Numbers 83 to 96 indicate DNA samples. NT = negative control. Samples no. 86 and 96 are negative. PCR band sizes lies between 378 and 740 bp. Positive controls were not working properly, therefore only negative control was used in this particular batch of DNA samples as a guide.

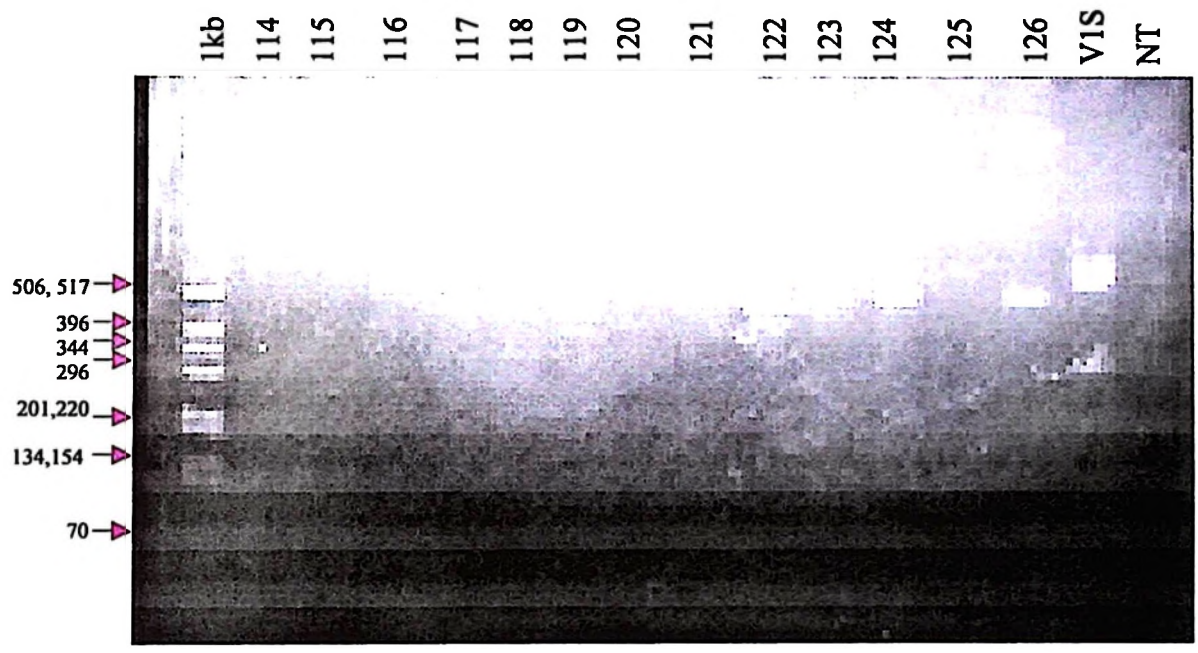

Figure 7

MSP2 gene PCR products run on 2% agarose gel. Numbers 114 to 126 indicate study samples. V1S = positive control and NT = negative control. Samples no. 115, 120 and 125 are negative PCR Band sizes lies between 378 and 740 bp.

4.2.2 PCR amplification of the DHFR and DHPS genes

To amplify the regions on the parasite DHFR gene containing point mutations associated with resistance to SP, M3 + F/ and F + M4 were used as primers. M3 + F/ were used to amplify regions on the DHFR locus containing point mutations at codons 51 and 164 while F+ M4 were used to amplifying regions on the DHFR locus containing point mutations at codons 59 and 108. For the DHPS locus, primers K + K/ were used to

amplify regions on the gene containing point mutations at codons 437 and 540. Where as, L + L/ primers were used to amplify regions on the DHPS locus containing point mutations at codon 581. The primers and primer sequences are shown in Table 1.

Since different primers were used to amplify regions in the DHFR and DHPS containing various point mutations associated with parasite resistance to SP, different DNA fragments with different band sizes were obtained (Fig 8 – 11). The fragment sizes were estimated in as previously described by Duraisingh *et al.* (1998). F + M4 amplification produced DNA amplicons of about 326 bp (Fig 8) while DNA amplicons of approximately 522 bp band size were obtained following DNA amplification by M3 + F/ primers (Fig 9). PCR amplification using K + K/ (Fig 10) and L + L/ primers (Fig 11) produced fragments of 438 and 161 bp, respectively on 2% agarose gel.

Of 141 samples analyzed 120 (85.1%) were successfully amplified using M3+ F/ primers while 96.5% (136/141) of samples were successfully amplified using F + M4. About 93.6% (132/141) samples were successfully amplified using K + K/ primers. Amplification of DNA samples using L +L/ produced 94.3% (133/141) success.

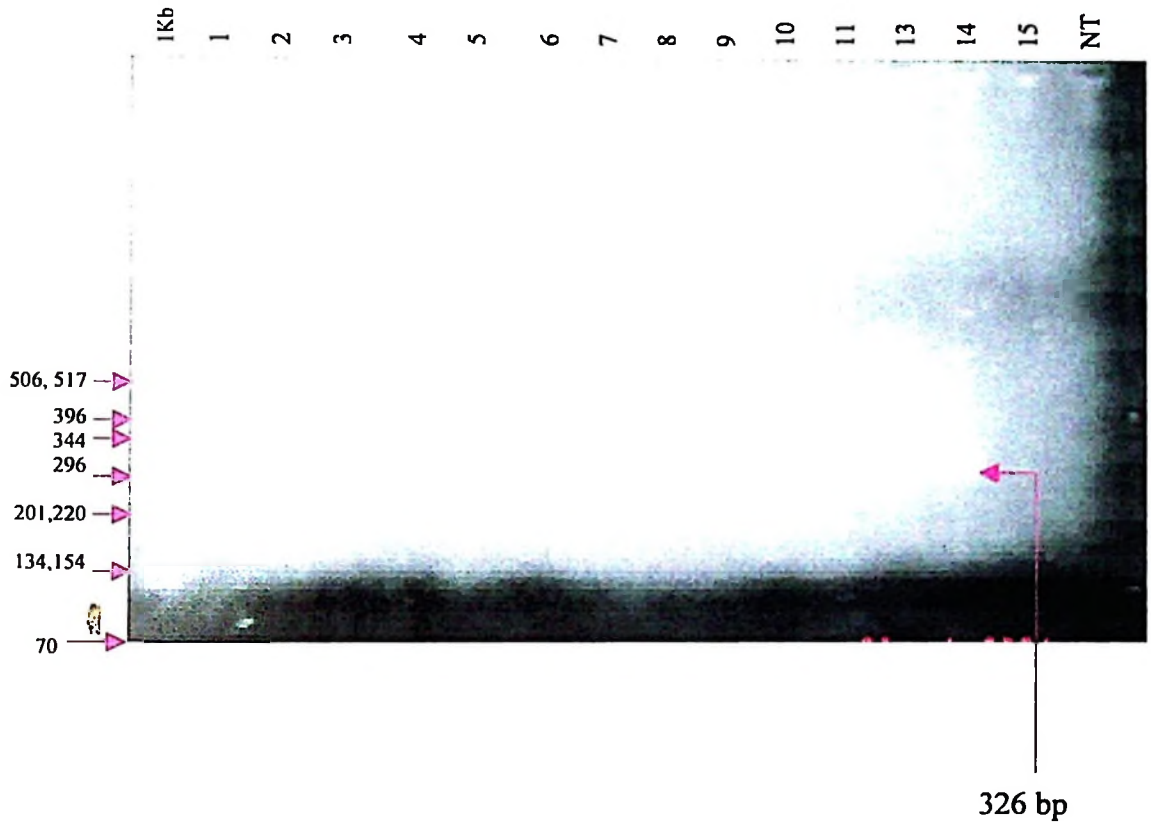

Figure 8

Representative DHFR gene PCR amplified fragments (using F + M4 primers), separated on 2% agarose, stained with ethidium bromide and photographed under UV light using Polaroid camera and electronically stored using a digital camera. 1Kb = DNA Ladder, NT = DNA negative control. Numbers 1 – 15 = DNA amplicons from nested PCR. Number 15 is a negative sample.

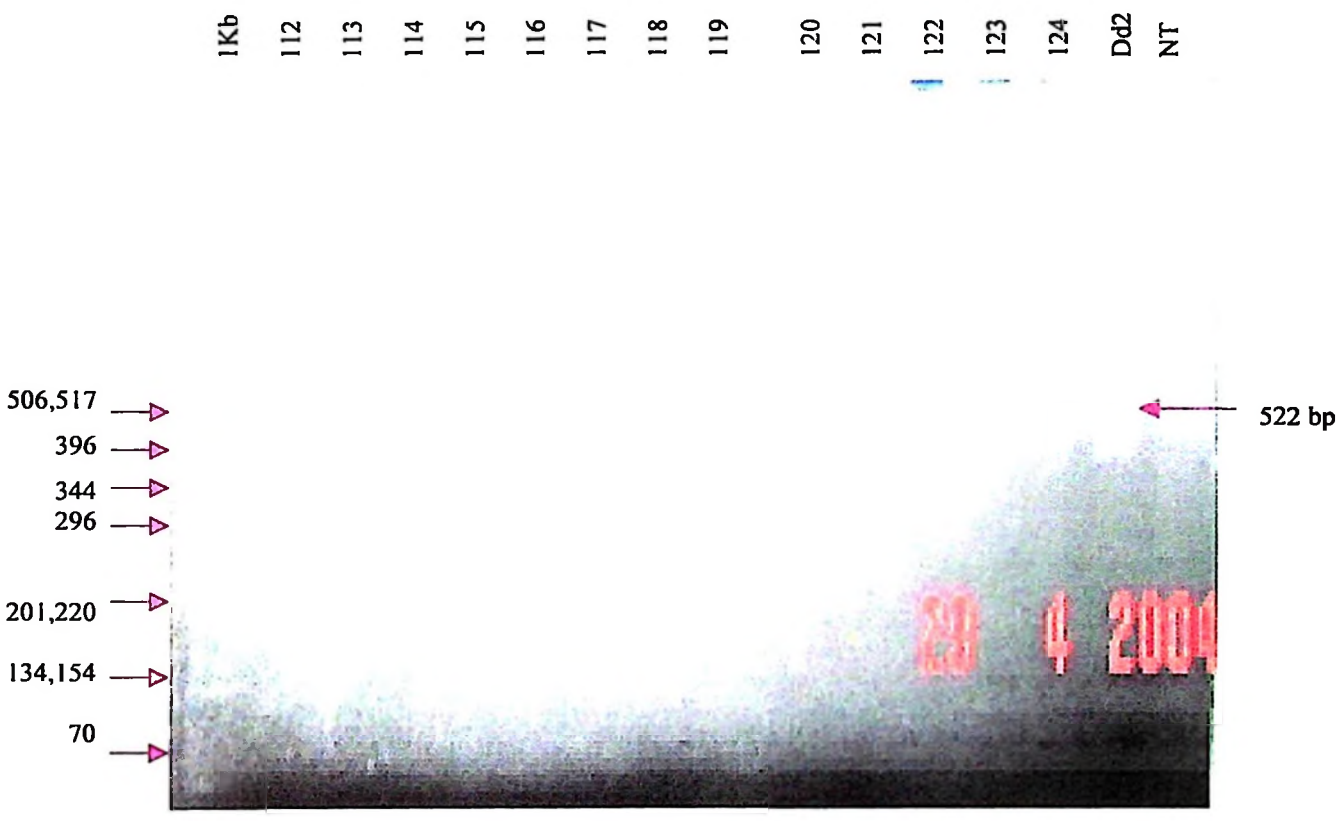

Figure 9

A representative 2% agarose gel showing approximately 522bp PCR amplicons resulted from amplification of DHFR gene with F + M3 primers. Samples are numbered 112 to 124. The first lane is a DNA Ladder, Dd2 = positive control, NT = Negative control. 112 and 120 are negative samples.

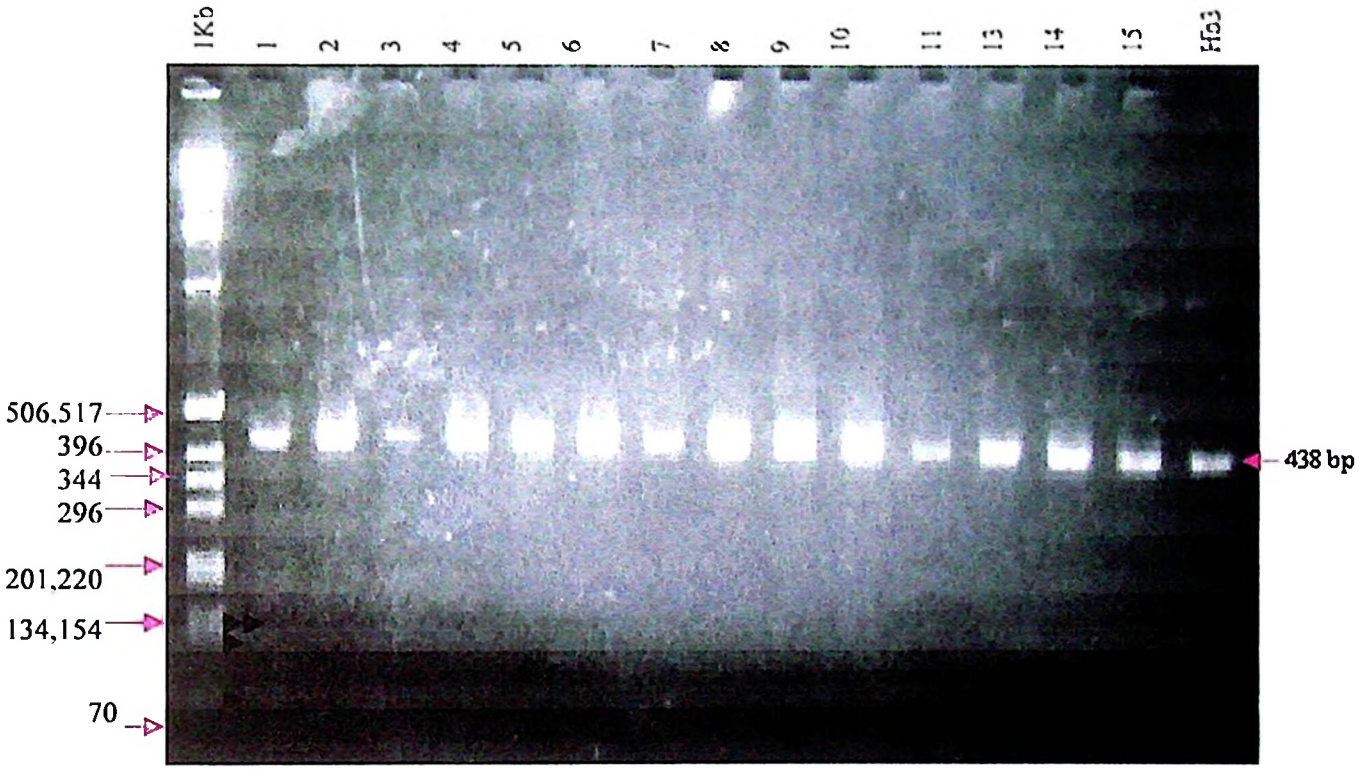

Figure 10

A 2% agarose gel showing representative PCR amplification of DHPS gene using K + K/ primers. Uncut bands = 438 bp, HB3 = positive control, 1 to 15 lane are samples.

