IN-VIVO AND MOLECULAR CHARACTERIZATION OF DRUG RESISTANT *Plasmodium falciparum* ISOLATES FROM SOLWEZI, ZAMBIA

BY

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A dissertation submitted to the University of Zambia in fulfillment of the award of the degree of Master of Science in Biochemistry.

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DEPARTMENT OF BIOMEDICAL SCIENCES
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DECLARATION

I, **Roy Mwenechanya,** hereby declare that this dissertation represents my own original work and has not been previously submitted for the award of a degree or any other qualification at this university or any other university.

Signature: _____ Date: 07/06/07

CERTIFICATE OF APPROVAL

This dissertation, of **Roy Mwenechanya,** is approved as fulfilling the requirements for the award of the Degree of Master of Science in Biochemistry of the University of Zambia.

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(Chairperson Board of Examiners) Signature 07-06-07 Date

ABSTRACT

The study assessed the prevalence of drug resistant malarial parasites in patients at Solwezi urban clinic in northwestern province of Zambia in February 2004, using the *in-vivo* and molecular methods. In the *in-vivo* method each patient was monitored for signs and symptoms of the disease following treatment with either sulfadoxine/pyrimethamine (fansidar®), lumefantrine/artemether (coartem®) or quinine. Molecular determination of mutations on the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*), associated with chloroquine resistance, and the dihydropteroate synthase gene (*dhps*) and the dihydrofolate reductase gene (*dhfr*), associated with sulfadoxine and pyrimethamine resistance, respectively, was also done on parasites obtained from the same patients, before treatment, on the day of recruitment to the study. The extracted *Plasmodium* DNA was amplified using nested PCR and the resulting secondary PCR products were digested with restriction enzymes. The digested DNA fragments were separated by electrophoresis using 2 – 2.5% agarose gels and visualized by using UV transillumination after staining with ethidium bromide.

In-vivo analysis revealed a 27% (n=74) fansidar® treatment failure rate. The molecular analysis revealed 11% (n=108) prevalence of the combination mutation at codons 437 and 108 of the *dhps* and *dhfr*, respectively, associated with fansidar® resistance. There was a significant association between the presence of a combination of mutations on both genes and the *in-vivo* S/P treatment response in patients (χ^2, P = 0.001). There was, however, no significant association between the

in-vivo response in patients that received sulfadoxine/pyrimethamine treatment and the presence of a single or combined mutation(s) on either the *dhps* (codon 437: χ^2, P = 0.143; codon 540: χ^2, P = 0.205; codon 437/540: χ^2, P = 0.454) or the *dhfr* (codon 108: χ^2, P = 0.390; codon 59: χ^2, P = 0.246; codons 108/59: χ^2, P = 0.969) alone. Molecular analysis showed a 97% (152) prevalence of the pre-requisite mutation K76T, for chloroquine resistance while the supporting mutation at codon 75 had a prevalence of 99% (n=155). The higher *in-vivo* treatment failure rate in comparison with the lower prevalence of mutation associated with resistance recorded could be attributed to other causes other than the presence of drug resistant parasites.

The study demonstrated that the collection of *P. falciparum* infected blood samples on filter papers and the use of restriction fragment length polymorphism molecular analysis is a viable method that can be employed in the identification of drug resistant *Plasmodium* isolates.

DEDICATION

I wish to dedicate this piece of work to my parents, Peter and Esnart Mwenechanya, who made it possible from the start, their patience along the way and prayers. I also wish to give a slice of this dedication to my late sister, Fanny, who would have been so proud of me for this undertaking. A slice should also go to my wife, Judy, for the encouragement and understanding.

ACKNOWLEDGEMENTS

Firstly, I wish to express my sincere gratitude to Dr. V. Konde, for making this study possible through his encouragement, advice and the facilitation of the funding. Thanks to Dr. H. Chitambo who introduced me to the School of Veterinary Medicine, University of Zambia (UNZA), for his guidance and encouragement and, to Dr. K. Choongo who was there at a time when I needed help. May I also thank the technical and academic staff of the Department of Biomedical Sciences, School of Veterinary Medicine, University of Zambia, and in particular the academic staff of the entire school, for accepting me as one of their own and affording me the opportunity to carry out the work.

Secondly, sincere thanks to Uncle Jorry and Aunty Yvonne who also contributed to my work in their own way and my brothers and sisters for their little patience they exercised during my work.

Lastly, I wish to thank the health workers I worked with during the collection of samples at Solwezi Urban Health Clinic, in particular the laboratory technician Mr. Chishimba. I also thank everyone who may have contributed to my work in one way or another.

May the Lord God Almighty bless and watch over us all.

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ABBREVIATIONS AND SYMBOLS

AIDS	Acquired Immuno Deficiency Syndrome
A	Adenine
ATP	Adenosine tri-phosphate
Ala	Alanine
α	Alpha
Å	Angstrom
Asn	Asparagine
Asp	Aspartic acid
Bti	*Bacillus thuringiensis var isrealensis*
bp	Base pairs
β	Beta
BSA	Bovine serum albumin
cm	Centimeters
CBoH	Central Board of Health
χ^2	Chi-Square
CQ	Chloroquine
CSP	Circumsporozoite Surface Protein
cDNA	Complementary deoxyribonucleic acid
Cys	Cysteine
C	Cytosine
°C	Degrees Celsius
dATP	Deoxyadenosine tri-phosphate
dCTP	Deoxycytidine tri-phosphate
dGTP	Deoxyguanosine tri-phosphate
dNTPs	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphophates
dTTP	Deoxythymidine tri-phosphate
dTMP	Deoxythymidylate
dUMP	Deoxyuridyrate

DDT	Dichlorodiphenyltrichloroethane
H_2-folate	Dihydrofolate
DHFR	Dihydrofolate reductase enzyme
dhfr	*Dihydrofolate reductase gene*
DHPS	Dihydropteroate synthase enzyme
dhps	*Dihydropteroate synthase gene*
DOT	Directly Observed Therapy
dH_2O	Distilled water
dsDNA	Double stranded deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
E-selectin	Endothelial selectin
FP	Ferriprotoporphyrin IX
G6PD	Glucose-6-phosphate dehydrogenase
GTP	Guanosine triphosphate
Glu	Glutamate
Gly	Glycine
G	Guanine
h	hour
HIV	Human Immuno-deficiency Virus
ICAM-1	Intracellular adhesion molecule-1
ITNs	Insecticide Treated Nets
IL 1	Interleukin 1
Ile	Isoleucine
Kb	Kilo base
Kg	Kilogram
Km	Kilometer
LSA-I	Liver Stage Antigens-I
K	Lysine
MSA	Merozoite surface antigens
mRNA	Messenger ribonucleic acid
m	Meter

µg	Microgram
µl	Micro litre
µM	Micromolar
ml	Millilitre
mm	Millimetre
MDR	Multi drug resistance
min	Minutes
NMCC	National Malaria Control Centre
NEB	New England Biolabs
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
#	Number
pABA	Para-aminobenzoic acid
P. falciparum	*Plasmodium falciparum*
Pf	*Plasmodium falciparum*
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter gene
*pf*CRT	*Plasmodium falciparum* Chloroquine Resistance Transporter protein
*pf*EMP	*Plasmodium falciparum* Erythrocytic Membrane Protein
+	Plus or and
PCR	Polymerase chain reaction
1°	Primary
Q	Quinine
RAPD	Randomly Amplified Polymorphic DNA
ROI	Reactive oxygen intermediates
RBC	Red Blood Cell
RFLP	Restriction Fragment Length Polymorphism
2°	Secondary
Ser	Serine
SUCHC	Solwezi Urban Clinic Health Center
S. aureus	*Staphylococcus aureus*
S/P or SP	Sulfadoxine/pyrimethamine
S	Svedberg units
Temp.	Temperature

H_4-folate	Tetrahydrofolate
THF	Tetrahydrofolate
T	Threonine
×	Times (unit of buffer concentration)
TBE	Tris Borate EDTA
Tris	Tris (hydrooxymethyl) aminomethane
TB	Tuberculosis
TNF-α	Tumour Necrosis Factor alpha
UNICEF	United Nations International Children's Education Fund
US$	United States Dollar
URTIs	Upper Respiratory Tract Infections
UV	Ultraviolate light
V	Volts
Val	Valine
Vol.	Volume
WHO	World Health Organization
yrs	Years

CHAPTER ONE

1 INTRODUCTION

1.1 Malaria

1.1.1 Occurrence of the disease

Malaria caused by *Plasmodium* species affects people in over 90 countries worldwide. According to the WHO (Bloland, 2001), 36% of the global population live in areas where there is risk of malaria transmission, 7% reside in areas where malaria has never been under meaningful control, and 29% live in areas where malaria was once transmitted at low levels or not at all, but where significant transmission has been re-established. Each year an estimated 300 (Perlman, 1987) to 500 million (Bloland, 2001) clinical cases of malaria occur, making it one of the most common infectious diseases of man worldwide. Malaria is a devastating disease with high morbidity and mortality, demanding a rapid, comprehensive response. The development and spread of drug-resistant strains of malaria parasites has been identified as a key factor exacerbating the resurgence of outbreaks and the numbers of clinical cases is likely to be on the increase (Barker *et al.*, 1989). Occurrence of drug resistant *P. falciparum* strains is one of the greatest challenges to malaria control today, so much that some antimalarial drugs like chloroquine are no longer recommended for use. In spite of current increase in attention and resource mobilization aimed at malaria control, which includes such initiatives as Roll Back Malaria, the Multilateral Initiative on Malaria and the introduction of new drugs for Malaria, research activities in most of these endemic countries still suffer from little technical capacity building in the detection of resistant malaria species.

In many areas of the world where malaria occurs, especially in sub-Saharan Africa, malaria is ranked among the most frequent and leading cause of morbidity and mortality among children and pregnant women. WHO estimates that more than 90% of the 1.5 to 2.0 million deaths attributed to malaria each year occur in African children. In addition to high morbidity and mortality, the economic effects of malaria infection are enormous. The latter are direct costs of treatment and prevention, as well as indirect costs which are due to loss of productivity from numerous morbidity and mortality rates in societies through frequent time diversion and other household resources (Bloland, 2001). The annual economic burden of malaria infection in 1995 was estimated at US$ 0.8 billion, for Africa alone (Foster and Phillips, 1998). This heavy toll can surely hinder economic and social development of the people in the region. Malaria is primarily prevalent in the tropical and subtropical regions of sub-Saharan Africa, Latin America and the Caribbean, and Asia (Figure 1.1).

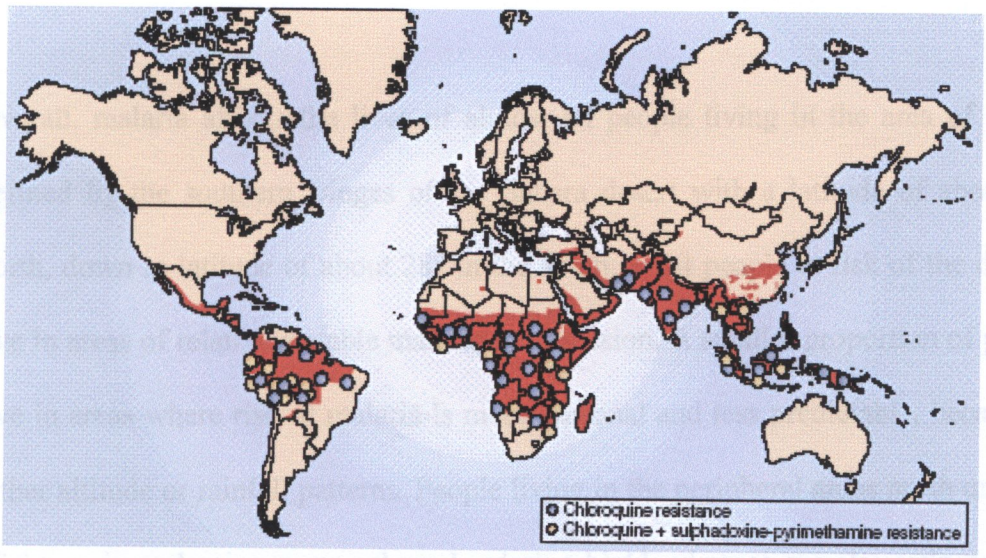

Figure 1.1 Approximate global distribution of malaria, showing places with documented chloroquine and sulfadoxine/pyrimethamine resistance (Source: Fidock *et al.*, 2004)

However, there are considerable variations in the actual intensity of transmission and risk of malaria infection in these regions. Highlands greater than 1500 m above sea level, and arid areas of less than 1000 mm rainfall/year, are typically at lower risk of malaria, although they can also be prone to epidemic malaria when parasitaemic individuals provide a source of infection when micro climatic conditions become favourable to mosquito development. Although urban areas are expected to be at lower risk, unplanned explosive population growth has contributed to the growing problem of urban malaria transmission in many countries (Bloland, 2001).