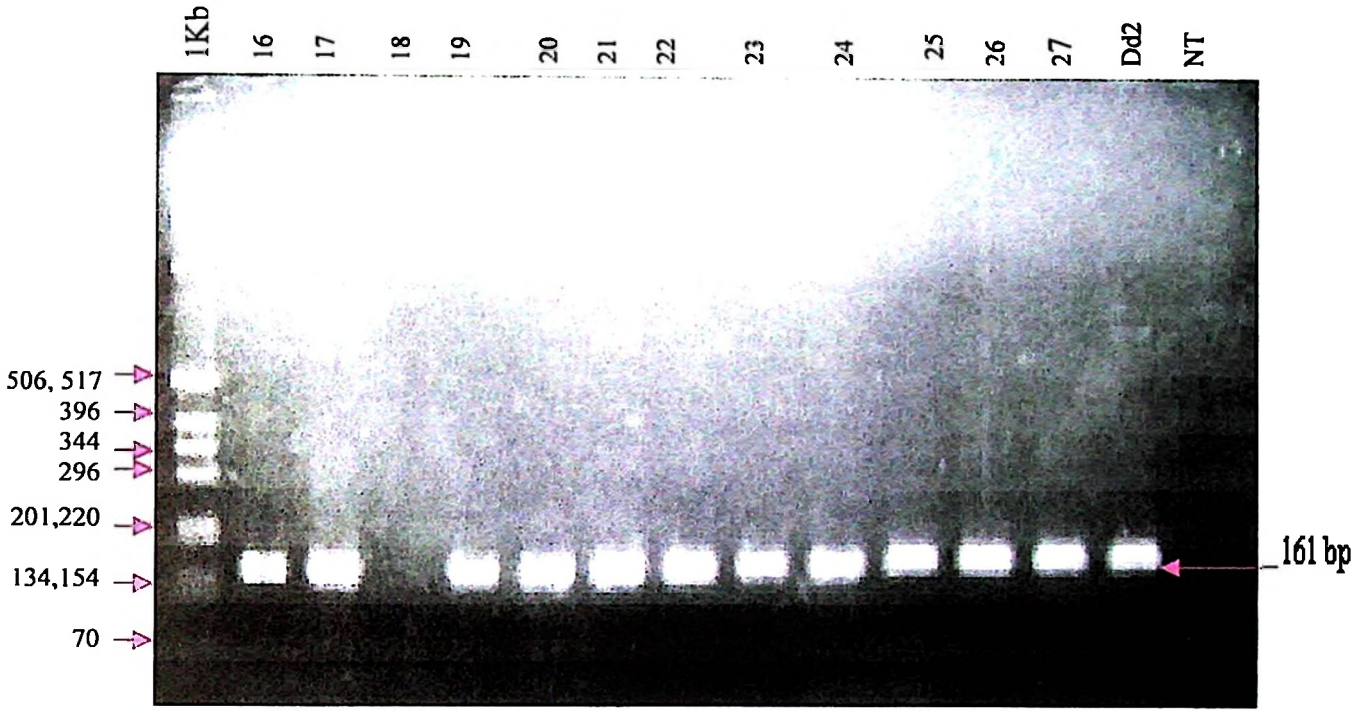

Figure 11

A 2% agarose gel showing representative PCR amplification of the DHPS gene using L +L/ primers. Uncut bands = 161 bp, Dd2 = positive control NT = Negative control. Sample 18 indicate negative DNA sample

4.2.3.0 RFLP analysis

4.2.3.1 Multiplicity of *P. falciparum* infections

The number of infecting genotypes in an isolate (multiplicity of infection) was detected by restriction digestion (RFLP) of the MSP2 gene (Beck *et al.*, 1997). For MSP2 genotyping, nested PCR products were digested with *Hinf*I (New England Biolabs) and restriction fragments separated on 10% PAA gel. Fragment sizes were estimated using a DNA size marker (1kb Ladder, Gibco BRL Life Technologies). In order to identify size differences of the DNA fragments from longitudinal studies, consecutive samples from day 0 to day 14 were run side by side on the same gel (Irion, 2000). Restriction digestion was successful in 81% (101/125) of PCR products. Figure 12 depicts the restriction fragment patterns of the MSP2 gene obtained. Restriction fragment patterns contained conserved fragments of 115 and 137 bp for FC27-type alleles. The variable fragments ranged from 150 – 378 bp to complete the pattern. For 3D7-type alleles, despite the conserved fragments of 70 and 108 bp, variable fragments of sizes ranging from 250 to 550 bp were obtained revealing this allelic family. The various fragment patterns obtained in this study indicative of either of the two allelic families are shown in Appendix 2 and 3.

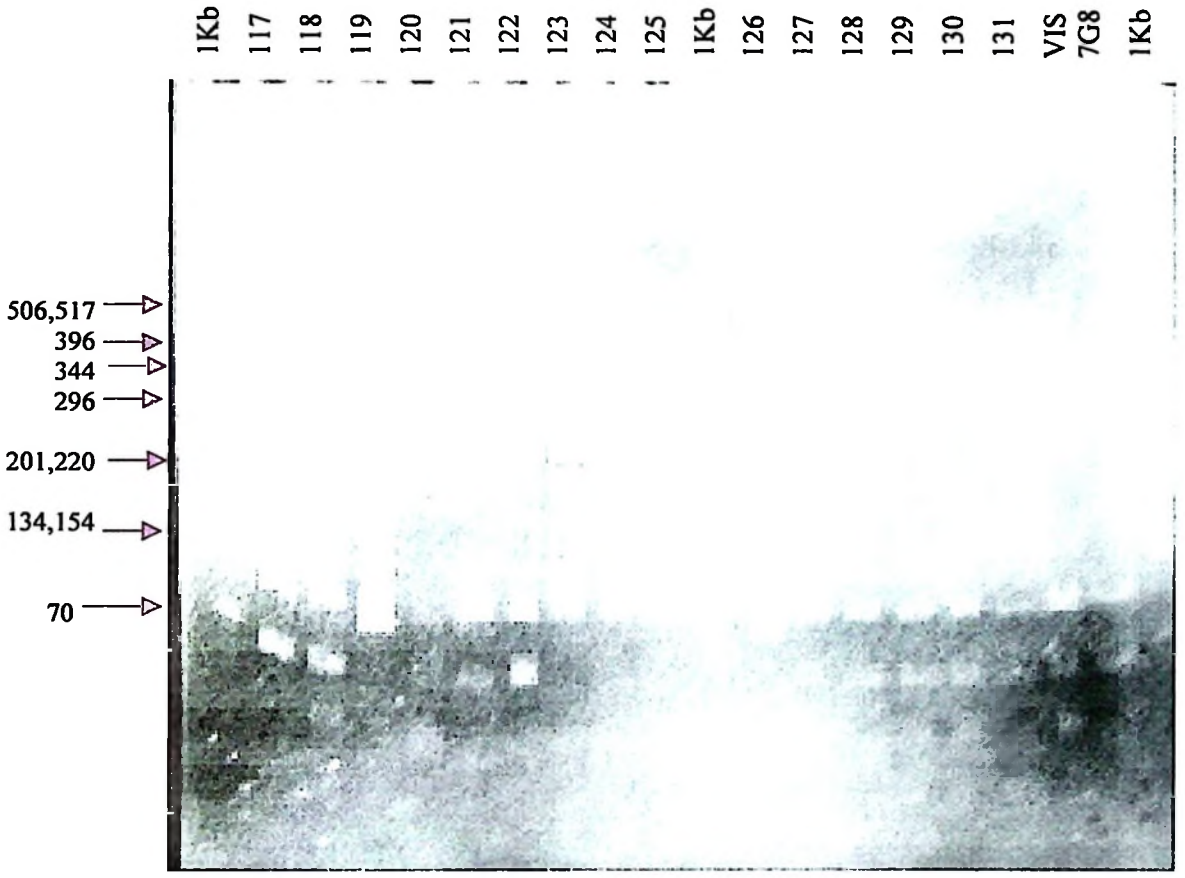

Figure 12

MSP2 RFLP products on 10% PAA gel. Lane 122 and 123 represents the 3D7 and FC27 allelic families, respectively. The patterns of fragment sizes are 70, 108, 290 for 3D7 and 115, 137, 260 bp for FC27 in that order. Lanes 119 and 126 indicate degraded DNA fragments (Due probaly to fluctuations in storge conditions particularly temperatures).

This study investigated the multiplicity of infections in the patients infected with *P. falciparum* malaria basing on the PCR-RFLP techniques (Fig 12 and 13). Considering individual allelic families (Table 5), 75 (74.3%) patients carried 3D7 allelic family genotypes in which 57 (56.4%) occurred as single alleles. Double 3D7 allelic family genotypes were detected in 14 (13.9%) while 3 (3.0%) had triple 3D7 genotypes. Only 1 (1.0%) possessed quintuple genotypes of the particular allelic family. On the other hand, the FC27 allelic family occurred in 62 (61.4%) patients in which 47 (46.7%) were single genotypes and 12 (11.9%) were double genotypes of the allelic family. Two (2.0%) of the patients had triple FC27 allelic family genotypes and 1 (1.0%) had quadruple genotypes of the particular allelic family (Table 5).

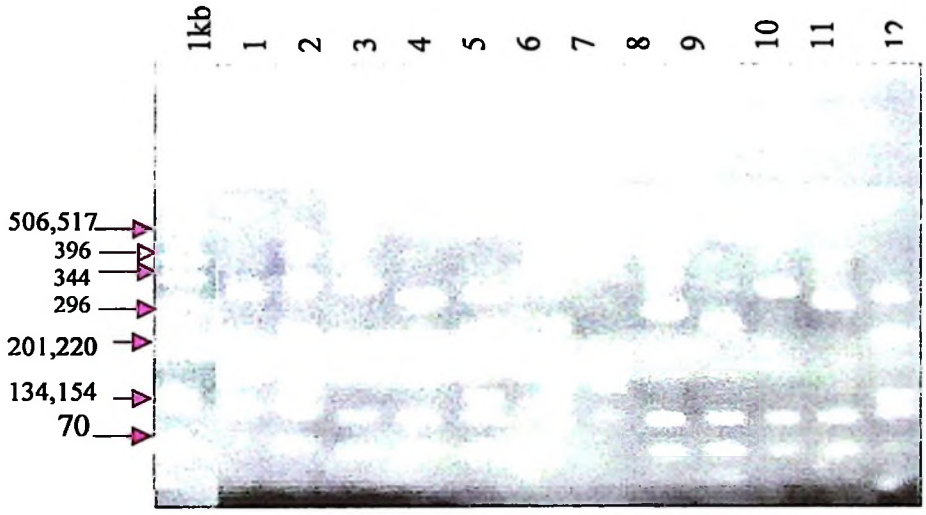

Figure 13

A 10% representative PAA gel for the MSP2 PCR-RFLP. Restriction patterns in lanes 2, 5 and 12 represents samples with mixed genotypes in a single patient defining the multiplicity of infection per infection.

Results (Table 6) also show that, 51 (50.5%) out of 101 patients carried single infection of either of the two allelic families. Double infection occurred in 32 (31.7%) of the patients. Twelve (11.9%) patients carried triple infection and six (5.9%) had multiple infection. Multiplicity of infection ranged from 1 to 6 infections per blood sample with mean multiplicity of 2.58 per infection excluding single infections (Appendix 4). With single infections included in estimating the multiplicity of infection, an average multiplicity of 1.78 infection per patient was obtained. Pure 3D7 allelic family genotypes were detected in 39 (38.6%) of all genotypes detected (n = 101) (Fig 11). Twenty-six (25.7%) of samples carried purely FC27 allelic family genotypes. About 36 (35.6%) carried mixed alleles of these two allelic families (Fig 14). Tables 5 and 6 show proportion, which is indicative of distribution of each allelic family in the sampled population. The Venn diagram (Fig 14) further summarizes the restriction analysis of the MSP2 genes and how these proportions have been derived. The proportions of each allelic family in the total samples indicate how diverse is the parasite in the population. About 20 different alleles of the 3D7-type and 16 different FC27-type alleles were detected in this study (Appendix 2 and 3). The alleles occurred as single or mixed with another or other alleles within the family or from another allelic family pinpointing parasite diversity.

Table 5: Frequency of different MSP2 alleles of *P. falciparum* found in this study in 101 PCR-RFLP successful samples of children from Mlimba

Allelic Family	(Allele)'	Single Infection	Double Infection	Triple Infection	Multiple Infection	Total
3D7	26 (25.7%)	57 (56.4%)	14 (13.9%)	3 (3.0%)	1 (1.0%)	101 (100.0%)
FC27	39 (38.6%)	47 (46.5%)	12 (11.9%)	2 (2.0)	1 (1.0%)	101 (100.0%)
Total	65	104	36	5	2	212

(Allele)' = Not of that particular allelic family but the counterpart.

NB: Infection is defined by various genotypes of the two allelic families as obtained from PCR-RFLP analysis.

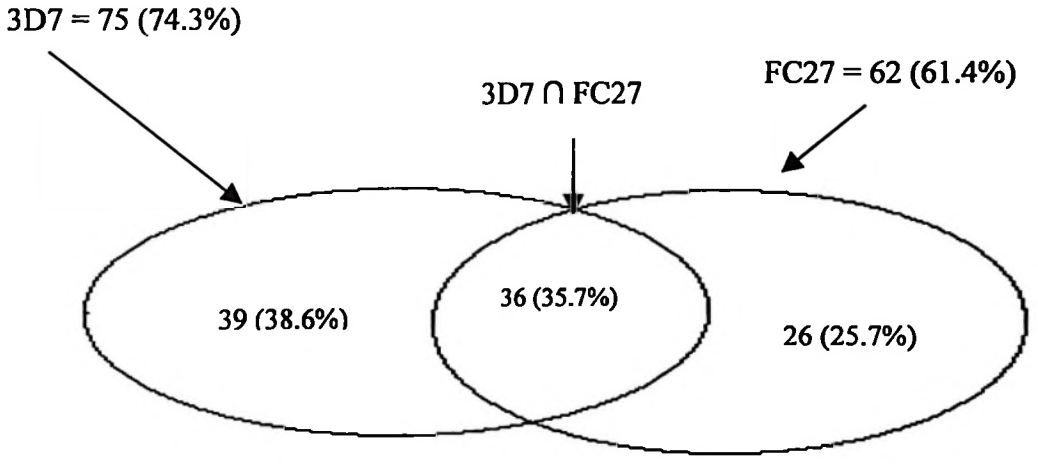

Figure 14: Venn Diagram

Distribution of MSP2 allelic families among isolates. 3D7 allelic family occurred in 75 isolates of which 39 contained the pure form of the allelic family and 36 occurred mixed with FC27 allelic family. Pure FC27 was detected in 26 isolates.

Table 6: Overall frequency of multiplicity of infections of the MSP2 genotypes of the *P. falciparum* 3D7 and FC27 allelic families from Mlimba

Infections	Frequency	Percent	Cumulative Frequency
Single	51	50.5%	50.5%
Double	32	31.7%	82.2%
Triple	12	11.9%	94.1%
Multiple	6	5.9%	100.0%
Total	101	100.0%	

Since this was a longitudinal molecular epidemiological study, restriction digestion was also done on follow up samples (Table 7). Fifteen follow up samples were subjected to restriction digestion basing on clinical results in attempt to detect recrudescent or new infections. These samples had parasitaemia at day 3 (D3), day 7 (D7), day 14 (D14) or in all these days of follow up. A diverse of results was obtained from RFLP of these samples (Table 7). Of the 15 follow up samples, only 3 samples had similar restriction fragment patterns with baseline samples. Of these one sample gave no RFLP digestion product on day 7. No DNA fragment could be determined in two samples (T08011 and T08018). One sample had D0 infection different from that of D3 but despite absence of DNA detected on D7, D14 infection was similar to that in D3. One sample had only D0 detectable restriction digest but not in the rest of follow up days. One sample had similar

D0 and D3 restriction fragment patterns belonging to 3D7 allelic family but in the next two days of follow up, double infections were detected one belonging to either of the two MSP2 allelic families. In this sample the original allelic family persisted with a new one belonging to the FC27 allelic family emerging. Another sample had baseline single infection similar to D3 and D7 belonging to 3D7 allelic family but D14 had a different infection belonging to FC27 allelic family. Two samples had similar restriction fragment patterns belonging to FC27 allelic family but none was detectable on the rest of follow up samples (D7 and D14). Restriction digestion analysis also showed existence of a single infection of 3D7-type in D0 and D3 in two samples but none could be detected on D7 and D14 samples. Another sample had triple infections two of 3D7-type and one of FC27 allelic family detectable at D0. In this sample, one of 3D7 allele was not detected on D3 but two, each belonging to either of the two allelic families. D7 sample possessed all triple infections detected at D0. No infection was detectable on D14. One sample harboured a single 3D7-type allele on D0; none could be detected on D3 and D7 and in D14, a single infection belonging to FC27 was detected.

Table 7: MSP2 PCR-RFLP product trend of detection from fifteen follow-up samples analysed post-medication in this study

ID	Day 0	Day 3	Day 7	Day 14	Remarks
T08006					S
T08011					ND
T08012					V
T08014					V
T08018					ND
T08022					NC
T08038					S
T08056					S
T08060					S
T08110					V
T08117					S
T08119					V
T08124					V
T08125					V
T08132					S

KEY:

V = Variable genotypes between follow-up days

S = Similar genotypes/infections in all three follow-up days

NC = Genotype obtained once and only in day zero

ND = Nothing detected

Shaded box = RFLP outcome obtained; Clear box = No RFLP outcome obtained

4.2.3.2 Drug resistance in *P. falciparum* infections

The regions of DHFR and DHPS genes flanking the regions of interest were amplified by PCR. To detect each variant responsible for the parasite resistance to SP, restriction digestion of PCR amplicons was done using specific restriction enzymes (Table 1). Figure 13 – 17 depicts representative PAA gels of the restriction digests used to detect the fragment patterns at each codon on the DHFR and DHPS domains.

The fragment patterns corresponding to the different variants are indicated and band sizes are as predicted from the sequences (Fig 15 – 19). PCR amplification of the DHFR gene with the primers F + M4 yields a PCR product sized 326 bp (Fig 8) on which the two alternative forms (Wild type and Mutant) of codon 108 can be discriminated. The restriction enzyme *Alu*I was used specifically to cut fragments distinguishing between wild-type (180 and 118 bp) and mutant (299 plus 27 bp) forms of the codon. The wild type indicates presence of serine (Ser) in the amino acid sequence of the enzyme system while the mutant form of the gene indicates the substitution by asparagine (Asn). Restriction digestion of the same PCR fragment with *Xmn*I distinguishes the variants on codon 59 of the gene wild type (189 and 137 bp) and mutant (162, 137 + 27 bp fragments). Mutation at this codon reflects substitution of amino acid cystine (Cys, wild type) by arginine (Arg, mutant) (Duraisingh *et al.*, 1998).

The M3 + F/ amplified PCR fragments (522 bp, Fig 7) was digested with restriction enzymes *TSP509*I and *Dra*I to distinguish variants at codon 51 and 164, respectively.