1.1.2 Malaria in Africa

About one million people in Africa die from malaria each year. The majority of infections in Africa are caused by *Plasmodium falciparum*, which is transmitted by a very effective vector, the female *Anopheles gambiae* mosquito. This mosquito is widespread in Africa and has proven difficult to control (WHO/UNICEF, 2003).

Overall, malaria affects the lives of almost all people living in the area of Africa defined by the southern fringes of the Sahara desert with a latitude of about 15° north, down to latitude of about 28° in the south. Most people at risk of the disease live in areas of relatively stable malaria transmission. A smaller proportion of people live in areas where risk of malaria is more seasonal and less predictable, because of either altitude or rainfall patterns. People living in the peripheral areas north or south of the main endemic areas or those bordering highland areas are also vulnerable to seasonal malaria outbreaks (Figure 1.2).

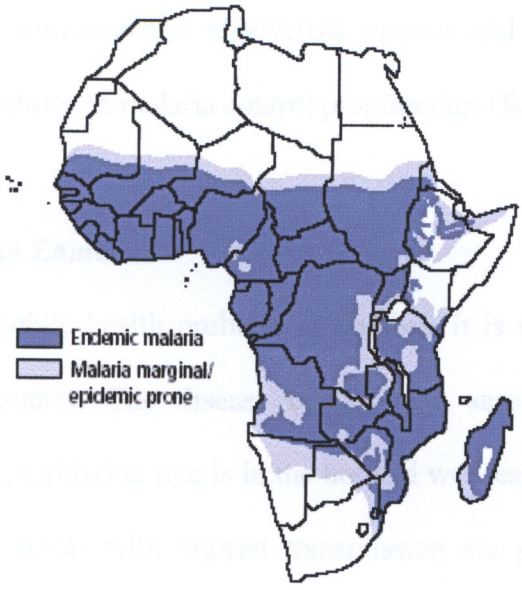

Figure 1.2 Distribution of endemic malaria in Africa (Source: WHO/UNICEF, 2003)

P. falciparum malaria causes at least 20% of all deaths in children under the age of five years in Africa, a mortality rate that is only similar to that due to respiratory diseases caused by a variety of infectious agents (WHO/UNICEF, 2003).

In all malaria-endemic countries in Africa, about 25 - 40% (average 30%) of all outpatient clinic visits are due to malaria. Diagnoses of most of these cases are done clinically without any laboratory confirmation. It is also reported that between 20% and 50% of all hospital admissions are as a result of malaria. It has further been recognized that high case-fatality rates can be attributed to late presentation, inadequate disease management and unavailability or stock-outs of effective drugs, making malaria a major cause of death among hospital in-patients (WHO/UNICEF, 2003). Since the 1980s, some of the important factors that have exacerbated malaria situation in Africa include development of drug resistance, frequent exposure of low-

immune populations, emergence of HIV/AIDS, climate and environmental changes and the general breakdown in malaria control programmes (WHO/UNICEF, 2003).

1.1.3 Malaria in Zambia

Malaria is a major public health problem in Zambia. It is endemic in all the nine provinces of the country. The disease occurs with seasonal and geographical variations. Highest transmission rate is in the hot and wet season, which occurs from November to April. Areas with highest transmission are provinces found in the northern parts of the country, while the urban and hilly areas are less affected. Approximately three million cases and 50 000 deaths are attributed to malaria annually (CBoH, Zambia. 2003a). It is estimated that about 20% of maternal deaths in Zambia are due to malaria caused by *P. falciparum*. Children below the age of five years and pregnant women are most affected with this disease (NMCC, 2003). Prevalence of malaria has tripled in the last three decades and this has been attributed to the development of chloroquine resistance by *P. falciparum* parasites. The first documented evidence of chloroquine resistance in Zambia was around 1983, this was followed by an increase in the incidence of the disease in the last 22 years coupled with an increase in case fatality. Other reasons that have been identified to contribute to escalating malaria incidence rates are reduced vector control activities such as indoor residual spraying in the urban areas, declining access to quality health care, increase in HIV/AIDS infected individuals and the general increase in poverty (NMCC, 2003). Chloroquine treatment failure levels averaged 60% by 2003, a figure which is far above the WHO recommended 25% treatment failure rate (WHO, 2001a) criteria recommended for effecting drug change (CBoH Zambia, 2003b). In

2003, chloroquine was phased out as the first-line drug for malaria in the country. In its place, coartem® (artemether-lumefantrine) was introduced as the first-line drug for uncomplicated malaria, but not for children below 10 kg body weight, until such a time coartem® efficacy and safety data become available. For this group, sulfadoxine/pyrimethamine (fansidar®) was recommended as the first-line drug, although resistance to the drug is already emerging. Quinine is now recommended as the second - line drug for all age groups where the first line-drug fails (CBoH, 2003b; NMCC, 2003).

1.2 Justification and objectives

While some malaria control strategies may help in reducing the disease burden, not all of them may be available to the malaria endemic countries. The most feasible control method that can be relied upon is the use of chemotherapy to treat patients combined with the use of insecticides to kill the vector mosquitoes and biological control to break the mosquito life cycle. It is imperative therefore that the efficacy of the available drugs and insecticides is monitored to safeguard against their indiscriminate use which may lead to development of resistance problems. One way of determining the efficacy of chemotherapy is by monitoring the presence of the indicators of resistance in the parasite. With globalization and efficient communication in the world, people are always on the move. Thus the malaria parasites will also always be spreading along. It is for this reason that global effort in mapping and understanding the extent of drug resistance in malaria parasite is concerted and well documented.

In this study, the molecular markers of drug resistance in *Plasmodium falciparum* were put in practice to screen for drug resistant *P. falciparum* parasites from the field. The results were expected to give the status of drug resistance problem and help to provide a basis for formulating well informed malaria treatment strategies. The study looked at possible existence of *P. falciparum* gene mutations associated with chloroquine (4-aminoquinoline) and fansidar® (sulfadoxine/pyrimethamine) resistance and how it relates to *in-vivo* efficacy of treatment.

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The main objectives of the study, therefore, were:

1.2.1 To observe the *in-vivo* efficacy of fansidar® treatment in confirmed malaria positive-patients.

1.2.2 To collect *P. falciparum* parasites from patients, before treatment, for DNA extraction and PCR amplification.

1.2.3 To determine *in-vitro* (molecular) characterization for the prevalence of *P. falciparum pfcrt, dhps* and *dhfr* mutations associated with chloroquine and fansidar® resistance.

1.2.4 To compare the association between *in-vivo* and *in-vitro* (molecular) analyses.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 The Genus *Plasmodium*

In humans, malaria infection is caused by four species of the intracellular apicomplexan protozoan parasites *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (Gutteridge and Coombs, 1977; Kreier, 1977). These protozoan parasites belong to the Genus *Plasmodium*, family plasmodiidea, class sporozoa and subclass coccida (Lwoff, 1951). Of these the widespread species being *P. falciparum* and *P. vivax* and the less common are *Plasmodium malaria* and *Plasmodium ovale* (Pampana, 1969). The Anopheles mosquito transmits the *Plasmodium* parasites by injecting sporozoites contained in their saliva into humans as they feed on blood (Kreier, 1977). The four species differ in geographical distribution, microscopic appearance, clinical features (periodicity of infection, potential for severe disease, and ability to cause relapses), and potential for development of resistance to antimalarial drugs. To date, drug resistance has only been documented in two of the four species, *P. falciparum* and *P. vivax* (Bloland, 2001).

The most clinically significant infections are caused by *P. falciparum* which is the most common virulent and lethal malaria causing parasite (Gutteridge and Coombs, 1977; Hartl, 2004). In Zambia, 95% of malaria infections are caused by *P. falciparum*, while the other 5% are caused by the other three species (NMCC, 2003).

2.1.1 The life cycle of *Plasmodium*

Plasmodium parasites have a complex life cycle (Figure 1.3) which is shared between a vertebrate host and an insect vector. The parasite enters the bloodstream of

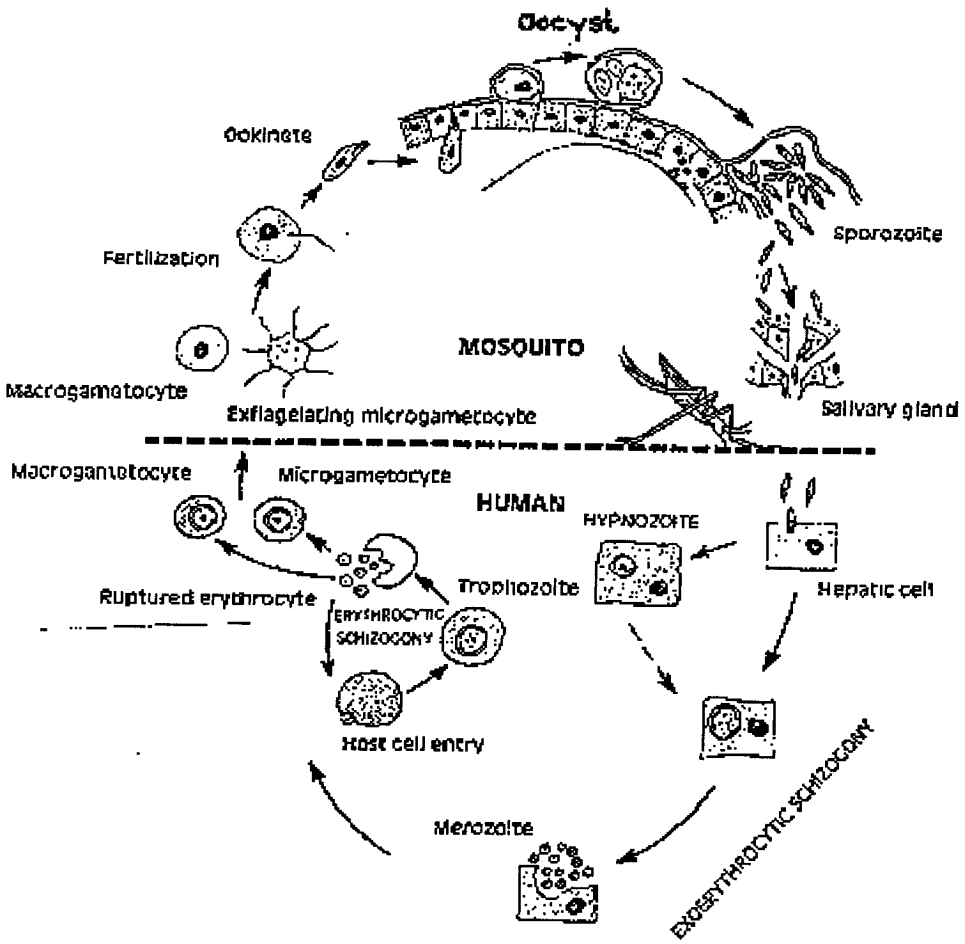

Figure 1.3 Schematic diagram of the life cycle of malaria parasites (Source: Fujioka and Aikawa, 2002)

the host through the bite of an infected female *Anopheles* mosquito (Kreier, 1977). Sixty out of the 380 anophelene species are able to carry the malarial parasite. It has been discovered that mosquito species that resist growth of *Plasmodium* parasites in their mid gut have a higher trypsin-like activity in their midguts. Sporogony within

the mosquito is governed by environmental temperature, as mosquitoes are poikilotherms (Lwoff, 1951; Pampana, 1969). Sporozoites, from the mosquito's salivary gland, that are injected into the human bloodstream travel to the liver and infect hepatic cells (Kreier, 1977). The sporozoites are 10-15 μm in length and about 1 μm in diameter. They have a thin outer membrane, a double inner membrane below which lie the subpelicular microtubules. Extending half the length of the body are three polar rings and long rhoptries. They have convoluted elongate bodies called micronemes, which run forward to the anterior of the sporozoite entering a common duct with the rhoptries. Mitochondria are located at their posterior end (Fujioka and Aikawa, 2002).