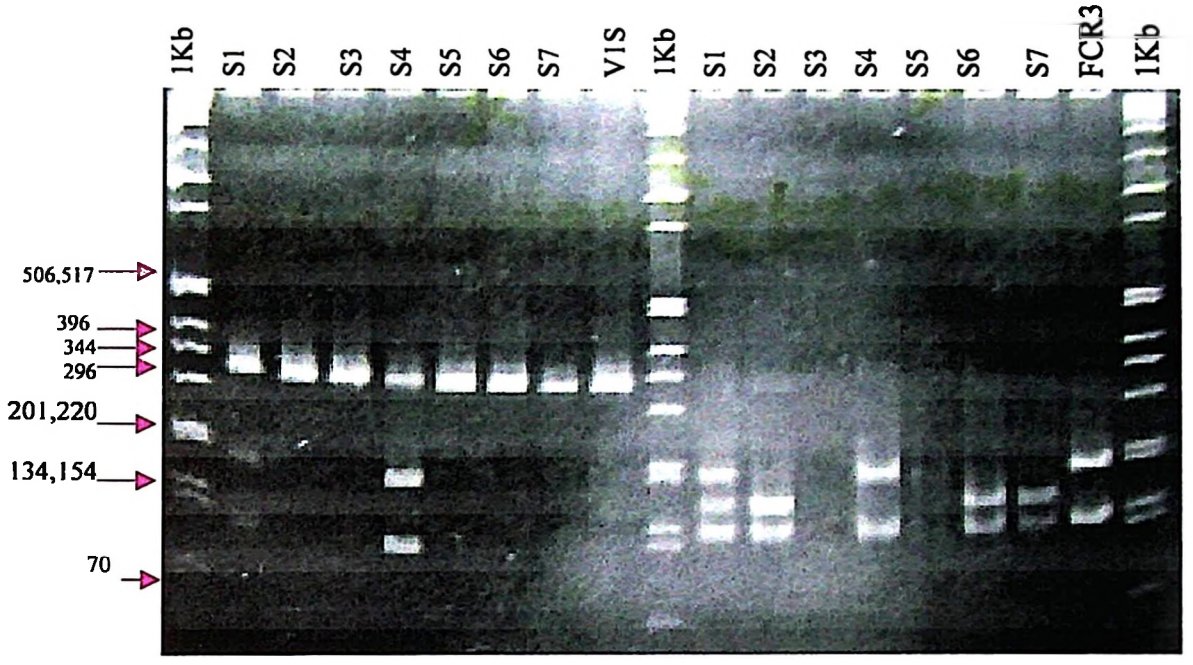

Figure 15

RFLP with *Alu*I (mutant 299 + 27 bp; wild type 180 + 118 bp i.e. S1 – S7 on the left), then 1Kb followed by *Xmn*I (mutant 162, 137 bp; wild type 189, 137 bp i.e. S1 – S7 on the right). In *Alu*I digests S4 is a mixture of wild type and mutant genotypes and in *Xmn*I digests S1 is a mixture of wild type and mutant genotypes. In both *Alu*I and *Xmn*I restriction digestion, V1S = Control Mutant genotype, FCR3 = Control wild type genotype.

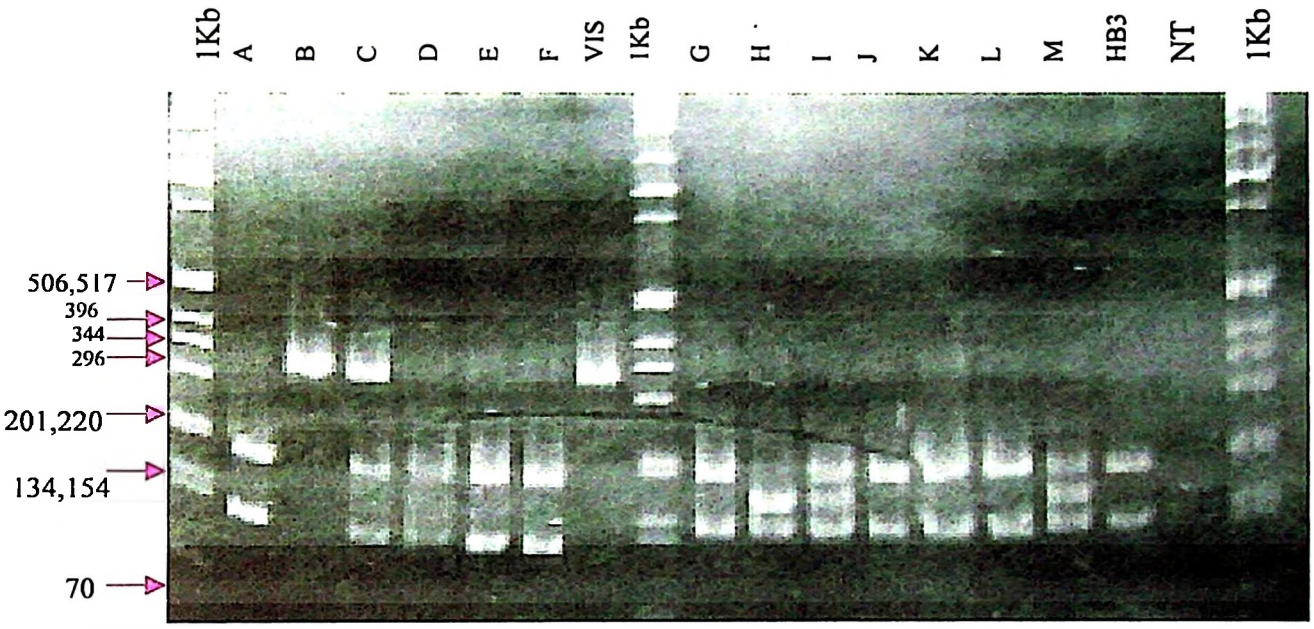

Figure 16

*Alu*I and *Xmn*I restriction digests (codons 108 and 59, respectively). In *Alu*I restriction digestion, A, D, E, F, are wild type genotypes of fragment size (162 and 137 bp). B is a mutant genotype (299 bp). C is mixed mutant and wild type genotypes. Molecular marker (1kb) is located at both ends of the gel and in the middle. *Xmn*I restriction fragments are located on the right after the middle 1kb marker. Lanes G, J, K, L are wild type genotypes (189, 137 bp) and lane H is a mutant genotype (162, 137 bp). Lanes I and M are mixed mutant and wild type genotypes.

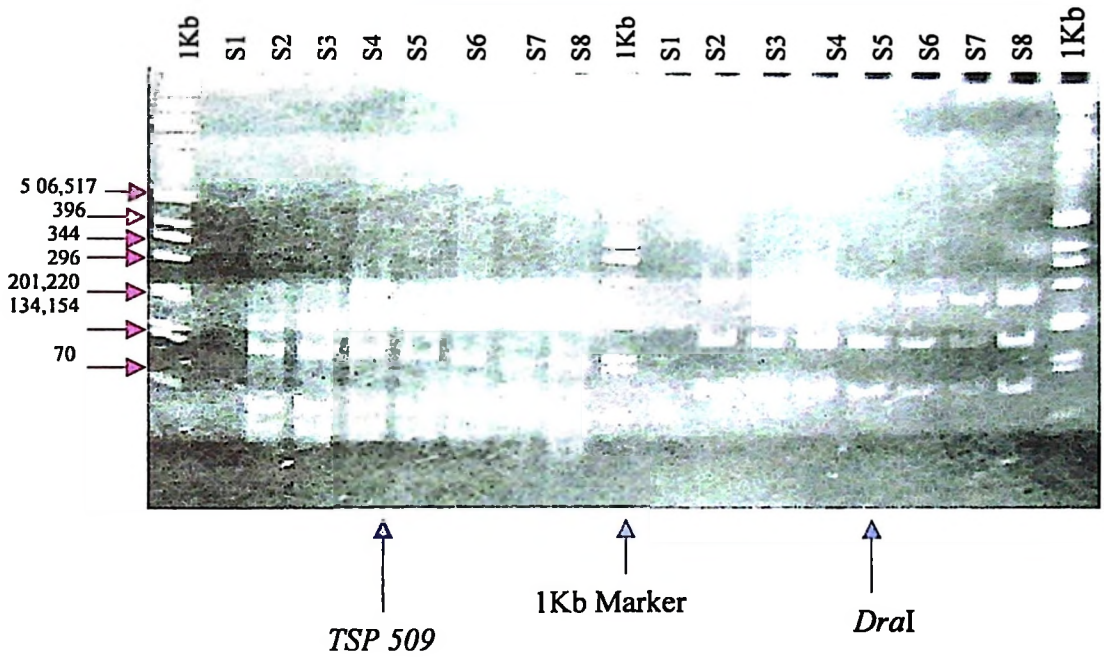

Figure 17

*TSP 509*I and *Dra*I DHFR restriction digests (codons 51 and 164, respectively). Different restriction enzymes produced different patterns of restriction fragments. S1 – S8 on the left of mid-1Kb Marker are for *TSP 509*I and those on the right are for *Dra*I. Fragment sizes for *TSP 509*I are 153, 120, 64 bp (wild type) and 217, 120, 64 bp (mutant); lanes S4, S6 and S8 are mutant genotypes, S2, S3, S5 are wild type while S1 and S7 are negative samples. All lanes in *Dra*I restriction fragments are wild types (size: 245, 171, 106 bp).

Restriction digestion by *TSP509I* produced fragments of sizes 153 and 120 bp indicative of presence of amino acid asparagine (wild type) and 217 and 120 bp indicating presence of amino acid isoleucine (Ile, mutant). On the other hand, restriction digestion with *DraI* produced fragments of sizes 245, 171 and 106 bp (wild type) and 145, 143, 106 and 28 bp (mutant) distinguishing polymorphism at codon 164. In this context wild type is denoted by occurrence of isoleucine (Ile) and mutant determined by occurrence of leucine (Le). The combination of all four restriction enzymes were used in this study to produce products useful in the detection of mutations ascribed to resistance to antifolate, SP by the malaria parasite basing on the DHFR gene.

Tests (PCR-RFLP) for polymorphisms on the DHPS gene are shown in Figure 18 and 19 based on PCR products of approximately 438 bp (Fig 10) obtained from amplification with the K and K/ primers. The variants at codon 437 were discriminated by restriction digestion using the restriction enzyme *AvaII* (Fig 18). In this case uncut fragment (438 bp) indicates wild type while mutations at this site is shown by a cut fragment sized 404+ bp. Mutation at this codon is indicative of substitution of glycine (Gly) for alanine (Ala). The K + K/ amplified PCR products have been used to detect polymorphisms occurring at codon 540. A restriction enzyme, *FokI* was used to digest and specifically discriminate variants at this site (Fig 19). Using this restriction enzyme, fragments sized 405 bp (wild type) and 320 and 85 bp (mutant) were obtained. The 405 bp fragment indicates presence of amino acid Lysine (Lys) while the 320 and 85 bp fragments reflect substitution of glutamate (Glu) for lysine at this codon.

Following amplification with L + L/ primers, a PCR product of size 161 was produced (Fig 11). These PCR fragments have used to describe polymorphism on the DHPS gene occurring at codon 581 using a restriction enzyme *Bst*UI (Duraisingh *et al.*, 1998). This restriction enzyme produces restriction digests of sizes 105, 33 and 23 bp (wild type) and 138 and 23 bp (mutant) (Fig 18). The former reflects presence of amino acid, alanine at this codon and the latter indicates substitution of glycine for alanine. Only larger fragments show up in Figure 18.

In regard to frequencies of mutation on the DHFR gene at codon 51, restriction digestion with *TSP509*I shows that 35 (29.7%) of the samples carried wild type variant at this codon, 74 (62.7%) mutant and 9 (7.6%) carried mixed wild type and mutant variants. Restriction digestion was successfully achieved in 118 samples on this particular codon.

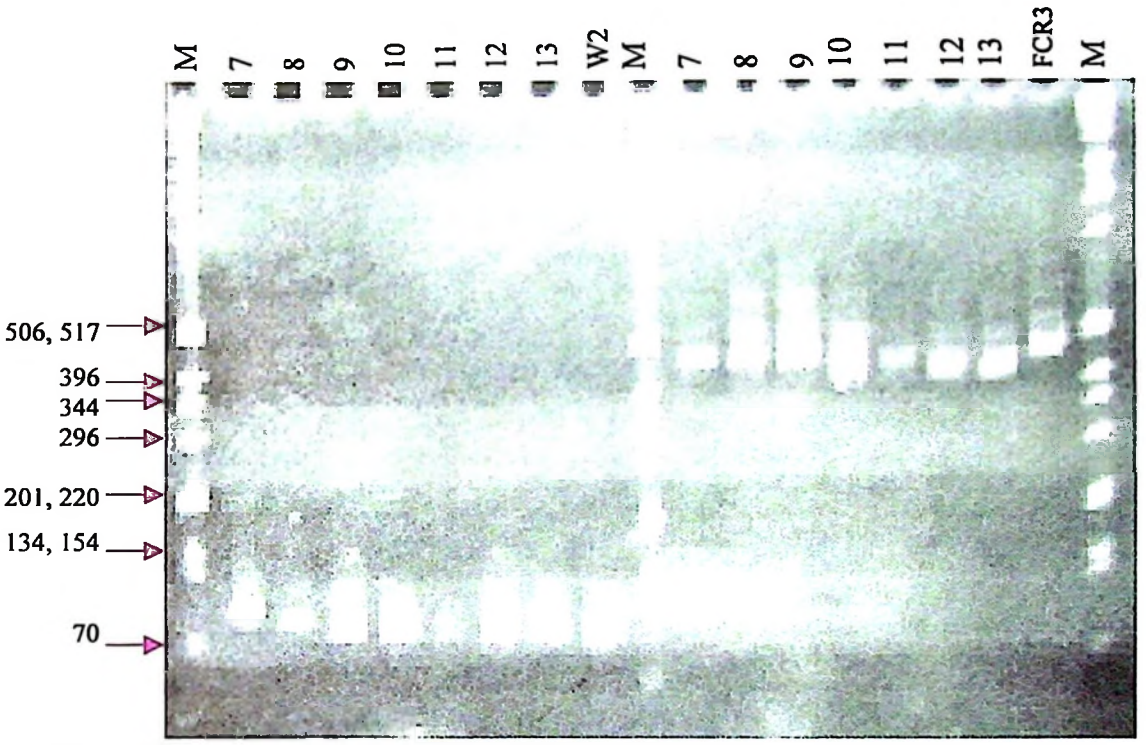

Figure 18

*BstU*I and *Ava*II restriction digests. The first lanes 7 to 13 before 1Kb ladder (M) in the midway, show restriction digests for *Bst*UI resulting from uncut fragment of 161 bp (Fig 11). All lanes resulting from *Bst*UI are wild type (105 bp). The lanes after the middle M are restriction digests resulting from *Ava*II digestion of uncut fragment sized 438 bp depicted in Fig 10. Lanes 7, 8, 9 and 11 are wild type (438 bp); 10, 12, 13 are mutant genotypes (404+ bp). W2 and FCR3 are control wild type in both cases.

Figure 19

*Fok*I restriction digestion. Restriction products consisting of point mutation have fragment sizes of 320 + 85 bp. Restriction products consisting of wild type genotypes have fragment sizes of 405 bp. Restriction digests 2, 6, 38, 41 are mutant genotypes and 35, 37, 39, 40 and 46 digests are wild type genotypes. Sample No 47 contains a mixture of wild type and mutant genotypes.

At codon 59, out of 130 samples digested with the *Xmn*I, 121 yielded restriction digests of interest. Of these, 36 (29.8%) were of wild type, 59 (48.8%) mutant and 26 (21.5%) mixed wild type and mutant. Restriction digestion with *Alu*I at codon 108 produced only 17 (14.1%) wild type, 81 (66.9%) mutant and 23 (19.0%) mixed variants out of 121 samples. Restriction digestion was also done with *Dra*I to detect possible mutations occurring at codon 164. Restriction digestion was successful in 104 samples and all consisted of wild type restriction digests at this codon. This is to say, of the all samples which yielded restriction digests 100% were wild type with regard to codon 164.

In detecting mutations occurring on the DHPS gene, three codons were considered basing on previous studies (Jelinek *et al.*, 2002; Pearce *et al.*, 2003; Bwijo *et al.*, 2003). A total of 128 samples were successful in regard to restriction digestion. To detect mutations at codon 437, *Ava*II was used to digest the PCR products, which resulted into 71 (55.5%) of samples being wild type, 56 (43.7%) mutant and one (0.8%) mixed variants at this point. Restriction digestion with *Fok*I at codon 540 resulted into 65 (54.5%) wild type, 47 (39.2%) mutant and 8 (6.3%) mixed variants out of 120 samples successfully restriction digested. The other mutation determined was that occurring at codon 581. The restriction enzyme, *Bst*U was employed and of the 128 samples digested, 126 (98.4%) samples were wild type, 1 (0.8%) mutant and 1 (0.8%) mixed variants. Table 8 shows the proportion of mutations at each codon considered essential for SP resistance in *P. falciparum* as determined in this present study. Proportions of point mutations on DHFR and DHPS gene related to parasite resistance to antifolates are summarised in Table 8.

Table 8: Proportions of point mutations on DHFR and DHPS gene related to parasite resistance to antifolates.

Resistance to	Gene locus	Mutations (%)	Wild type (%)	Mixed (%)	Total
Pyrimethamine	DHFR 51	74 (62.7%)	35 (29.7%)	9 (7.6%)	118
	DHFR 59	59 (48.8%)	36 (29.8%)	26 (21.5%)	121
	DHFR 108	81 (66.9%)	17 (14.1%)	23 (19.0%)	121
	DHFR 164	0 (0.0%)	104 (100.0%)	0 (0.0%)	104
Sulphadoxine	DHPS 437	56 (43.7%)	71 (55.5%)	1 (0.8%)	128
	DHPS 540	47 (39.2%)	65 (54.5%)	8 (6.3%)	120
	DHPS 581	1 (0.8%)	126 (98.4%)	1 (0.8%)	128
Total		318	454	68	840

The overall rsults show that only 11 (8.5%) of samples possessed wild type genotypes at all known codons of interest on both DHFR and DHPS genes investigated in this study (Table 9). Single mutations were detected in 6 (4.6%) of samples. Double mutations were detected in 18 (13.8%) samples while triple mutations were detected in 21 (16.2%) of samples. Quadruple mutations were detected in 9 (6.9%) of samples with 24 (18.5%) of all samples having quintuple mutations. Thirteen (10.0%) of samples possessed at least one wild type and one mutant genotype. About 14 (10.8%) of samples harboured at least single mutation and a mixed genotype. Restriction digestion also showed that 4 (3.1%) of samples harboured at least mixed genotypes at one codon plus quadruple mutations. Five (3.8%) of samples possessed at least one mutation and two mixed genotypes at any codon in either of the DHFR and DHPS domains. Three mixed genotypes and at least one mutation was observed in 4 (3.1%) of samples. One (0.8%) sample possessed mixed variants at four loci plus at least one mutation at any known codon of interest. Table 8 shows the overall proportions of known DHFR and DHPS point mutations examined in this study. As may be observed in Table 10, highest frequencies of mutations were those occurring in the DHFR than in the DHPS domain.

Table 9: Overall proportions of known DHFR and DHPS mutations investigated in this study

Item	Frequency	Percentage	Percent Cum.
Wild Type	11	8.5%	8.5%
Single Mutation	6	4.6%	13.1%
Double Mutation	18	13.8%	26.9%
Triple Mutation	21	16.2%	43.1%
Quadruple Mutation	9	6.9%	50.0%
Quintuple Mutation	24	18.5%	68.5%
Mixed (Mutant, Wild type)	13	10.0%	78.5%
Mixed + at least single Mutation	14	10.8%	89.2%
Mixed + Quadruple Mutation	4	3.1%	92.3%
2 Mixed + at least one mutation	5	3.8%	96.2%
3 Mix + at least 1 mutation	4	3.1%	99.2%
4 Mix + at least 1 mutation	1	0.8%	100.0%
Total	130	100.0%	

Table 10: Various DHFR/DHPS combinations obtained from restriction analysis to determine point mutations in genes responsible for SP resistance.

Category	Number	Percentage (%)	Percent Cum.
tDHFR	44	36.40	36.40
dDHPS	18	14.90	51.30
tDHFR/dDHPS	24	19.80	71.10
dDHFR/dDHPS	10	8.30	79.40
tDHFR/sDHPS	4	3.30	82.70
dDHFR/sDHPS	7	5.80	88.50
tDHFR/nDHPS	14	11.50	100.00
Total	121	100.0	

Key: tDHFR = Triple mutants on DHFR gene

dDHFR = Double mutants on DHFR gene

dDHPS = Double mutants on the DHPS gene

sDHPS = Single mutant on the DHPS gene

nDHPS = No mutation on the DHPS gene

On an average of 121 restriction digestion successful products (Table 10), a total of 44 (36.4%) contained triple mutant on the DHFR gene of which 24 (19.8%) occurred as triple DHFR/double DHPS mutants (quintuple mutation). A combination of double mutation in both DHFR and DHPS genes (quadruple mutation) was observed in 10 (8.3%) of all samples. Combined triple DHFR-single DHPS mutations appeared in 4

(3.3%) samples while double DHFR-single DHPS occurred in 7 (5.8%) of samples. Restriction analysis also revealed 14 (11.5%) samples containing triple DHFR with no mutations on the DHPS gene.

Mixed genotypes (wild type/mutant) at loci considered important causative of high SP resistance in *P. falciparum* was also detected in this study. A total of 68 mixed genotypes on either DHFR or DHPS genes were detected (Table 11). Of these mixed genotypes, 15 (22.1%) occurred as triple DHFR mixed variants and 16 (23.5%) existed as double DHFR mixed variants. A proportion of 2.9% of mixed variants was detected at two codons on the DHPS domain. Mixed genotypes at one codon on the DHFR domain were about 27 (39.7%) while those occurring at one codon on the DHPS domain were 8 (11.8%) of all mixed variants. Triple mixed variants on the DHPS domain were not detected in this study. The proportion of mixed genotypes in this perspective is very important due to the selection of mutant variants following medication leading to perpetuation of drug resistant strains in the parasite population while sensitive strains get rid off.

Table 11: Proportions of mixed infections detected in this study

Category	Variants detected	Total No. of variants	Percentage (%)
smDHFR	27x1	27	39.7
smDHPS	8x1	8	11.8
dmDHFR	8x2	16	23.5
dmDHPS	1x2	2	2.9
tmDHFR	5x3	15	22.1
tmDHPS	0x3	0	0.00
Total		68	100

Key: smDHFR = Mixed variants detected at a single codon on the DHFR domain

smDHPS = Mixed variants detected at a single codon on the DHPS domain

dmDHFR = Mixed variants detected at two codons on the DHFR domain

dmDHPS = Mixed variants detected at two codons on the DHPS domain

tmDHFR = Mixed variants detected at three codons on the DHFR domain

tmDHPS = Mixed variants detected at three codons on the DHPS domain

Like in MSP2 gene, restriction digestion was also done on follow up samples, which were diagnosed malaria positive on day 14 or with malaria symptoms and positive PCR results regardless whether they had parasitaemia on day 7 or not. The aim was to establish recrudescence or new infections among infected patients with resurgence assuming that PCR is more efficient in detecting low parasitaemias.

Of the 15 follow up samples with positive PCR results (Table 12), 8 samples had detectable restriction digests at D7 of which 7 (87.5%) retained the mutant genotypes detected in D0 (baseline) and D3 samples (SR). In these samples, at least double mutations were detected on the DHFR and DHPS domains. One (12.5%) of the restriction digests had mixed genotype at codon 540 of the DHPS locus instead of the mutant genotypes, which were detected in D0 and D3 samples (MR). The point mutations on DHFR gene were predominantly those occurring at codon 59 and 108 associated with substitution of arginine for cystine and asparagine for serine respectively. The double mutations in the DHPS gene were those involving codon 437 and 540 associated with substitution of glycine for alanine and glutamate for lysine respectively. Similar to RFLP for MSP2, two samples (T08011 and T08018) had no detectable DNA restriction fragments. In one sample, mixed variants were detected on codon 51 and 108 in the DHFR domain and wild type genotypes in the DHPS domain on D0. In this sample, no restriction digests were detected on days 3, 7 and 14. Three samples harboured triple DHFR and single DHPS detected on days 0 and 3. In these samples, no restriction digests were detected on days 7 and 14 (DT). Mixed triple DHFR and wild type DHPS were detected on D0 in one sample (RM). In this sample no restriction digests were observed on D3 and 7. On D14, triple DHFR mutants were detected following SP use. Table 12 summarises the trend of PCR-RFLP outcome during follow-up.

Table 12: Trend of PCR-RFLP product as detected for drug resistance markers from fifteen follow-up samples analysed post-medication in this study

ID	Day 0	Day 3	Day 7	Day 14	Remarks
T08006					SR
T08011					
T08012					SR
T08014					SR
T08018					
T08022					DO
T08038					DT
T08056					SR
T08060					MR
T08110					SR
T08117					DT
T08119					SR
T08124					SR
T08125					RM
T08132					DT

Shaded box = RFLP product obtained; Clear box = No RFLP product obtained

SR = Similar restricton products to day zero

MR = Mixed genotypes detected during follow-up instead of mutants detected on D0

RM = Mutant genotypes detected during follow-up instead of D0's mixed genotypes

DT = Restriction fragments detected twice, on D0 and D3

DO = Restriction fragments detected once and only on D0

CHAPTER FIVE

5.0 DISCUSSION

5.1 Clinical data

The clinical data (Table 2) were used as a basis and guidance towards subsequent molecular analysis in this longitudinal molecular epidemiological study. The data were kindly provided by IHRDC. From that study, early treatment failure was observed in 30.8% (53/172) of the patients during follow up. One (0.6%) patient developed dangerous signs of severe malaria and was excluded from the study. Six (3.5%) of patients were lost from the study during follow up. Thirteen (7.7%) patients were lost from the study during follow up at day 7. Late treatment failure was encountered in 10 (5.9%) of the patients (Table 3). Adequate clinical response was observed in 146 (84.6%) of all patients. A total of 142 patients persisted up to day 14 of the study. Of these, 128 (81.1%) attained adequate clinical response following SP treatment and 14 (8.9%) developed either recrudescent or new infection. No samples were available from one patient hence was eliminated in subsequent analysis. This reduced the number of samples to 141. These are the ones, which were subsequently used in molecular studies.

The 141 patients represent children aged less than five years, the age most vulnerable to falciparum malaria (WHO, 2003). From the preliminary data (Table 2, 3 & 4), early treatment failure was observed in D3 (30.8%) and late treatment failure (5.9%) in D7. Fourteen (8.9%) of patients were either having recrudescent parasite or new infection at D14. These combined clinical observations (from clinical data) provided clues for molecular studies on the causal-outcome relationship regarding SP treatment

in follow up samples. The treatment failures observed in this study (clinical data) from day 3 to day 14 were most probably attributable to parasite resistance to SP (Kyabayinze *et al.*, 2003). Hence the essence of the molecular work which was a concern in this particular study.

Parasite recurrence in SP treated individuals has been linked to many factors (Felger *et al.*, 1999). Such factors include overwhelmed immunity, multiple concurrent infections and drug resistance. In this study detection of polymorphisms in both the MSP2 and the drug target genes were done in accordance to Felger *et al.* (1999) and Duraisingh *et al.* (1998), respectively. PCR was performed to confirm whether the samples contained the parasite DNA gene loci of interest or not. Before the sample was declared negative the PCR was repeated at least twice. Primers used in this study (Table 1) have been used by Duraisingh *et al.* (1998) and are stipulated in 'The Guidelines for Protocols Involving Molecular Markers of Drug Resistance and Multiplicity of Infections' part IV (MIM/TDR, 2003) which has been adopted in this study. The standard protocol can also be obtained from [http://www.nlm.nih.gov/adrn/sop_molecular_markers.pdf].

5.2 Multiplicity of infection

To obtain desirable fragment containing the region responsible for polymorphisms in the MSP2 gene, the primers S2/S3 and S1/S4 were used as primary and nested primers, respectively. These primers have been developed to amplify the 5' and 3' conserved region immediately flanking the central polymorphic part of the MSP2 gene (Felger *et al.*, 1999). The PCR amplification was expected to produce DNA

fragments varying from 378 bp to 740 bp (Figure 6 & 7). The primers proved efficiency as they amplified about 87.9% of the samples used in this particular study. The unamplified 12.1% of the samples may be attributable to low parasitaemia undetectable by PCR, microscopical errors (e.g wrong determination of *plasmodium species*) or mislabelling of slides or blood samples. As stipulated by Färnert *et al.* (1997), failure to obtain PCR products in microscopically positive blood samples is also attributable to sequestration of parasites at time of sampling but could possibly be a result of wrong positivity of blood samples. Insensitivity in MSP2 gene amplification in some samples might have been resulted from point mutations at position where the MSP2-specific primers anneal (Kassberger *et al.*, 2002) thus impairing successful results.

*Hinf*I digestion of PCR amplicons produces restriction fragments, distinguishing the 3D7 and FC27 allelic families of the highly polymorphic MSP2 gene. The restriction enzyme has a high discriminatory power. However, additional digestion with *Dde*I increases resolution especially where multiplicity of infection is very high (Felger *et al.*, 1999). Separation of the digests in 10% polyacrylamide gel allows discrimination of a multitude of different MSP2 alleles (Felger *et al.*, 1993; Felger *et al.*, 1994; Owusu-Agyei *et al.*, 2002) and to determine the number of concurrent genotypes per blood sample (multiplicity of infection). The method provides the highest resolution at this particular locus and also new alleles can easily be determined (Irion *et al.*, 1998). Basing on previous studies (Felger *et al.*, 1993; Felger *et al.*, 1994; Felger *et al.*, 1999; Owusu-Agyei *et al.*, 2002) the restriction enzyme *Hinf*I was selected and used in this study and has provided good results.

In this study the high resolution of PCR-RFLP genotyping of MSP2 gene (Hill and Babiker, 1995) was exploited to detect multiple clones from individual parasite infections concurrently present in a blood sample. In previous studies (Felger *et al.*, 1999), the single locus PCR-RFLP typing using MSP2 gene was found to have adequate discriminatory power with an average of five infection per child detected. In this study the overall restriction digestion showed that 51 (50.5%) out of 101 patients carried single infection of either of the two allelic families. Double infection was observed in 32 (31.7%) of the patients, 12 (11.9%) patients carried triple infection and 6 (5.9%) had multiple infections (Table 6).

Multiplicity (number of genotypes per infection) of up to 6 infections per patient was observed in this study (Appendix 4). However, only two patients (2%) harboured six infections per blood sample (Appendix 4). About 50 (49.5%) of patients harboured 2 – 6 infection at the time the blood samples were taken, leading to an average of 2.58 infections per patient (Appendix 4). This is slightly lower than what was reported by Babiker *et al.* (1999) of multiplicity of 3.2 – 3.4 per patient. In general, the multiplicity of infection ranged from single to six infections per patient. Similar observations were reported by Babiker *et al.* (1999) in samples collected from patients in Michenga, a village along Kilombero valley, which is endemic to the disease. However, in contrast to that study, multiple infection was found in nearly 50% of the samples in Mlimba when compared with 85% obtained in Michenga. Including single infections in estimating the multiplicity of infections, an average multiplicity of 1.78 infection per patient was obtained. This implies that on average each sampled patient in Mlimba division had at least two infections at the time when

blood sample was taken. The parasite population changes with time and season. In addition, Babiker *et al.* (1999) reported differences in multiplicity of infection between areas with seasonal transmission and those with stable transmission (endemic areas), the multiplicity being higher in the latter due to genetic recombination. Felger *et al.* (1999) observed that all PCR products representing recombination between the 3D7 and FC27 allelic families contained family-specific 36 and 96 bp repeats which were classified as FC27. These fragments were detected in this study and were classified as FC27 alleles. In addition, the large proportion of patients with single infection lowered the overall multiplicity of infection in this study. Multiplicity of infections is also dependent on the typing method and age group of patients from which the blood sample is obtained. Beck *et al.* (1997) and Smith *et al.* (1999) reported a higher load of infection among children aged between 1 – 9 years old with multiple clones of an average of 5 clones per patient. But another study in the same area by Felger *et al.* (1999) revealed an average of 2.1 clones among infants which is close to what was obtained in this study (average of 1.78 clones per infection).

In view of individual allelic families (Table 5, Fig 13), 75 (74.3%) patients carried 3D7 allelic family in which 57 (56.4%), 14 (13.9%), 3 (3.0%) and 1 (1.0%) of the parasite occurred as single, double, triple and quintuple infections of the particular allelic family, respectively. The FC27 allelic family occurred in 62 (61.4%) patients in which 47 (46.7%), 12 (11.9%), 2 (2.0%) and 1 (1.0%) were single, double, triple and quadruple infections, respectively of this particular allele. This shows that, of the two polymorphic MSP2 gene allelic families, the 3D7 was more prevalent in the

study population than the FC27. However, the composition of alleles in each family in this study (Appendix 2 and 3) was similar to that reported by Felger *et al.* (1999) who conducted a similar study in the Kilombero valley five years ago along which Mlimba is situated. This suggests that the parasite population in this area might have become stable which may be attributable to endemicity of the disease in the area resulting to frequent cross-mating between parasites. This is important in disease intervention studies as it allows clones with novel genotypes to be produced, bringing together genes which additively generate phenotypes such as multiple drug-resistance (Babiker *et al.*, 1999).

Allelic diversity in the MSP2 gene detected in this study was high with 20 different 3D7 family alleles and 16 different FC27 family alleles observed. This indicates that, at the MSP2 gene, 3D7 and FC27 alleles were nearly equally distributed (ratio = 5/4) although the 3D7 alleles was relatively higher in proportion than FC27 (Appendix 4). Similar results were reported by Smith *et al.* (1999) in Namawala, another area lying along the northern edge of flood plains of the Kilombero River.

Results from restriction digestion of follow up samples (Table 7) showed that some alleles either transiently disappeared, or additional alleles appeared and eventually disappeared again in some samples during follow up. Similar results were reported by Aubouy *et al.* (2003b) in Sudan in a similar study. In the present study, similar RFLP fragment patterns were observed from baseline to D14 samples in three patients during follow up. Of these, one sample had no RFLP digests on D7. The re-occurrence of similar fragments in the aforementioned three samples may be coupled

with a recrudescing parasite. However infection with a new clone of similar genotype is not uncommon in a stable parasite population due to cross-mating as was reported in previous studies by Babiker *et al.* (1999). The absence of restriction digests in one sample on D7 might be due to absence of parasite DNA sample possibly accounted with low parasitaemia following medication. No DNA fragment could be determined in two samples (sample T08011 and T08018). The absence of RFLP digests in these two samples might have been due to lack of available DNA for effective restriction digestion.