Sporozoites remain in hepatic cells (safe from an immune response) for 9 -16 days. A developing sporozoite form numerous daughter nuclei by undergoing multiple asexual fission (schizogony), producing the schizont or cryptozoite (Pampana, 1969; Kreier, 1977). Within this structure the mitochondrion also enlarges during maturation and buds off to produce many small mitochondria. Finally numerous apical complexes are formed next to the outer membrane giving rise to the formation of the merozoites. The merozoites are shorter than the sporozoite and the rhoptries have a teardrop shape with oval micronemes. After being released from the liver, some merozoites may continue to re-infect other liver cells in *P. vivax* and *P.ovale* and can lie dormant and are called hypnozoites (Daily, 2006) or may attach to and penetrate erythrocytes (Kreier, 1977). The rhoptries facilitates the entry of the meroziotes into the (red blood cells) RBCs (Perkins, 1989). The parasite directs formation of a "knob" on a red blood cell's (RBC) surface that secures the infected

cell to a blood vessel wall. This is vital to the parasite, as the erythrocyte would simply be transported to the spleen and destroyed, along with the parasite. Also, the body's immune system recognizes foreign proteins generated and destroys infected cells. The parasite overcomes this obstacle by producing phenotypic, polymorphic variants of particular surface proteins so that if the immune system recognizes one variant, others exist to avoid detection and elimination. A build up of attached erythrocytes onto a vessel wall can block circulation, resulting in hypoxia and even coma or death in cerebral malaria (Newton *et al.*, 2000).

The merozoites attachment to the surface of the red cell by a special binding receptor causes the red cell membrane to invaginate forming a food vacuole within the red cell. The merozoites digest the red cell haemoglobin producing a large food vacuole within it which produce the characteristic signet ring stage within the red cell, and the merozoites at this stage are called trophozoites (Lwoff, 1951; Kreier, 1977). The ring forms of the parasites are the most commonly observed in a blood smear of *P. falciparum* malaria. The trophozoites devour 75% of the RBC's haemoglobin in order to replicate its nucleus. As the trophozoite matures, the vacuole disappears and it enters into erythrocytic schizogony stage. At this stage the parasite is called a schizont and it is seldom seen in blood smears. Cytoplasm and cell membranes are then placed around each nucleus to form 12-28 merozoites. The parasite ingests haemoglobin by the process of pinocytosis via the cytostome. It is then degraded by two aspartic proteases (plasmepsin I and plasmepsin II) and one cysteine protease, a falcipain, (Sijwali *et al.*, 2001) in the acidic digestive vacuoles of the malaria parasite. This result in the production of the toxic chemical called heme, the parasite

polymerises the heme to harmless hemozoin (Gutteridge and Coombs, 1977). The depletion of antioxidants, an acidic environment (pH 4.5-5.0) and the presence of ferriprotoporphyrin IX in the Fe^{+3} state are some of the chemical and biochemical conditions necessary for this process to occur.

Eventually, the erythrocytes lyse (from osmotic fragility and infection), thus releasing even more merozoites which re-infect new red cells (Lwoff, 1951; Kreier, 1977). This cycle continues a number of times. The rupturing of the red cells is coincident with the recurring malarial symptoms. Pyrogens are also released, causing severe cycles of chills and fever every 36 h with subtertian malaria (*P. falciparum*), every 48 h with tertian malaria (*P. vivax*) and every 72 h with quartan malaria (*P. malariae*). These time spans correspond to the infection and breakdown of erythrocytes and may result in anaemia (Gutteridge and Coombs, 1977; Kreier, 1977).

Merozoites may also differentiate into gametocytes which do not rupture the erythrocyte (Kreier, 1977). When another feeding mosquito ingests these gametocytes, they develop into male and female gametes, the microgametocytes and the macrogametocytes, respectively (Lwoff, 1951; Kreier, 1977). The gametocytes usually appear many in number and are commonly observed in blood smears from patients with *P. falciparum* malaria. If the gametocytes are not ingested by a mosquito in a blood meal, they eventually die by being phagocytosed by the host's reticulo-endothelial system. In the stomach of the mosquito, the microgametocytes rapidly develop into microgametes and separate in a rapid explosive fashion known

as exflagellation (Kreier, 1977). Exflagellation transformation is triggered by an increase in pH which occurs in the mosquitoes gut. The nucleus of the microgamates divides to form between six to eight daughter nuclei which are associated with the elements of a developing axoneme. The microgamete membrane is disrupted and forms around the individual flagella and nucleus. The rapidly moving microgametes, whose life span is very short, break free and locate and penetrate the macrogamete to form a diploid zygote also called ookinete (Lwoff, 1951; Kreier, 1977) which possesses no rhoptries or micronemes. The ookinete penetrates the peritrophic membrane and the intestinal wall of the insect gut and forms the oocyst within the host cells on the haemocoel side of the gut wall. As the oocyst develops it ruptures and bursts out of the host cells. The first division is a reduction division forming a number of haploid nuclear masses called sporoblasts. The sporoblasts in turn divide numerous times to form the sporozoites, which are released into the haemocoel. The rupture of the oocyst releases kinetes which migrate to the salivary gland, to reproduce by a process called sporogony before releasing the sporozoites which are injected into another host, beginning the cycle (Lwoff, 1951; Kreier, 1977).

2.1.2 The structure of *Plasmodium falciparum*

The cell structure of *Plasmodium falciparum* takes on different forms and shapes depending on the stage of its life cycle at which it is, as discussed under its life cycle and as shown in figure 1.3. However, the key structural features are shown in figure 1.4.

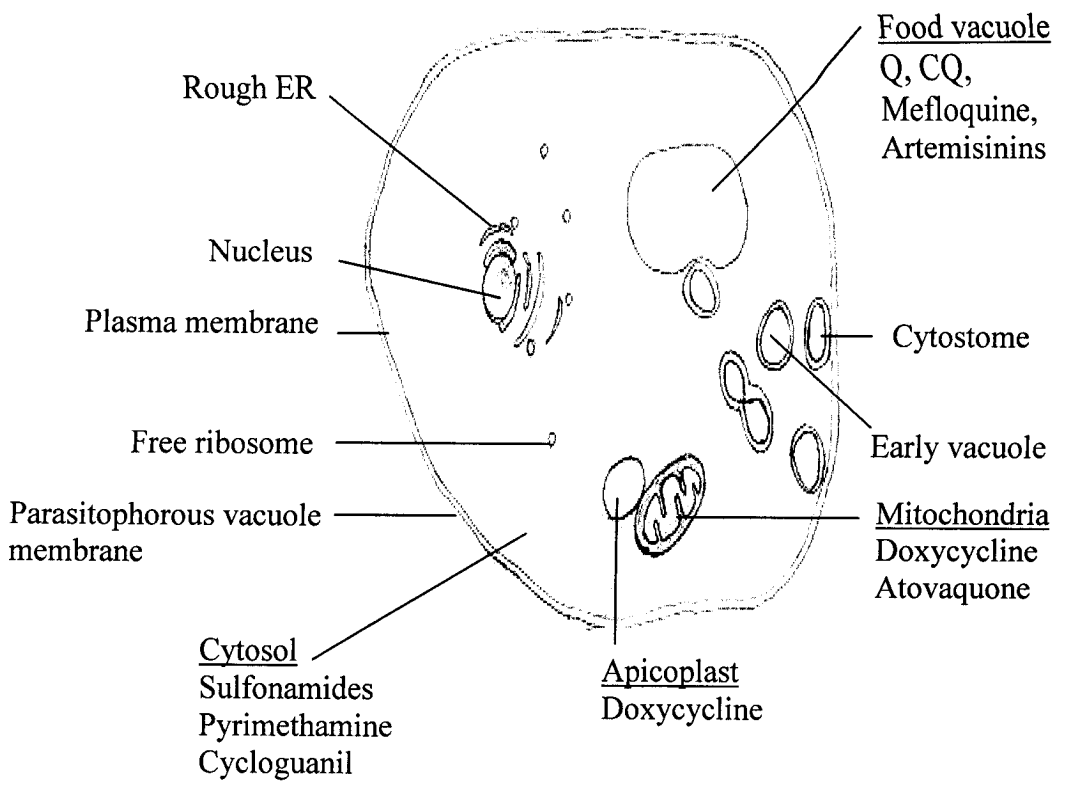

Figure 1.4 Key intracellular structural features of intra-erythrocytic *Plasmodium falciparum* trophozoite. (underlined structures/organelles are the site of action of the classes of drugs or drugs shown, Q – Quinine, CQ – Chloroquine) (Source: Warhurst, 2002; Fidock, *et al.*, 2004)

2.1.3 The food vacuole

The food vacuole (see Figure 1.4) is one of the organelles found in the cytoplasm of *P. falciparum.* It is surrounded by a double membrane. The outer one, which is in contact with the host cell cytoplasm, is called the parasitophorous vacuole membrane. The inner membrane, which surrounds the cytoplasm of the parasite, is called the parasite plasma membrane (Wiser, 2003; Fidock, *et al.*, 2004).

The parasite takes up the host cell stroma by pinocytosis during its early ring stage resulting in double membrane vesicles whose parasitophorous vacuole membrane

rapidly disappear and the digestion of haemoglobin takes place within these small vesicles. Mature parasites develop a special organelle called the cytostome which is used for taking up host cytoplasm. The large food vacuole is formed by fusion of small pigment-containing vesicles. Gametocytes, which are characterized by small pigment-containing vesicles found throughout their cytoplasm, are unable to form the large food vacuole (Wiser, 2003). Double-membrane vesicles derived from the base of the cytostome fuse with the food vacuole (Fidock, *et al.*, 2004). The parasitophorous vacuole membrane lyses leading to the release of haemoglobin into the food vacuole (Wiser, 2003). Thus the fusions of the vesicles with the food vacuole lead to the enlargement of the latter.

The food vacuole resembles a lysosome by having an acidic pH of 5.0 to 5.4 and contains protease activities, although other acid hydrolases, e.g. nucleases, have not yet been identified (Wiser, 2003). Since the microenvironment of the erythrocyte is almost exclusively made of protein, in particular haemoglobin, therefore other acid hydrolases would most likely not be needed. Three out of four major classes of proteases, representing several distinct protease activities, have been identified in the food vacuole and these include the plasmepsins, falcipains and falcilysin (Wiser, 2003; Fidock, *et al.*, 2004). These enzymes are involved in a semi-ordered process involving the sequential digestion of haemoglobin. Apparently, haemoglobin is not completely digested into free amino acids in the food vacuole as no exopeptidases activity has been identified in it, although several *Plasmodium* species have been found to possess neutral amino peptidase activity (Florent, 1998; Wiser, 2003). Therefore, small peptides are pumped out of the food vacuole into the parasite

cytoplasm where they are digested (Wiser, 2003). Thus, the food vacuole is the site for host haemoglobin digestion to provide the parasite with the amino acids derived from the digested haemoglobin (Lwoff, 1951). It is for this reason that the food vacuole is exploited for the many of the antimalarial drugs as it contains enzymes that are unique to the food vacuole (Warhurst, 2002).

2.2 The disease

The causative agents of malaria are transmitted through bites from infected female *Anopheles* mosquitoes (Kreier, 1977; Newton *et al.*, 2000). The time from the initial *P. falciparum* infection and the first clinical signs of the disease is called the incubation period, which differs according to the species of *Plasmodium*. Malaria caused by *P. falciparum* generally has incubation periods ranging from 7-14 days (Pampana, 1969). The incubation period may take longer if a person is or has taken medication as prophylaxis, or in persons who develop partial immunity through previous exposure to *P. falciparum* (WHO, 2001a).