In multiple infections, the most abundant allele in the blood sample suppresses amplification of an allele, which is less abundant (Contamin *et al.*, 1995). This can explain the different infections in one sample between D0 and D3. It is most likely that application of drug in D0 killed most of the parasite clones abundant (but sensitive to the study drug) in the peripheral blood of the patient so that on D3 the less abundant but slightly resistant parasite clones emerged. The absence of restriction digests on D7 is attributable to absence of DNA template for restriction digestion (a weak PCR amplicon was observed for this sample). The allele detected on D14 but similar to that on D0 might possibly be a new infecting parasite clone not necessarily the same parasite detected on D0 (Greenwood, 2002). Equally true, infection detected on D14 might have been the same as that was detected on D0 possibly resolvable by DNA sequencing (Felger, *et al.*, 1999). Interestingly, D7 and D14 samples in this patient were microscopically diagnosed negative for the parasite. Hence detectable PCR-RFLP products on D14 quantify the efficiency of molecular

tools in diagnosing the disease at low parasitaemia as compared to microscopy (Nsobya *et al.*, 2004).

Presence of detectable restriction digests on D0 and its absence on days 3, 7 and day 14 might possibly be attributed to low or no parasitaemia in the follow up days (Contamin *et al.*, 1995; Smith *et al.*, 1999). RFLP products were also detected on D0 and D3 but not on D7 and D14 in four samples (Table 7). In these samples, the parasite clones might have been sensitive to the drug hence was subsequently cleared (Aubouy *et al.*, 2003b). Nevertheless, presence of low parasitaemias probably was the cause of failure in the detection of restriction digests in follow up days except on days 0 and 3 (Irion, 2000). In one patient similar restriction fragment patterns belonging to 3D7 allelic family was observed in D0 and D3 samples but double infections were detected on D7 and D14 samples, one belonging to each of the two MSP2 allelic families. In these samples the original allelic family persisted with a new one belonging to the FC27 allelic family emerging. Probably the patient was infected with a new infection or the effect of the drug suppressed the previously abundant parasite clone to a level that enabled the previously less abundant clone in the blood express.

It was also observed in this study that a similar infection belonging to 3D7 allelic family was detected on days 0, 3 and 7. However a different infection belonging to FC27 was observed in the same patient on D14. It is possible that treatment with SP slowly cleared the previous parasite clone and a new one emerged by day 14. One of the follow up samples in which infection recurred had triple infections detected on D0. Two of the infections were of 3D7-type and one of FC27 allelic family. In this

patient, one of 3D7 allele, which was detected on D0, was not observed on D3 instead, two infections that belonged to either of the two allelic families were detected. However, all triple infections that were detected on day 0 were also observed on D7. No infection was detectable on D14. The allele, which was detected on D0 but not on D3 but reappearing on D7, most likely was suppressed by the drug (SP) to the level that was not detectable in the presence of other abundant alleles on D3 (Babiker *et al.*, 1999; Aubouy *et al.*, 2003b). Alternatively, SP treatment might have cleared the infection thus the similar allele that reappeared on D7 probably was a new infection. In one of patients, a single infection belonging to 3D7-allelic family was detected on D0. In this patient, no restriction digests were detected on days 3 and 7 but a single infection belonging to FC27 was observed on D14. This indicates that treatment with SP cleared the parasite clone that was detected on D0 and a new parasite clone infected the patient and thus detected on D14.

It is apparent from this study that genotyping of the highly polymorphic MSP2 gene is of great use in assessing the multiplicity of infections and discriminating between recrudescent and a new infection.

5.3 Drug resistance target genes

Several previous studies have investigated the association between mutations in the DHFR and DHPS genes and the parasitologic and/or clinical response to SP medication at individual level (Wang *et al.*, 1997a; Wang *et al.*, 1997b; Basco *et al.*, 2000; Nzila *et al.*, 2000; Omar *et al.*, 2001). Most of these studies produced tangible results regarding the use of DHFR and DHPS genotypes as resistance marker genes

for SP (Alifrangis *et al.*, 2003). The present study investigated the frequency of point mutations in the DHFR and DHPS genes in Mlimba division of Kilombero district. This malaria-endemic area is situated along Kilombero River with perennial transmission of the disease. A multiplex PCR was done in 141 DNA samples and an average of 121 (91.64%) samples were amplified successfully using various primers (Table 1). To establish the level of SP resistance-associated point mutations in Mlimba, 4 protein-coding regions on the DHFR gene and three protein-coding regions on the DHPS gene was investigated. Various controls were employed to ensure against misinterpretations that might be caused by incomplete digestion (Duraisinigh *et al.*, 1998). A choice of the DHFR codons 51, 59, 108 and 164 and DHPS codons 437, 540 and 581 used in the present study was based on earlier studies (Mshinda, 2000; Jelinek *et al.*, 2002; Bwijo *et al.*, 2003; Pearce *et al.*, 2003). According to these studies, point mutations at these loci are said to be highly associated with SP resistance by *P. falciparum* in Africa. However, it was observed from this study (clinical data) that SP treatment cleared infection in 81.1% of the patients who completed the study. This treatment success is nearly consistent with what was reported (82%) by Aubouy *et al.* (2003a) in Bakoumba village in Haut-Ogooué province of Southeast Gabon. According to that study, DHFR mutations that lead to high-level *in vitro* resistance to pyrimethamine plus one or two DHPS mutations were reported to be not sufficient to induce *in vivo* failure of SP treatment in young children. This could also be probable outcome from the present study. Nevertheless, the reported over 60% semi-immune population of Tanzania (Kitua, 2000) might have contributed to clearance of infection in large proportion of patients observed in this study.

RFLP was done on successful PCR amplicons and the proportions of point mutations on both DHFR and DHPS genes associated with SP resistance are shown in Table 8. In addition, Table 9 shows overall proportions of obtained point mutations in the SP resistant markers investigated in this study. Combinations of mutant alleles in the DHFR and DHPS genes are shown in Table 10.

This study investigated mutations in the DHFR and DHPS genes, which are associated with SP resistance in Mlimba 2 years, after similar studies by Mshinda in 2000. In this study, it was found that 66.9% and 62.7% of infections carried mutation on DHFR gene at codons 108 and 51, respectively. These values are slightly higher that reported by Mshinda (2000) of 50%. This could be attributed to stepwise selection of mutation at these codons following use of antimalarial, SP at sub-dosage levels e.g. two patients sharing a single dose prescribed for a single patient (personal observation). In most malaria-endemic areas of Africa, resistance to antimalarial drugs is attributable to pharmacological case management of the disease occurring at informal level (Winstanley *et al.*, 2002; WHO, 2003). In this case patients purchase chaotic mixture of branded antimalarials and anti-pyretics, with poor compliance with dosing schedules contributing to selection of mutations for drug resistance due to sub-optimal dosages. In addition, the extensive use of different types of antifolates with mechanism of action similar to SP like trimethoprim and sulphamethoxazole (TS) combination (e.g. septrin) in treating other infections over time, probably accounts for the increased selection of mutations ascribed to SP resistance. Pearce *et al.* (2003) reported the widespread use of septrin in Northern Tanzania to indirectly select for DHFR and DHPS resistance mutations.

The observed mutation (66.9%) in the DHFR gene on codon 108 was higher than what was reported by Jelinek *et al.* (2002) in West Africa (54.0%) but slightly lower than that which was observed in Central Africa (72.4%), South Africa (68.9%) and East Africa (72.9%). The possible explanation for this decrease could be due to the high proportion of mixed genotype infections (mutant and wild type, 19.0%) at this codon which was observed in this study. These genotypes were counted separately. Pearce *et al.* (2003) reported the increase of mixed genotypes in codons whose mutations are associated with SP resistance in an area of high endemicity. This proportion of mixed genotypes gives rise to the DHFR mutations of about 85.9% if at all these genotypes were additive. The DHFR mutation in codon 51 was also higher than that reported previously by Jelinek *et al.* (2002) in Africa. The DHFR mutation at codon 59 was also higher than previously reported in Africa and all together explains the significance of these mutations in causing higher SP resistance in *P. falciparum*. No mutation was detected at codon 164 in DHFR gene in this study and augment previously described studies by Hastings *et al.* (2002). Generally, mutations in the three codons in the DHFR domain were higher (Table 8) than those on the DHPS domain suggesting that mutations on DHFR domain precedes those on DHPS domain in conferring parasite resistance to SP.

The point mutations in DHPS gene observed in this study are far higher than what has been observed in previous studies in East and South Africa (Jelinek *et al.*, 2002) and Eastern Iran (Jafari *et al.*, 2003). This can possibly be explained by the widespread use of Septrin in Tanzania, which indirectly selects mutations for SP resistance. Takahashi *et al.* (2000) further reported that the use of antifolates such as co-

trimoxazole for prophylaxis or medication against other infections than malaria indirectly selected mutations for resistance to the drug. According to that report, the double mutations at DHPS were predominantly selected. Nevertheless, the DHPS mutations observed in this study were relatively lower than that observed in Central and West Africa by Jelinek *et al.* (2002). The possible explanation for this is the geographical difference which is reported to influence the occurrence of mutant forms in DHPS gene (Biswas, 2004). The difference in selection of mutation between areas may also be attributable to differences in patterns of drug use between areas (Pearce *et al.*, 2003). However, Biswas (2004) reported the DHPS mutations in Tanzania to range between 30 – 34%, which is less than that observed in this study (39.2 – 43.7%). This signifies the increasing selection of these mutant alleles with time in Tanzania with frequent use of antifolates.

Molecular investigation of SP resistance mutant alleles in the DHFR and DHPS gene in this study produced an overall proportion of point mutations which reflect the existence of high resistance in the study area (Table 9). Only 8.5% of infections carried pure wild type genotype and 4.6% of the samples showed single mutant alleles. Quintuple mutations were highest (18.5%) followed by triple (16.2%) and double mutations (13.8%). Quadruple mutations occurred in 6.9% of samples. About 41 (31.5%) of infections had at least one mixed (mutant, wild type) genotypes attributable to endemicity of the disease. In endemic areas disease transmission is reported to be high consequently giving rise to high proportion of mixed clones in one infection (Babiker *et al.*, 1999).

The co-occurrence of point mutations in the two DHFR and DHPS loci was also examined in this study (Table 10). About 44 (36.4%) of samples contained triple mutant on the DHFR gene. The proportion of triple DHFR found in this study was relatively similar to that reported by Mshinda (2000) in the same place (41%) but was about twice to that reported by Mugittu *et al.* (2004) of 18.6% (Fig 2). However, the proportion escalates if the mixed triple DHFR variants (22.06%) and mixed double DHFR variants 16 (23.53%) detected in the present study (Table 11) was counted inclusively. The proportion of these mutations was less than that obtained by Pearce *et al.* (2003) in Hai district, Northern Tanzania (>70%). This is because resistance to SP is already unprecedentedly high in Northern Tanzania as reported previously due to widespread use of SP. In these areas, SP resistance was also reported to increase dramatically due to movement of resistance from Muheza (Pearce *et al.*, 2003). Mugittu *et al.* (2004) reported a prevalence of 80.3% in Mkuzi (Fig 2), an area in Muheza District where pyrimethamine was used for prophylactic and/or therapetic trials at different periods from 1950s to 1994 (Clyde, 1954; Clyde and Shute, 1957; Rønn *et al.*, 1996). The observed differences in levels of SP resistance between Mlimba and Hai might also be attributable to differences in patterns of drug use between these two communities. Pearce *et al.* (2003) also observed the difference in patterns of drug use between communities to probably contribute to the differences in development of drug resistance among Tanzanian communities. In Muheza District Hospital, for instance, SP was implimented as first-line drug in children less than 5 years of age since 1984 (Mutabingwa *et al.*, 2001).

Triple-DHFR/double-DHPS mutants (quintuple mutation) occurred in 19.8% of all samples (Table 10). This frequency of quintuple mutation is high as compared to previous studies in Mlimba and Idete (STI report, 2000) which was reported as rare event. This proportion (19.8%) was almost similar to that recently reported by Mugittu *et al.* (2004) (Fig 2). The increase in proportion of quintuple mutations in Mlimba might be due to selection of mutation at these loci with time following use of SP in treating malaria. However, the proportion of quintuple mutation in Mlimba (Table 10) was less than that reported by Pearce *et al.* (2003) in Hai and Pare areas of Northern Tanzania (30 – 63%). The proportion of quintuple mutation was also less than that generally reported by Jelinek *et al.* (2002) (42.9%) in East Africa, and that by Bwijo *et al.* (2003) in Maonga and Chimbala villages of Salima District, Malawi (78%). The lower frequency of quintuple mutation than that previously reported in Tanzania and other areas of Africa is partly attributable to prior development of resistance in those areas compared to the area in this particular study. In Malawi the study was carried 7 years after introduction of SP as a first-line drug while this study was carried shortly (2 years) after introduction of SP as a first-line drug which provided a relatively shorter time for selection of SP resistance mutations. But also can be linked to the proportion of genotypes at similar point mutation sites possessing mixed variants. In those previous studies, the mixed variants were not reported. These could increase the proportion of quintuple mutation if they were counted inclusively.

Greater use of alternative antimalarial drugs such as amodiaquine might possibly have contributed to less selection pressure against SP-associated point mutations in this study. Reduced use of antimalarials due to poor access to health facilities or

raised levels of acquired immunity (Pearce *et al.*, 2003) probably contributed to the low level of SP-associated point mutations observed in this study. In addition, samples from different populations are expected to give different results. For instance the study carried in Malawi by Bwijo *et al.* (2003) involved children of < 6 years, school children of 6 – 15 years and adults living in the school's neighbourhood with long exposure to disease and medication raising the possibility of selecting resistance mutations against the drug. In this present study, case selection involved children < 5 years of age with less prior exposure to the disease.

The double-DHPS mutation was detected in 19.8% of samples where it occurred as triple-DHFR/dDHPS mutations (Table 10). A double-DHFR/double-DHPS (quadruple mutation) was observed in 8.3% of all samples. Triple-DHFR/single-DHPS mutations were found in 3.3%, double-DHFR/single-DHPS occurred in 5.8% of samples. It was also found that about 11.5% infections contained triple-DHFR with wild type on the DHPS gene. The random selection of these mutations might generally, be a consequent of country-wide use of SP as a second-line antimalarial drug several years before it was implemented as an interim first-line drug in Tanzania by 2001.

In this study, the prevalence of double-DHPS mutation which is considered to be a prerequisite for resistance to sulphonamides (Kublin *et al.*, 2002) was found to be 6x that reported by Mugittu *et al.* (2004) in the same place (Fig 2). This proportion (19.8%) was also higher than that reported in Kyela and Masasi areas of Tanzania but nearly equal to that reported in Butimba (Fig 2) but less than that reported in Mkuzi

areas of Tanzania. However, this prevalence (19.8%) was higher than that generally reported by Jelinek *et al.* (2002) in East Africa of 5.7%. This might be attributed to the effect of septrin, an antifolate extensively used in Tanzania as antibiotic agent, indirectly and preferentially selecting double mutations on the DHPS locus. Takahashi *et al.* (2000) reported the use of co-trimoxazole as prophylaxis against opportunistic infections in HIV-infected individuals to largely select mutations on DHPS locus than on the DHFR locus. Similar effects can result from septrin, which is essentially similar to co-trimoxazole in composition as previously stated. Both are antifolates basically composed of trimethoprim-sulphamethoxazole (TS) with similar effects to SP (Khalil *et al.*, 2003; Bwijo *et al.*, 2003). Observation (Table 10) also showed that 52.1% of infections harboured at least one mutation on the DHPS locus. This reflected that probably the effect or the prevailing use of septrin in the area was high. The high proportion of double DHPS mutations when coupled with triple mutations in DHFR can result into quintuple mutation rendering SP questionable as first-line antimalarial drug in endemic areas like Mlimba.

Existence of mixed genotypes (wild type/mutant) in the DHFR and DHPS loci was also detected in this study. Sixty-eight samples (Table 11) contained mixed genotypes in either DHFR or DHPS genes. Of these mixed genotypes, 5 (7.3%) had triple DHFR mixed genotypes and 8 (11.8%) possessed double DHFR mixed genotypes. Only 1 (1.5%) of restriction products possessed double DHPS mixed genotypes. The rest possessed single mixed genotypes in either DHFR or DHPS genes and are presented in Table 11. The detection of mixed genotypes in the DHFR and DHPS found in this study is important due to its influence on the overall proportion of point

mutations in baseline samples which upon drug pressure, the wild type got cleared with mutant genotypes persisting in longitudinal follow up samples. Most recrudescent infection detected in follow up cases came from pure mutant and mixed genotypes in baseline samples as parasites with wild type genotypes are sensitive and were cleared post-medication by the study drug.

Of the 15 follow up samples with positive PCR results (Table 12), only 8 samples had detectable restriction digests at D7 of which six (87.5%) retained the mutant genotypes detected in baseline and D3 samples. In these samples, it was observed that at least double mutations were detected in DHFR and DHPS genes. One (12.5%) of the samples harboured mixed genotypes on codon 540 of the DHPS locus instead of the mutant genotype, which was obtained in baseline and D3 samples. In this sample, probably a new infection arose at D7 of the study. On day 14, the wild type variant detected on D7 was cleared and only mutant variants remained. In other samples, variants similar to what was previously detected in the same patient on D7 were observed on D14. The point mutations on DHFR gene were predominantly those occurring at codon 59 and 108 and the double mutations in the DHPS involved codons 437 and 540 signifying the role of antifolate combination, SP in selecting point mutations at these genes. Similar results were reported by Khalil *et al.* (2003) in Sudan post TS (co-trimoxazole) medication. In this context the results reflected that, probably the antifolates SP and TS were concurrently used in the study area prior to this study.

Results also showed that one sample, harboured mixed variants on codon 51 and 108 in the DHFR domain and wild type genotypes in the DHPS domain on D0 (Table 12). Nevertheless, no restriction digests could be detected on days 3, 7 and 14. In this sample, probably there was no enough DNA template available in the sample for restriction digestion (Aubouy *et al.*, 2003a). Similarly, the use of SP might have slowly cleared the parasite in three samples which harboured triple DHFR and single DHPS on days 0 and 3 but not in days 7 and 14. For one sample that harboured mixed triple DHFR and wild type DHPS on D0 which disappeared on D3 and D7 but reappearing again on D14, probably a new infection emerged on day 14. Alternatively, the use of SP might have cleared the sensitive parasite clone detected on D0 and suppressed the mutant strain on D3 and D7. The latter could be the one, which later expressed in the sample on D14.

In summary, the study results showed that, at D14, of the fifteen supposed recrudescent infections, 7 (46.7%) were recrudescent and 2 (13.3%) were probably new infections. Four infections (26.7%) possessed parasite clones, which later were suppressed from D3 to D14 by the study drug. Failure of detection of restriction digests in these samples could be due to low DNA template for effective restriction digestion. No restriction digests could be determined in two samples (T08011 and T08018) probably due to absence of target DNA in these samples. The high proportion of resurgence at D14 might possibly suggest the association between point mutations in the DHFR and DHPS genes with recurrent infection.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study used MSP2 genes and the DHFR and DHPS genes as measures of multiplicity of infections and SP resistance markers in *P. falciparum*, respectively. The three marker genes were also used to establish recrudescent and new infecting malaria parasites. The findings from this study showed that the marker gene were suitable for use in the aimed study objectives. Genotyping of these genes enabled establishment of multiplicity of infections and level of recrudescence in the study area. From this study we cannot rule out that clonal disappearance during follow up was the result of treatment activity, and its reappearance due to re-infection by new mosquito bite. Generally, disappearance of clones indicates sensitive clones while recrudescence reflects drug resistance. The malaria parasite in Mlimba was highly polymorphic and diverse due to high number of different alleles detected in MSP2 allelic families.

High proportion of point mutations associated with SP resistance was detected in baseline samples indicating that there is extensive use of antifolates other than SP, which indirectly contributed in selecting mutations towards SP resistance. In addition, selection of mutations against SP might have resulted from abuse of the drug in this area. However, despite the high proportion of SP resistance point mutations detected in this study, most of infections cleared at day 14. Probably population immunity in Mlimba against the disease is high enough to combat the infection. Nevertheless, it is possible that the point mutation investigated in this study alone, are not sufficient in

conferring total SP resistance. It can be suggested from this study that, the DHFR and DHPS remain to be useful markers of SP resistance and important tools for monitoring drug resistance in malaria endemic areas. However, there is still a necessity to advocate the rational use of the drug and conduct continued surveillance to monitor resistance. In addition, the evaluation of different alternative treatments, especially combination therapies should go faster so that an effective, safe and affordable antimalarial drug is obtained. This will aid not only to replace SP as an interim first-line drug in Tanzania but also to reduce the disease burden in the country and contribute to the universe in fighting against the disease.

Generally, it can be concluded that the impact of quintuple mutation on SP resistance is weighed down by host immunity in endemic areas although may not suggest continued use of the drug for treatment against malaria. The impact of other drugs with similar mechanisms of action used as antibiotics in selecting mutations responsible for SP resistance need be studied especially for co-trimoxazole, which is currently used as a prophylaxis against opportunistic infections in HIV-infected individuals. The information obtained will be of direct and immediate relevance to current HIV and malaria control policies in Tanzania and possibly in Africa and the universe. In addition, it will add to our basic knowledge of the molecular basis of antifolate-resistant malaria.

6.2 Recommendations

There is a need for reviewing the policy on the use of SP as a first-line drug for treating malaria in Tanzania. Short-acting antimalarials such as chlorproguanil-dapsone combination (LapDap) and atovaquone-proguanil (Malarone®) may be rewarding albeit thorough clinical trials are still needed to evaluate the possible harmful side effects of these proposed drugs. In deployment of a new antimalarial drug for treating malaria, the effect of other drugs with similar modes of action to the drug, used in treating other infections than malaria have to be considered to preclude the possibilities of early development of resistance to the drug due to cross-resistance. The findings from this study propose that *in vivo* studies be further performed to confirm that the high frequency of SP resistance alleles is indicative of treatment failure. Improvement of health services with adequate drugs and skilled medical staffs from village levels may reduce uncontrolled and inappropriate use of the drug, consequently reducing the chances of selecting SP resistance mutations against malaria.

7.0 REFERENCES

Alifrangis, M., Enosse, S., Khalil, I.F., Tarimo, D.S., Lemnge, M.M., Thompson, R., Bygbjerg, I.C. and Rønn, A.M. (2003). Prediction of *Plasmodium falciparum* resistance to sulphadoxine/pyrimethamine *in vivo* by mutations in the dihydrofolate reductase and dihydropteroate synthase genes: A comparative study between sites of differing endemicity. *American Journal of Tropical Medicine and Hygiene* 69 (6): 601 – 606.

Al-Yaman, F., Genton, B., Reeder, J.C., Anders, R.F. and Alpers, M.P. (1997). Evidence that recurrent *Plasmodium falciparum* infection is caused by recrudescence of resistant parasites. *American Journal of Tropical Medicine and Hygiene* 56: 436 – 439.

Aubouy, A., Jafari, S., Huart, V., Migot-Nabias, F., Mayombo, J., Durand, R., Bakary, M., Le Bras, J. and Deloron, P. (2003a). DHFR and DHPS genotypes of *Plasmodium falciparum* isolates from Gabon correlate with *in vitro* activity of pyrimethamine and cycloguanil, but not with sulphadoxine-pyrimethamine treatment efficacy. *Journal of Antimicrobial Chemotherapy* 52: 43 – 49.

Aubouy, A., Migot-Nabias, F. and Deloron, P. (2003b). Polymorphism in two merozoite surface proteins of *Plasmodium falciparum* isolates from Gabon. *Malaria Journal* 2 (1): 12.

Babiker, H., Randford-Cartwright, L., Sultan, A., Satti, G. and Walliker, D. (1994). Genetic evidences that RI choloroquine resistance of *Plasmodium falciparum* is caused by recrudescence of resistant parasites. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 88: 328 – 331.

Babiker, H.A., Ranford-Cartwright, L.C. and Walliker, D. (1999). The epidemiology of multiple Plasmodium falciparum infections: Genetic structure and dynamics of Plasmodium falciparum infections in the Kilombero region of Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93 (1): 11 – 14.

Basco, L. (2002). Molecular epidemiology of malaria in Cameroun. XII. *In vitro* drug assays and molecular surveillance of chloroquine and proguanil resistance. *American Journal of Tropical Medicine and Hygiene* 67 (4): 383 – 387.

Basco, L.K., De Pecoulas, P.E., Wilson, C.M., Le Bras, J. and Mazabraud A. (1995). Point mutations in the dihydrofolate reductase-thymidylate synthase gene and pyrimethamine and cycloguanil resistance in *Plasmodium falciparum. Molecular & Biochemical Parasitology* 69: 135 – 138.

Basco, L.K., Tahar, R. and Ringwald, P (1998). Molecular basis of *in vivo* resistance to sulphadoxine-pyrimethamine in Africa adult patients infected with *Plasmodium falciparum* malaria parasites. *Antimicrobial Agents and Chemotherapy* 42: 1811 – 1814.

Basco, L. and Ringwald, P. (2000). Molecular epidemiology of malaria in Younde, Cameroun. VI. Sequence variation in the *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase gene and *in vitro* resistance to pyrimethamine and cycloguanil. *American Journal of Tropical Medicine and Hygiene* 62 (2): 271 – 276.

Basco, L.K., Tahar, R., Keundjian, A., and Ringwald, P. (2000). Sequence variations in the genes encoding dihydropteroate synthase and dihydrofolate reductase and clinical response to sulphadoxine-pyrimethamine in patients with acute

uncomplicated falciparum malaria. *Journal of Infectious Diseases* 182: 624 – 628.

Beck, H.-P., Felger, I., Huber, W., Steiger, S., Smith, T., Weiss, N., Alonso, P.L. and Tanner, M. (1997). Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the trial of the malaria vaccine SPf66. *Journal of Infectious Diseases* 175: 921 – 926.

Berglez, J., Iliades, P., Sirawaporn, W., Coloe, P. and Macreadie, I. (2004). Analysis in *Eschelichia coli* of *Plasmodium falciparum* dihydropteroate synthase (DHPS) alleles implicated in resistance to sulphadoxine. *International Journal for Parasitology* 34: 95 – 100.

Biswas, S. (2004). Association of antifolate resistance *in vitro* and point mutations in dihydrofolate reductase and dihydropteroate synthetase gene of *Plasmodium falciparum*. *Journal of Postgraduate Medicine* 50: 17 – 20.

Bousema, J.T., Gouagna, L.C., Meutstege, A.M., Okech, B.E., Akim, N.I.J., Githure, J.I., Beier, J.C. and Sauerwein, R.W. (2003). Treatment failure of pyrimethamine-sulphadoxine and induction of *Plasmodium falciparum* gametocytaemia in children in western Kenya. *Tropical Medicine and International Health* 8 (5): 427 – 4 30.

Bwijo B., Kaneko, A., Takechi, M., Zungu, I.L., Moriyama, Y., Lum, J.K., Tsukahara, T., Mita, T., Takahashi, N., Bergqvist, Y., Björkman, A. and Kobayakawa, T. (2003). High prevalence of quintuple mutant DHPS/DHFR genes in *Plasmodium falciparum* infections seven years after introduction of sulphadoxine and pyrimethamine as first line treatment in Malawi. *Acta Tropca* 85: 363 – 373.

Clyde, D.F. (1954). Observations on monthly pyrimethamine (Daraprim) prophylaxis in an East African village. *East African Medical Journal* 3: 41 – 46.

Clyde, D.F. and Shute, G.T. (1957). Resistance of Plasmodium falciparum in Tanganyika to pyrimethamine administered at weekly intervals. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 51: 505 – 513.

Contamin, H., Fandeur, T., Bonnefoy, S., Skouri, F., Ntoumi, F. and Mercereau-Puijalon, O. (1995). PCR typing of field isolates of *Plasmodium falciparum. Journal of Clinical Microbiology* 33: 944 – 951.

Cortese, J.F. and Plowe, C.V. (1998). Antifolate resistance due to new and known *Plasmodium falciparum* dihydrofolate reductase mutations expressed in yeast. *Molecular Biochemistry and Parasitology* 94: 205 – 214.

Cowman, A.F. (1998). Malaria: Parasite Biology, Pathogenesis, and Protection. In: *The Molecular Basis of Resistance to the Sulphones, Sulphonamides, and Dihydrofolate Reductase Inhibitors. (Edited by Irwin W. Sherman).* ASM Press, Washington, D.C. pp. 317 – 339.

Curtis, J., Duraisingh, M.T. and Warhurst, D.C. (1998). In vivo selection for a specific genotype of dihydropteroate synthase of *Plasmodium falciparum* by pyrimethamine-sulfadoxine but not chlorproguanil-dapsone treatment. *Journal of Infectious Diseases* 177: 1429 – 1433.

Danis, M. and Bricaire, F. (2003). The new drug combinations: their place in the treatment of uncomplicated *Plasmodium falciparum* malaria. *Fundamental and Clinical Pharmacology* 17 (2): 155 – 160.

De Pecoulas, P.E., Basco, L.K., Le Bras, J. and Mazabraud, A. (1996). Association between antifolate resistance *in vitro* and DHFR gene point mutation in

Plasmodium falciparum isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 90: 181 – 182.

Diourte, Y., Djimde, A., Doumbo, O.K., Sagara, I., Coulibaly, Y., Dicko, A., Diallo, M., Diakite, M., Cortese, J.F., Plowe, C.V. (1999). Pyrimethamine-sulfadoxine efficacy and selection for mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase in Mali. *American Journal of Tropical Medicine and Hygiene* 60 (3): 475 – 8.

Duraisingh, T..M., Curtis, J. and Warhurst, D.C. (1998). *Plasmodium falciparum*: Detection of polymorphisms in the Dihydrofolate Reductase and Dihydropteroate Synthase Genes by PCR and Restriction Digestion. *Experimental Parasitology* 89: 1 – 8.

Eisen, D., Billman-Jacobe, H., Marshall, V. F., Fryauff, D. and Coppel, R. L. (1998). Temporal variation of the merozoite surface protein-2 gene of *Plasmodium falciparum*. *Infection and Immunity* 66: 239 – 246.

Färnert, A., Snounou, G., Rooth, I. and Björkman, A. (1997). Daily dynamics of *Plasmodium falciparum* sub-populations in asymptomatic children in a holoendemic area. *American Journal of Tropical Medicine and Hygiene* 56: 538 – 547.

Felger, I., Tavul, L. and Beck, H.P. (1993). *Plasmodium falciparum*: A rapid technique for genotyping the merozoite surface protein 2. *Experimental Parasitology* 77: 372 – 375.

Felger, I., Tavul, L., Kabintik, S., Marshall, V., Genton, B., Alpers, M. and Beck, H-P. (1994). *Plasmodium falciparum*: Extensive polymorphisms in merozoite

surface protein 2 alleles in an area with endemic malaria in Papua New Guinea. *Experimental Parasitology* 79: 106 – 116.

Felger, I., Marshal, V. M., Reeder, J. C., Hunt, J. A., Mgone, C. S. and Beck, H. P. (1997). Sequence diversity and molecular evolution of the merozoite surface antigen 2 of *Plasmodium falciparum. Journal of Molecular Evolution* 45: 154 – 160.

Felger, I., Irion, A., Steiger, S. and Beck, H.-P. (1999). Epidemiology of multiple *Plasmodium falciparum* infections: Genotypes of merozoite surface protein 2 of *Plasmodium falciparum* in Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93 (1): 3 – 9.

Fenton, B., J. T., Clark, C. M., Khan, J. V., Robinson, D., Walliker, R., Ridley, J. G., Scaife, and McBride, J. S. (1991). Structural and antigenic polymorphism of the 35- to 48-kilodalton merozoite surface antigen (MSA-2) of the malaria parasite *Plasmodium falciparum. Molecular and Cellular Biology* 11: 963 – 974.

Foley, M., Ranford-Cartwright, L.C., Babiker, H.A. (1992). Rapid and simple method for isolating malaria DNA from fingerprick samples of blood. *Molecular and Biochemical Parasitology* 53: 241 – 244.

Greenwood, B. (2002). The Molecular Epidemiology of Malaria. *Tropical Medicine and International Health* 7 (12): 1012 – 1021.

Gyang, F.N., Peterson, D.S. and Wellems, T.E. (1992). *Plasmodium falciparum*, rapid detection of dihydofolate reductase mutations that confer resistance to cycloguanil and pyrimethamine. *Experimental Parasitology* 74: 470 – 472.

Hankins, E.G., Warhurst, D.C. and Sibley, C.H. (2001). Novel alleles of the *Plasmodium falciparum* DHFR highly resistant to pyrimethamine and chlorproguanil, but not to WR99210. *Molecular and Biochemical Parasitology* 117: 91 – 107.

Hastings, M.D., Bates, S.J., Blackstone, E.A., Monks, S.M., Mutabingwa, T.K. and Sibley, C.H. (2002). Highly pyrimethamine-resistant alleles of dihydrofolate reductase in isolates of *Plasmodium falciparum* from Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 96: 674 – 676.

Hill, W.G and Babiker, H.A. (1995). Estimation of numbers of malaria clones in blood samples. *Proceedings of the Royal Society of London, Series B, Biological Sciences* 262: 249 – 257.

Hoffmann, E.H., da Silveira, L.A., Tonhosolo, R., Pereira, F.J., Ribeiro, W.L., Tonon, A.P., Kawamoto, F. and Ferreira, M.U. (2001). Geographical patterns of allelic diversity in the *Plasmodium falciparum* malaria-vaccine candidate, merozoite surfaces protein 2. *Annals of Tropical Medicine and Parasitology* 95: 117 – 132.

Irion, A., Beck, H.P., and Felger, I. (1997). New repeat unit and hot spot of recombination in FC27-type alleles of the gene coding for the *Plasmodium falciparum* merozoite surface protein 2. *Molecular and Biochemical Parasitology* 90: 367 – 370.