2.2.1 Clinical signs and symptoms

There are variations in the severity of the symptoms of the disease depending on regions. In regions where malaria is endemic, people are believed to be semi-immune and may be infected with malaria but show no symptoms (Kreier, 1977). The common symptoms of malaria, especially those in the early stages of the disease are common to other diseases caused by bacteria, viruses, or other parasites. These symptoms may include fever, which may be periodic, chills, headache, sweats, fatigue, nausea and vomiting (WHO, 2000). The symptoms may appear in cycles and

may occur and disappear at different intensities and with different lengths of time especially at the beginning of the illness (Pampana, 1969; Kreier, 1977). The symptoms may not follow a typical cyclic pattern and the cyclic pattern of malaria symptoms is due to the parasites' development, reproduction and release from the red blood cells and liver cells in the human body. The cycle of symptoms is one of the major indicators of the presence of the disease. The other common symptom of malaria are dry cough, muscle and/or back pain, enlarged spleen, and impaired function of the brain or spinal cord, leading to seizures, or loss of consciousness, although this latter condition is rare (WHO, 2000).

2.2.2 Cerebral malaria

Cerebral malaria collectively involves the clinical manifestations of malaria caused by *Plasmodium falciparum* that induces changes in mental status and coma (WHO, 2000). It is an acute widespread disease of the brain, which is accompanied by fever. The clinical manifestations of cerebral malaria are numerous, but there are three primary symptoms common to both adults and children. There is an impaired consciousness with non-generalized convulsions, neurological sequelae, and a coma that persists for 24-72 hours, initially rousable and then unrousable (Newton *et al.*, 2000). The cause of cerebral malaria is not well understood. Currently there are two major hypotheses to explain its aetiology. These are the mechanical and humeral hypotheses. The mechanical hypothesis states that a specific interaction between a *P. falciparum* erythrocytic membrane protein (*pf*EMP-1) and ligands on endothelial cells, such as ICAM-1 or E-selectin, reduces microvascular blood flow and induces hypoxia (Kaul et al., *1998; Newton et al., 2000). This selective cytoadherence of*

parasitized RBCs and non-parasitized RBCs, also known as rosetting, can apparently better account for cerebral malaria's characteristic coma condition. The humoral hypothesis suggests that a malaria toxin may be released that stimulates the macrophages to release TNF-∝ and other cytokinins such as IL-1. The cytokinins themselves are not harmful, but they may induce additional and uncontrolled production of nitric oxide (Clark *et al.*, 1992; Newton *et al.*, 2000). Nitric oxide would diffuse through the blood brain barrier and impose similar changes on the synaptic function, as do the general anesthetics and high concentration of ethanol, leading to a state of reduced consciousness (Newton *et al.*, 2000).

2.2.3 Malaria in pregnancy

Severe malaria infection during pregnancy is often a result of *P. falciparum* infection. The impact of the other three human malaria parasites namely *P. vivax*, *P. malariae*, and *P. ovale*, is less clear. Every year at least 30 million women in malaria endemic areas of Africa become pregnant (WHO/UNICEF, 2003; WHO, 2004).

The symptoms and complications of malaria during pregnancy vary according to the intensity of malaria transmission and the level of acquired immunity of the pregnant woman at risk. Since the intensity of malaria transmission may vary within the same country from relatively stable or endemic transmission to unstable or epidemic transmission, the clinical picture of malaria in pregnant women can range from asymptomatic to a severe and life-threatening illness (WHO/UNICEF, 2003; WHO, 2004).

The physiological changes of pregnancy and the pathological changes due to malaria have a synergist effect on the course of each other. *P. falciparum* malaria is often common in the first and the second pregnancies (Akotet *et al.*, 2003). Malaria complications are more common and severe in hyperendemic areas for *P. falciparum* infections. Physiological changes associated with pregnancy exacerbate malaria infection. There is a generalized immunosuppression in pregnancy with reduction in gamma globulin synthesis and inhibition of the reticulo-endothelial system, resulting in decrease in the level of antimalarial antibodies and loss of acquired immunity to malaria (Riley *et al.*, 1989; Meeusen *et al.*, 2001). These immunological changes make pregnant women become more prone to malaria infection and enhanced parasitemia. Changes in the humeral milieu, increase in the body fluid volume, decrease in haemoglobin level and other changes add to the severity of the clinical malaria outcome (Fievet *et al.*, 1995).

In epidemic or low (unstable) malaria transmission areas, adult women tend to have low or no acquired immunity and thus, usually become ill when infected with *P. falciparum*. Maternal death is attributed directly to severe malaria or indirectly to complications due to malaria-related severe anaemia (Hammerich, *et al.*, 2002; WHO, 2004). Malaria may also manifest itself in a range of various other pregnancy effects which may include low birth weight, spontaneous abortion, and neonatal death (WHO/UNICEF, 2003; WHO, 2004).

Pregnant women in endemic areas tend to be at higher risk of developing severe malaria than non-pregnant women living in the same area. In areas of high and

moderate and/or stable malaria transmission, most adult women develop sufficient immunity (WHO/UNICEF, 2003). In such cases, even during pregnancy, *P. falciparum* infection does not usually result in fever or other clinical symptoms. In these areas, the principal impact of malaria infection is malaria-related anaemia in the mother and the presence of parasites in the placenta which may impair foetal nutrition, contributing to low birth weight and poorer infant survival and development (McGregor *et al.*, 1983; Greenwood *et al.*, 1992; WHO, 2004). In Africa, in areas with stable malaria transmission, *P. falciparum* infection during pregnancy is estimated to cause an estimated 75 000 to 200 000 infant deaths each year (WHO/UNICEF, 2003).

2.2.4 Intrinsic resistance to malaria

Certain host genes and human genetic diseases are known to confer innate protection against malaria. The point in the life cycle of the malarial parasite at which the parasite can better be stopped in humans is at the phase of red blood cell invasion and multiplication. Red blood cells are constantly created and destroyed. Therefore any mutation that somehow may lead to the destruction of the infected red blood cells could eliminate the malaria parasite.