Irion, A., Felger, I., Abdulla, S. Smith, T., Mull, R., Tanner, M., Hartz, C. and Beck, H.P. (1998). Distinction of recrudescence from new infections by PCR-RFLP analysis in a comparative trial of CGP 56 697 and chloroquine in Tanzanian children. *Tropical Medicine and International Health* 3: 490 – 497.

Irion, A. (2000). Molecular diversity and immunological properties of the *Plasmodium falciparum* merozoite surface protein 2 (MSP2). Unpublished Dissertation for Award of PhD degree at University of Basel, Basel, Switzerland, pp. 11 – 124.

Jafari, S., Le Bras, J., Asmar, M. and Durand, R. (2003). Molecular survey of *Plasmodium falciparum* resistance in south-eastern Iran. *Annals of Tropical Medicine and Parasitology* 97 (2): 119 – 124.

Jelinek, T., Ronn, A.M., Curtis, J., Duraisingh, M.T., Lemnge, M.M., Minha, J., Bygbjerg, I.C. and Warhurst, D.C. (1997). High prevalence of mutations in the dihydrofolate reductase gene of *Plasmodium falciparum* in isolates from Tanzania without evidence of an association to clinical SP resistance. *Tropical Medicine and International Health* 2: 1075 – 1079.

Jelinek, T, Peyer-Hoffmann, G., Mühlberger, N., Wichmann, O., Wilhelm, M., Schmide, N., Grobusch, M.P., von Sonnenburg, F., Gascon, J., Laferl, H., Hartz, C., Alifrangis, M., Burchard, G., McWhinney, P., Schulze, M., Kollaritsch, H., da Cunha, S., Beřan, J., Kern, P., Gjørup, I. and Cuadros, J. (2002). Molecular surveillance of drug resistance through imported isolates of *Plasmodium falciparum* in Europe. *Malaria Journal* 1:11

Kassberger, F., Birkenmaeir, A., Khattab, A., Kremsner, P.G. and Klinkert, M.-Q. (2002). PCR typing of *Plasmodium falciparum* in matched peripheral, placental and umbilical cord blood. *Parasitology Research* 88: 1073 – 1079.

Khalil, I., Rønn, A.M., Alifrangis, M., Gabar, H.A., Satti, G..M.H. and Bygbjerg, I.C. (2003). Dihydrofolate reductase and dihydropteroate synthase genotypes associated with *in vitro* resistance of *Plasmodium falciparum* to

pyrimetamine, trimethoprim, sulphadoxine and sulphamethoxazole. *American Journal of Tropical Medicine and Hygiene* 68 (5): 586 – 589.

King, M.W., (August 2003). Medical Biochemistry. *mking@medicine.indstate.edu* [http://www.indstate.edu/thcme/mwking/vitamins.html] site visited on 20th May 2004.

Kitua, A.Y. (2000). Malaria of *P. falciparum*: Facts on Unsolved Problems. In: *Report of the Interdisciplinary Workshop and PhD Course* (Edited by Blegvad, L. *et al.*). 17 – 25 January 2000, Arusha Tanzania, pp 5 – 6.

Kofoed, P.E, Alfrangis, M., Poulsen, A., Rodrigues, A., Gjedde, S.B., Ronn, A., and Rombo, L. (2004). Genetic markers of resistance to pyrimethamine and sulfonamides in *Plasmodium falciparum* parasites compared with the resistance patterns in isolates of *Escherichia coli* from the same children in Guinea-Bissau. *Tropical Medicine & International Health* 9 (1): 171 – 177.

Konate, L., Zwetyenga, J., Rogier, C., Bischoff, E., Fontenille, D., Tall, A., Spiegel, A., Trape, J-F. and Mercereau-Puijalon, O. (1999). The epidemiology of multiple *Plasmodium falciparum* infections: variation of *Plasmodium falciparum* MSP1 block 2 and MSP2 allele prevalence and of infection complexity in two neighbouring Senegalese villages with different transmission conditions. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93 (1): 21 –28.

Krawetz, S.A., Pon, R.T. and Dixon G.H. (1989). Increased efficacy of Taq Polymerase catalysed PCR. *Nucleic Acids Research* 17: 819.

Kublin, J. G., Witzig, R. S., Shankar, A. H., Zurita, J. Q., Gilman, R. H., Guarda, J. A., Cortese, J.F. and Plowe, C.V. (1998). Molecular assays for surveillance of antifolate-resistant malaria. *Lancet* 351: 1629 – 1630.

Kublin, J.G., Dzinjalamala, F.K., Kamwendo D.D., Malkin, E.M., Cortese, J.S., Martino, L.M., Mukadam, R.A.G., Rogerson, S.J., Lescano, A.G., Molyneux, M.E., Winstanley, P.A., Chimpeni, P., Taylor, T.E. and Plowe, C.V. (2002). Molecular markers for failure of sulphadoxine-pyrimethamine and chlorproguanil-dapsone treatment of *Plasmodium falciparum* malaria. *Journal of Infectious Diseases* 185: 380 – 388.

Kun, J.F., Lehman, L.G., Lell, B., Schmidt-Ott, R. and Kremsner, P.G. (1999). Low-dose treatment with sulfadoxine-pyrimethamine combinations selects for drug-resistant Plasmodium falciparum strains. *Antimicrobial Agents and Chemotherapy* 43 (9): 2205 – 2208.

Kyabayinze, D., Cattamanchi, A., Kamya, M.R., Rosenthal, P.J. and Dorsey, G. (2003). Validation of a simplified method for using molecular markers to predict sulphadoxine-pyrimethamine treatment failure in African children with falciparum malaria. *American Journal of Tropical Medicine and Hygiene* 69 (3): 247 – 252.

Lowe, T., Sharefkin, J., Yang, S.Q. and Dieffenbach, C.W. (1990). A Computer Program for Selection of Oligonucletide Primers for PCR. *Nucleic Acids Research* 18: pp. 1757 – 1761.

Mathews, C.K., Van Holde, K.E. and Ahern, K.G. (2000). *Biochemistry*. Addison Wesley Longman, Inc. San Francisco, USA. 1186pp.

Méndez, F., Munos, A. and Carrasquilla, G. (2002). Determinants of treatment response to sulphadoxine-pyrimethamine and subsequent transmission potential of in falciparum malaria. *American Journal of Epidemiology* 156: 230 – 238.

MIM/TDR, (January, 2003). Antimalarial drug resistance network (ADRN): Guideline for protocols involving antimalarial drug resistance. [http://www.nlm.nih.gov/adrn/sop_molecular_markers.pdf] site visisted on 18/1/2003.

Ministry of Health (1998) *Health Statistics Abstract, Vol. 1 Morbidity and Mortality Data*. Dar es Salaam, United Republic of Tanzania

Ministry of Health (1999). Summary report of the Task Force on Antimalarial Drug Policy. Dar es Salaam, United Republic of Tanzania.

Mookherjee, S., Howard, V., Nzila-Mouanda A, Watkins, W. and Sibley, C.H. (1999) Identification and analysis of dihydrofolate reductase alleles from Plasmodium falciparum present at low frequency in polyclonal patient samples. *American Journal of Tropical Medicine and Hygiene* 61 (1): 131 – 140.

Mshinda, H. (2000). The status of, and development of, resistance to antimalarials in an area of high perennial transmission. Unpublished Dissertation for Award of PhD Degree of University of Basel, Basel, Switzerland, pp. 165.

Mugittu, K., Ndejembi, M., Malisa, A., Lemnge, M., Premji, Z., Mwita, A., Nkya, W., Kataraihya, J., Abdulla, S., Beck, H-P. and Mshinda, H. (2004). Therapeutic efficacy of sulfadoxine-pyrimethamine and prevalence of resistance markers in Tanzania prior to revision of malaria treatment policy:

Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase mutations in monitoring *in vivo* resistance. *American Journal of Tropical Medicine and Hygiene* (2004, In Press).

Mutabingwa, T., Nzila, A., Mberu, E., Nduati, E., Winstanley, P., Hills, E. and Watkins, W. (2001). Chlorproguanil-dapsone for treatment of drug-resistant falciparum malaria in Tanzania. *Lancet* 358: 1218 – 1223.

Nagesha, H.S., Din, S., Casey, G.J., Susanti, A.I., Fryauff, D.J., Reeder, J.C. and Cowman, A.F. (2001). Mutations in the pfmdr1, dhfr and dhps genes of Plasmodium falciparum are associated with *in-vitro* drug resistance in West Papua, Indonesia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 95: 43 – 49.

Nsobya, S.L., Parikh, S., Kironde, F., Lubega, G., Kamya, M.R., Rosenthal, P.J. and Dorsey, G. (2004). Molecular evaluation of the natural history of asymptomatic parasitaemia in Ugandan children. *Journal of Infectious Diseases* 189: 2220 – 2226.

Nzila, A.M., Nduati, E., Mberu, E.K., Hopkins, S.C., Monks, S.A., Winstanley, P.A. and Watkins, W.M. (2000). Molecular evidence of greater selective pressure for drug resistance exerted by the long-acting antifolate pyrimethamine/sulphadoxine compared with the shorter-acting chlorproguanil/dapsone on Kenyan *Plasmodium falciparum*. *Journal of Infectious Diseases* 181: 2003 – 2028.

Nzila, A.M., Mberu, E.K. and Sulo, J. (2002). Towards an understanding of the mechanism of pyrimethamine-sulphadoxine resistance in *Plasmodium falciparum*: genotyping of dihydrofolate reductase and dihydropteroate

synthase of Kenyan parasites. *Antimicrobial Agents and Chemotherapy* 44: 991 – 996.

Omar, S.A., Adagu, I.S., Warhurst, D.C. (2001). Can pre-treatment screening for DHPS and DHFR point mutations in *Plasmodium falciparum* infections be used to predict sulphadoxine-pyrimethamine treatment failure? *Transactions of the Royal Society of Tropical Medicine and Hygiene* 95: 315 – 319.

Owusu-Agyei, S., Smith, T., Beck, H-P., Amenga-Etego and Felger, I (2002). Molecular epidemiology of *Plasmodium falciparum* infections among asymptomatic inhibitants of a holoendemic malarious area in northern Ghana. *Tropical Medicine and International Health* 7 (5): 421 – 428.

Patel, O., Satchel, J., Baell, J., Fernley, R., Coloe, P., and Macreadie, I. (2003). Inhibition studies of sulphonamide-containing folate analogues in yeast. *Microbiology and Drug Resistance* 9: 140 – 146.

Pearce, R.J., Drakeley, C., Chandramohan, D., Mosha, F. and Roper, C. (2003). Molecular Determination of Point Mutation Haplotypes in the Dihydrofolate Reductase and Dihydropteroate Synthase of *Plasmodium falciparum* in Three Districts of Northern Tanzania. *Antimicrobial Agents and Chemotherapy* 47 (4): 1347 – 1354.

Peterson, D.S., Milhous, W.K. and Wellems, T.E. (1990). Molecular basis of differential resistance to cycloguanil and pyrimethamine in Plasmodium falciparum malaria. *Proceedings of the National Academy of Sciences, USA* 87: 3014 – 3022.

Philips, R.S. (2001). Current Status of Malaria and Potential for Control: Clinical Microbiology Reviews. *American Society for Microbiology* 14 (1): 208 – 226.

Plowe, C.V., Djimde, A., Bouare, M., Doumbo, O. and Wellems, T.E. (1995). Pyrimethamine and proguanil resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *American Journal of Tropical Medicine and Hygiene* 52 (6): 565 - 568.

Plowe, C.V., Djimde, A., Wellems, T.E., Diop, S., Kouriba, B. and Doumbo, O.K. (1996). Community pyrimethamine-sulfadoxine use and prevalence of resistant Plasmodium falciparum genotypes in Mali: a model for deterring resistance. *American journal Journal of Tropical Medicine and Hygiene* 55 (5): 467 - 71.

Plowe, C.V., Joseph, F.C., Abdoulaye, D., Okey, C.N., William, M.W., Peter, A.W., Jose, G.E.F., Rene, E.M., Juan, C.A., Jose, L.C., Darrick, C. and Ogobara, K.D. (1997). Mutations in *Plasmodium falciparum* Dihydrofolate Reductase and Dihydropteroate Synthase and Epidemiologic Patterns of Pyrimethamine-Sulfadoxine Use and Resistance. *Journal of Infectious Diseases* 176: 1590 – 6.

Plowe, C.V. (2003). Monitoring antimalarial drug resistance: making the most of the tools at hand. *The Journal of Experimental Microbiology* 206: 3745 – 3752.

Primrose, S.B., Twyman, R.M. and Old, R.W. (2001). Principles of Gene Manipulation. In: *Basic Techniques. (Edited by Osney Mead.)*. Oxford OX2 0EL, London. 390pp.

Rallon, N.I., Osario, L.E., Giraldo, L.E. (1999). Lack of association between ASN-108 mutation in the dihydrofolate reductase gene and *in vivo* resistance to

sulfadoxine/pyrimethamine in *Plasmodium falciparum. American Journal of Tropical Medicine and Hygiene* 61: 245 – 248.

Ranford-Cartwright, L. C., Taylor, R. R., Asgari-Jirhandeh, N., Smith, D. B., Roberts, V., Robinson, P. E. I., Babiker, H. A., Riley, E. M., Walliker, D. and McBride, J. S. (1996). Differential antibody recognition of FC27-like *Plasmodium falciparum* merozoite surface protein MSP2 antigens which lack 12 amino acid repeats. *Parasite Immunology* 18: 411 – 420.

Ranford-Cartwright, L. C., Johnston, K.L., Abdel-Muhsin, A.M., Khan, B.K. and Babiker, H.A. (2002). Critical comparison of molecular genotyping methods for detection of drug resistant *Plasmodium falciparum. Transaction of the Royal Society of Tropical Medicine and Hygiene* 96 (5): 568 – 572.

Reeder, J.C., Rieckmann, K.H., Genton, B., Lorry, K., Wines, B. and Cowman, A.F. (1996). Point mutations in the dihydrofolate reductase and dihydropteroate synthetase genes and *in vitro* susceptibility to pyrimethamine and cycloguanil of *Plasmodium falciparum* isolates from Papua New Guinea. *American Journal of Tropical Medicine and Hygiene* 55: 209 – 213.

Rønn, A.M., Msangeni, H.A., Mhina, J., Wernsdorfer, W.H. and Bygbjerg, I.C. (1996). High level of resistance of *Plasmodium falciparum* to sulfadoxine-pyrimethamine in children in Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 90: 179 – 181.

Roper, C., Pearce, R., Bredenkamp, B., Gumede, J., Drakeley, C., Mosha, F., Chandramohan, D. and Sharp, B. (2003). Antifolate antimalarial resistance in southeast Africa: a population-based analysis. *Lancet* 361: 1174 – 1181.

Sambrook, J., Fritch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory mannual 2nd Edition. Cold spring Harbour Laboratory Press, New York. pp. 6.0 – 6.62.

Saul, A., Lord, R., Jones, G., Geysen, H. M., Gale, J. and Mollard, R. (1989). Cross-reactivity of antibody against an epitope of the *Plasmodium falciparum* second merozoite surface antigen. *Parasite Immunology* 11: 593 – 601.

Schellenberg, D., Kahigwa, E., Drackeley, C., Malende, A., Wigayi, J., Msokame, C., Aponte, J.J., Tanner, M., Mshinder, H., Menendez, C. and Alonso, P. (2002). The safety and efficacy of sulfadoxine, pyrimethamine, amodiaquine, and their combination in the treatment of uncomplicated *Plasmodium falciparum* malaria. *American Journal of Tropical Medicine and Hygiene* 67: 17 – 23.

Scythe, J.A., Peterson M.G., Compel, R.L., Saul, A.J., Kemp, D.J., Anders, and R.F. (1990). Structural diversity in the 45-kilodalton merozoite surface protein antigen of *Plasmodium falciparum. Molecular and Biochemical Parasitology* 39: 227 – 234.

Smith, T., Beck, H.-P., Mwankusye, S., Felger, I., Fraser-Hurt, N., Iron, A., Alonso, P.L., Teuscher, T. and Tanner, (1999). The epidemiology multiplicity of *Plasmodium falciparum* infections 3. Age dependence of the multiplicity of *Plasmodium falciparum* infections and other malariological indices in an area of high endemicity. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93 (1): 11 – 14.

Smythe, J. A., Coppel, R.L., Day, K. P., Martin, R. K., Oduola, A. M. J., Kemp, D. J. and Anders, R. F. (1991). Structural diversity in the *Plasmodium falciparum*

merozoite surface antigen 2. *Proceedings of the National Academy of Sciences of USA* 88: 1751 – 1755.

Snewin, V. A., Herrera, M., Sanchez, G., Scherf, A., Langsley, G., and Herrera, G. (1991). Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in *Plasmodium falciparum* wild isolates from Colombia. *Molecular and Biochemical Parasitology* 49: 265 – 275.

Snounou, G., Whu, X., Siripoon, N., Jarra, W., Thaithong, S., Brown, K.N. and Viriyakosol, S. (1999). Biased distribution of MSP1 and MSP2 allelic variants in *Plasmodium falciparum* populations in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93: 369 – 374.

Swiss Tropical Institute (STI) Report (1999 - 2000). An Overview of STI on Malaria. pp 10 – 22.

Takahashi, T., Hosoya, N., Endo, T., Nakamura, N., Sakashita, H., Kimura, K., Ohnishi, K., Nakamura, Y., and Iwamoto, A. (2000). Relationship between Mutations in Dihydropteroate Synthase of ***Pneumocystis carinii*** f. sp. ***hominis*** Isolates in Japan and Resistance to Sulfonamide Therapy. *Journal of Clinical Microbiology* 38 (9): 3161 – 3164.

Triglia, T., Menting, J.G., Wilson, C. and Cowman, A.F.. (1997). Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum. Proceedings of the National Academy of Sciences of USA* 94: 13944 – 13949.

Triglia, T., Wang, P., Sims, P.F.G., Hyde, J.E. and Cowman, A.F. (1998). Allelic exchange at the endogenous genomic locus in *Plasmodium falciparum* proves

the role of dihydropteroate synthase in sulfadoxine-resistant malaria. *European Molecular Biology Organization Journal* 17: 3807 – 3815.

Wang, P., Brooks, D.R., Sims, P.F.G. and Hyde, J.E. (1995). A mutation-specific PCR system to detect sequence variation in the dihydropteroate synthetase gene of *Plasmodium falciparum. Molecular Biochemistry and Parasitology* 71: 115 – 125.

Wang, P., Lee, C. S., Bayoumi, R., Djimde, A., Doumbo, O., Swedberg, G., Dao, L. D., Mshinda, H., Tanner, M., Watkins, W. M., Sims, P. F. and Hyde, J. E. (1997a). Resistance to antifolates in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. *Molecular and Biochemical Parasitolology* 89: 161 – 177.

Wang, P., Read, M. Sims, P.F. and Hyde, J.E. (1997b). Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization. *Molecular Microbiology* 23: 979 – 986.

Warsame, M., Kilimali, V.A., Wernsdorfer, W.H., Lebbad, M., Rutta, A.S. and Ericcson, O. (1999). Resistance to chloroquine and sulphadoxine-pyrimethamine in *Plasmodium falciparum* in Muheza District, Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 93: 312 – 313.

Watkins, W.M., Mberu, E.K., Winstanley, P.A. and Plowe, C.V. (1997). The efficacy of antifolate antimalarial combinations in Africa: A predictive model based on

pharmacodynamic and pharmacokinetic analyses. *Parasitology Today* 91: 125 – 132.

Weisman, S., Wang, L., Billman-Jacobe, H., Nhan, D.H., Richie, T.L. and Coppel, R.L. (2001). Antibody Responses to Infections with Strains of *Plasmodium falciparum* Expressing Diverse Forms of Merozoite Surface Protein 2. *Infection and Immunity* 69 (2): 959 – 967.

Wernsdorfer, W.H. and Noedl, H. (2003). Molecular markers for drug resistance in malaria: use in treatment, diagnosis and epidemiology. *Current Opinion on Infections Diseases* 16: 553 – 558.

Winstanley, P.A., Ward, S.A. and Snow, R.W. (2002). Clinical status implication of antimalarial drug resistance. *Microbes and Infection* 4: 157 – 164.

Wiser, M.F. (March 2004). Malaria epedemiology, and host-parasite interaction. [http://www.tulane.edu/~wiser/protozoology/notes/malaria.html#epi] site visited on 4/6/2004.

Wooden, J. M., Hartwell, L. H., Vasquez, B. and Sibley, C. H. (1997). Analysis in yeast of antimalaria drugs that target the dihydrofolate reductase of *Plasmodium falciparum. Molecular and Biochemical Parasitology* 85: 25 – 40.

World Health Organisation (1991). Basic malaria microscopy, Part I & II, WHO, Geneva, Switzerland.

World Health Organization (1996). Assessment of therapeutic efficacy of antimalarial drugs: For uncomplicated falciparum malaria with intense transmission. WHO/TDR, Geneva, Switzerland.

World Health Organization (2001). Assessment of Therapeutic Efficacy of Antimalarial Drugs for Uncomplicated Falciparum Malaria (Working Draft). WHO/TDR, Geneva, Switzerland. 31pp.

World Health Organisation (WHO) (2003). Microbiology & Immunology: BS2024: Malaria. [http: www-micro.msb.le.ac.uk/224/malaria.html] site visited on 13th June 2003.

Zwetyenga, J., Rogier, C., Tall, A., Fontenille, D., Snounou, G., Trape, J-F. and Mercereau-Puijalon, O. (1998). No influence of age in infection complexity and allele distribution in *Plasmodium falciparum* infection in Ndiop, a Senegalese village with seasonal. *American Journal of Tropical Medicine and Hygiene* 59: 726 – 735.

APPENDICES

Appendix 1: WHO-set criteria for the study and for participating patients used in the study as adopted from MIM/TDR Guidelines (2003).

Criteria for inclusion

Criteria for patient inclusion included:

- Age between 6 and 59 months (i.e., under 5 years).
- Signs/symptoms of acute uncomplicated falciparum malaria (WHO, 1987).
- Mono-infection with *P. falciparum*, with asexual blood stage parasitaemia in the range of 2,000 to 100,000 asexual parasites per μL.
- Willingness to give consent to participate.
- Absence of severe illness.
- Presence of axillary temperature of ≥37.5 °C but < 39.5 °C

Criteria for exclusion

- Presence of signs of severe complicated falciparum malaria (WHO, 1989).
- Cerebral malaria (unrousable coma)
- Vomiting > twice within preceding 24 hours
- One convulsion within preceding 24 hours
- Inability to drink or breast-feed, or to take oral medication.
- Pulmonary oedema
- Circulatory collapse/shock
- Spontaneous bleeding/disseminated intravascular coagulopathy (DIC)
- Macroscopic haemoglobinuria (urine red or coca cola colour)

- Jaundice
- Other manifestations of complicated malaria (hyperparasitaemia: >5% RBC infected, hyperpyrexia (axillary temperature > 39.5° C).
- A clear history of adequate treatment with drugs expected to be effective in the study area in the preceding 72 hours.
- History of taking drugs with antihistaminic actions, i.e., chlorpheniramine, promethazine, chlorpromazine) within the past 2 days at sites where chloroquine is the first line antimalarial drug.
- Presence of underlying diseases (cardiac, renal, hepatic diseases, malnutrition, gastrointestinal diseases).
- History of allergy to study drug.
- Inability to come for the stipulated follow-up visits, or difficulty in accessing the health facility, or any situation or condition which may compromise ability to comply with the trial procedures.
- Severe normocytic anaemia (haemoglobin < 5g/dL, or haematocrit < 15%)
- Hypoglycaemia (< 40mg/dL)

Criteria for withdraw of a patient from the study

- Danger signs/symptoms of severe malaria as per the WHO definition (WHO, 1990), or a clinical requirement for parenteral therapy.
- Any serious adverse events or severe adverse events requiring withdrawal from the study
- Vomiting after repeated doses.

- Need to use medicaments with antimalarial activity, for the treatment of infections other than malaria during study period (e.g., tetracycline, doxycycline, cotrimoxazole) during treatment with antimalarial.
- Need to use drugs with antihistaminic activity, i.e., chlorpheniramine, promethazine, chlorpromazine, etc., during study period.
- Withdrawal of consent by patient/parent/guardian.
- If the patient is lost to follow up

Criteria for Discontinuation

If at any time the progress of the child was unsatisfactory, the child's parent/guardians was instructed to bring the child to the clinic/health centre.

Objective criteria for discontinuation included:

- Danger signs/symptoms of severe malaria as per the WHO definition (WHO 1990), or a clinical requirement for parenteral therapy.
- Day 3 Parasite count $\geq$ 25% of day 0 count.
- Parasitaemia present on day 7
- Recurrent parasitaemia within 28 days of enrollment.
- Need to use medicaments with antimalarial activity for the treatment of infections other than malaria (e.g., tetracycline, doxycycline, cotrimoxazole) during treatment with antimalarial.
- Need to use drugs with antihistaminic activity, i.e., chlorpheniramine, promethazine, chlorpromazine, etc., during trial medication period.
- Withdrawal of consent by patient/parent/guardian.
- If the patient is lost to follow up

Study Procedures

Pre-treatment assessments

Prior to antimalarial treatment, the following assessments was performed:

- Clinical assessments: Physical examination and monitoring of vital signs symptoms and body temperature (axillary temperature: ^{o}C).
- Finger prick blood sample for microscopic (malaria parasitaemia) examination
- Finger prick blood sample for molecular markers spotted on well absorbing filter paper - Whatman 3 (following detailed protocol in section IV on molecular assays).

Special attention was paid to storage conditions of samples as per storage protocols described in the guidelines, to prevent deterioration

Antimalarial Treatment

- Treatment doses were based on the patient's body weight.
- All treatment doses were given under supervision, and the patient was observed for at least 1hour post administration to ascertain retention of the drug. If the patient vomited within the first 30 minutes post administration, the treatment was repeated with the same dose. If the patient vomited between 30 minutes and 1 hour after dosing, half dose was administered. Children with persistent vomiting was excluded from the study and referred urgently to the appropriate health facility after giving the first dose of the parenteral drug of choice such as quinine drips and injection. These data was recorded in the *Patient Record File (PRF)*.

Antimalarial drug formulation and dose strength

- Sulphadoxine/pyrimethamine was dispensed orally as tablets (500 mg sulphadoxine, 25 mg pyrimethamine).

The study medication was stored at room temperature in cool dry lock up cupboards protected from light.

Recommended treatment regimens

Treatment with sulphadoxine/pyrimethamine (SP) was given as a single dose equivalent to *25 mg/kg body weight based on sulphadoxine component, given once daily).* Fractions of the tablets were rounded up to the nearest quarter.

Concomitant medication

- Care was taken that no folates or drugs of such kind were given to patients receiving sulphadoxine/pyrimethamine at any time during the follow-up.
- Iron was given where necessary at the discretion of the treating clinician, following local recommendations.
- Blood transfusion was given as per local recommendations and patients requiring blood transfusions were withdrawn from the study.
- All other adjunct treatments were administered as required during the trial period. For example, the administration of analgesics such as paracetamol on day 0, 1 and 2 was effected and is permissible if the patient's condition warrants such medication. However, drugs such as tetracycline, quinoline antibiotics, chloramphenicol, cotrimoxazole and rifampicin were not administered during the trial period.

- Any use of concomitant medications was recorded in patient's record chart in details (dose, date and time given and stopped, generic/trade name, etc.).

Alternative treatment of Drug Failures

- Any patients who failed treatment with the trial medication were treated with an effective alternative antimalarial drug. Quinine was given as recommended in the case of poor response to sulphadoxine/pyrimethamine and was given as oral dose of 10 mg (sulphate) per kg body weight every 8 hours for 7 days.
- If the patient developed any signs of severe or complicated malaria or any of the general danger signs during the follow-up period, he or she was given the first dose of the rescue drug and taken urgently to the appropriate health facility.
- Once a patient was treated as a treatment failure, he/she was discontinued from the study. All protocol investigations up to and including the day of treatment failure was done. Thereafter, no other investigation protocol was done but follow up was made on the patient till cure.

Assessment of efficacy

This was done as follows:

- Cumulative cure rate by Day 14

Treatment failure was regarded by the occurrence of any of the following:

Early Treatment Failure (ETF)

- Development of danger signs or severe malaria on Day 1, Day 2 or Day 3, in the presence of parasitaemia.
- Axillary temperature $\geq 37.5^0$C on Day 3 in the presence of parasitaemia.
- Parasitaemia on Day 3 $\geq$ 25% day 0 count.

Late Treatment Failure (LTF)

- Development of danger signs or severe malaria on any day from Day 4 to Day 14:
- Axillary temperature $\geq 37.5^0$C in the presence of parasitaemia on any day from Day 4 to Day 14 without previously meeting the criteria of early treatment failure.

Success Criteria

- Cumulative cure rate by Day 14

Adequate Clinical Response (ACR)

- Absence of parasitaemia on Day 14 irrespective of axillary temperature, without previously meeting any of the criteria of early treatment failure;
- Axillary temperature $\leq 37.5^0$C irrespective of the presence of parasitaemia, without previously meeting any of the criteria of early or late treatment failure.

Appendix 2: Details of diverse number of MSP2 genotypes of *P. falciparum* 3D7 allelic family from Mlimba

MSP2 Genotypes	*Hinf*I restriction fragments (bp)	Number of isolates
1	51, 70, 108	8
2	70, 108, 201	3
3	70, 108, 250	6
4	70, 108, 266	2
5	51, 70, 108, 286	4
6	51, 70, 108, 290	3
7	51, 70, 108, 310	7
8	51, 70, 108, 330	9
9	51, 70, 108, 344	1
10	70, 108, 370	5
11	51, 70, 108, 386	13
12	70, 108, 396	4
13	51, 70, 108, 400	21
14	70, 108, 470	1
15	70, 108, 490	1
16	51, 70, 108, 500	2
17	70, 108, 510	4
18	70, 108, 517	1
19	70, 108, 530	1
20	51, 70, 108, 550	3

Appendix 3: Details of the MSP2 genotypes of the *Plasmodium falciparum* FC27 allelic family from Mlimba

MSP2 Genotypes	*Hinf*I restriction fragments (bp)	Number of isolates
1	1 x 96, 115, 137	14
2	115, 137, 150	3
3	115, 137, 162	3
4	1 x 96, 115, 137, 162	1
5	115, 137, 198	7
6	1 x 96, 115, 137, 198	1
7	115, 137, 200	4
8	115, 137, 234	9
9	115, 137, 270	15
10	1 x 96, 115, 137, 270	1
11	115, 137, 306	8
12	1 x 96, 115, 137, 306	3
13	115, 137, 334	1
14	115, 137, 342	1
15	115, 137, 378	9
16	1 x 96, 115, 137, 378	2

Appendix 4: Summary of multiplicity of infections determined in this study

Sample	3D7	FC27	Multiplicity	REMARKS
T08001	1	0	1	Single
T08002	2	1	3	Triple
T08006	1	0	1	Single
T08007	1	0	1	Single
T08008	1	2	3	Triple
T08009	0	1	1	Single
T08012	0	1	1	Single
T08013	0	1	1	Single
T08014	2	0	2	Double
T08015	1	0	1	Single
T08016	1	0	1	Single
T08017	2	0	2	Double
T08018	2	1	3	Triple
T08019	0	2	2	Double
T08021	0	1	1	Single
T08022	1	1	2	Double
T08024	2	0	2	Double
T08026	1	0	1	Single
T08027	3	1	4	Multiple
T08029	3	3	6	Multiple
T08030	1	1	2	Double
T08032	1	1	2	Double
T08033	1	1	2	Double
T08034	2	1	3	Triple
T08035	1	1	2	Double
T08036	1	1	2	Double
T08037	0	1	1	Single
T08038	0	1	1	Single
T08040	1	1	2	Double
T08041	5	1	6	Multiple
T08043	2	0	2	Double
T08044	1	2	3	Triple
T08045	1	2	3	Triple
T08046	1	1	2	Double
T08048	1	0	1	Single
T08050	2	1	3	Triple
T08052	1	1	2	Double
T08053	1	2	3	Triple
T08057	1	1	2	Double
T08059	3	1	4	Multiple

T08060	1	0	1	Single
T08063	0	1	1	Single
T08068	1	0	1	Single
T08069	1	0	1	Single
T08070	1	1	2	Double
T08071	1	1	2	Double
T08072	1	1	2	Double
T08073	1	1	2	Double
T08074	1	0	1	Single
T08075	0	1	1	Single
T08076	1	1	2	Double
T08077	2	1	3	Triple
T08078	0	1	1	Single
T08079	0	2	2	Double
T08080	1	0	1	Single
T08081	1	0	1	Single
T08082	0	3	3	Triple
T08083	0	2	2	Double
T08084	0	1	1	Single
T08087	0	2	2	Double
T08088	1	0	1	Single
T08089	1	0	1	Single
T08090	1	0	1	Single
T08091	1	4	5	Multiple
T08092	0	2	2	Double
T08093	1	0	1	Single
T08094	2	0	2	Double
T08095	1	0	1	Single
T08099	1	0	1	Single
T08100	0	1	1	Single
T08101	0	1	1	Single
T08104	0	1	1	Single
T08112	1	0	1	Single
T08114	1	0	1	Single
T08116	1	1	2	Double
T08117	1	0	1	Single
T08118	1	0	1	Single
T08119	0	1	1	Single
T08120	1	0	1	Single
T08121	2	1	3	Triple
T08122	1	0	1	Single
T08123	0	1	1	Single
T08124	1	1	2	Double
T08125	0	1	1	Single

T08126	1	2	3	Triple
T08127	0	2	2	Double
T08128	1	0	1	Single
T08129	2	0	2	Double
T08130	2	0	2	Double
T08131	1	0	1	Single
T08132	1	0	1	Single
T08133	0	1	1	Single
T08134	1	0	1	Single
T08136	1	0	1	Single
T08138	1	0	1	Single
T08139	0	1	1	Single
T08140	1	0	1	Single
T08142	2	2	4	Multiple
T08143	1	1	2	Double
T08144	1	1	2	Double
T08145	0	1	1	Single
Total	99	81	180	

Mean multiplicity excluding single infections =

$$\frac{\text{Total infections excluding single infections}}{\text{Number of patients with multiple clones of infections}} = 129/50 = 2.58$$

Mean multiplicity including single infections = $\frac{\text{Total infecting clones per patient}}{\text{Number of patients}}$

= 180/101

= 1.78