The Duffy antigen on the red blood cell membrane were observed to be the molecules used by *P. vivax* to enter the red blood cell (Wertheimer and Barnwell, 1989). The high association of Duffy antigen null red blood cells in some groups of people with the sickle cell trait suggested that the Duffy antigen might provide some protection against malaria (Gelpi and King, 1976). Later investigations have shown

that the Duffy antigen is the receptor which the merozoites of *P. vivax* use to enter red blood cells. Therefore people who lack the Duffy antigen (FY*O allele) are resistant to *P. vivax* (Hamblin and Di Rienzo, 2000). It has further been observed that the Duffy null phenotype is most common in people whose ancestors derive from regions in Africa where *P. vivax* malaria is endemic (Voet and Voet, 1990).

Sickle cell anaemia is an inherited molecular disease resulting from mutant haemoglobin. A molecule of haemoglobin is made up of two α and two β (globin) chains. Mutant haemoglobin results from a mutation at position six of one of the β chains leading to replacement of glutamic acid with valine at that position (Voet and Voet, 1990). The mutation causes sickle haemoglobin or haemoglobin S to aggregate into filaments of sufficient size and stiffness to deform the erythrocytes. Sickle cell haemoglobin provides the best example of a change in the haemoglobin molecule that impairs malaria parasite growth and development. The initial hints of a relationship between the two came with the realization that the geographical distribution of the gene for haemoglobin S and the distribution of malaria in Africa virtually overlap (Voet and Voet, 1990). A further hint came with the observation that people indigenous to the highland regions of the continent did not display the high expression of the sickle haemoglobin gene like their lowland neighbours in the malaria belts (Voet and Voet, 1990; Harlt, 2004). Although sickle cell trait provides a survival advantage over people with normal haemoglobin in regions where malaria is endemic, the trait does not necessarily provide absolute protection. Rather people, especially children, when infected with *P. falciparum* are more likely to survive the acute illness of malaria if they have the sickle cell trait. Only people with the

heterozygote trait for sickle cell trait are protected against malaria. Sickle cell trait is the genetic condition selected for in regions of endemic malaria. The precise mechanism by which sickle cell trait imparts resistance to malaria is not known but, a number of factors involved in varying degrees to imparting the defence against malaria have been identified (Voet and Voet, 1990). Effects of sickle cell trait in red blood cells infected with the *P. falciparum* parasite cause deformation of red blood cells, presumably because the parasite reduces the oxygen tension within the red blood cells to very low levels because of the parasite metabolism. Deformations of *P. falciparum* infected sickle red blood cells marks them as abnormal and are targets for destruction by host phagocytosis (Luzzatto *et al.*, 1970). Destruction of infected sickle trait red blood cells reduces the parasite burden making such people more likely to survive acute malarial infections.

Thalassemias are a group of inherited human genetic diseases resulting from impaired synthesis of haemoglobin's four subunits. These anaemia are called thalassemias because they commonly occur in the region surrounding the Mediterranean sea, although they are also found in Central Africa, India and the Far East (Voet and Voet, 1990). The imbalance in globin chain production, characteristic of thalassemia, produces membrane oxidation by hemichromes and other molecules that generate reactive oxygen species (Sorensen, *et al.*, 1990; Grinberg *et al.*, 1995). Reactive oxygen species are known to injure and kill malaria parasites. Haemoglobin H (ß-globin tetramers) occurs most often in people with three-gene deletion alpha-thalassemia (Zhu *et al.*, 1993). The compound heterozygous condition of two-gene deletion alpha thalassemia and haemoglobin Constant Spring also produces red blood

cells that contain haemoglobin H (Derry *et al.*, 1988). However, two-gene deletion-alpha thalassemia also protects the host from malaria. The process is difficult to demonstrate with *in-vitro* cultures of malaria parasites. Alpha thalassemia may protect against malaria in part by altering the immune response to parasitized red blood cells (Luzzi *et al.*, 1991).

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme in the Hexose Monophosphate Shunt or the Pentose Phosphate Pathway. The shunt generates reduced glutathione that protects sulfhydryl groups of haemoglobin and the red blood cell membrane from oxidation by oxygen radicals (Voet and Voet, 1990; Stryer, 1995). Deficiency of G6PD in humans is the most common congenital defects of the shunt. The increase in high frequency of alleles that produce red blood cells deficient in G6PD activity is one of the most dramatic examples of the selective pressure of malaria on humankind (Ruwende *et al.*, 1995; Tishkoff *et al.*, 2001). Individuals with G6PD deficiency have lower levels of reduced Nicotimide Adenine Dinucleotide Phosphate (NADPH) in their red blood cells, which is needed to maintain reduced glutathione (Stryer, 1995). The lower levels of reduced glutathione will result in an increased sensitivity to oxidative stress since glutathione peroxidase participates in the detoxification of reactive oxygen intermediates (ROI). The increased levels of ROI due to the parasite's metabolism combined with the decreased ability of G6PD-deficient red blood cells to remove ROI will result in a premature lysis of the infected red blood cells and therefore confer some protection against malaria. The parasite not only needs to protect itself against ROI but it also needs to insure that the host red blood cell is not damaged before it completes red blood cell schizogony. In

fact, it has been suggested that the parasite may supply the host red blood cells with glutathione to increase its reducing capacity. Reactive oxygen species are formed continually as red blood cells take up oxygen from the lungs and release it to the peripheral tissues. Malaria parasites are easily damaged by these ROI (Friedman, 1979). Glucose-6-phosphate dehydrogenase prevents oxidation of the haeme group. In its absence, hemichromes and other species that generate ROI accumulate in red blood cells (Janney *et al.*, 1986). *P. falciparum* grows poorly in red blood cells that are deficient in G6PD (Roth *et al.*, 1983).

2.3 Control methods

Various malaria control strategies are targeted either on the vector or the parasite. Insecticides are used to target the vector whereas the various chemotherapeutic treatments are used to target the parasites in the host. Attempts to eradicate malaria by chemotherapy and insecticides has only been partially successful because most of these control measures have now been seriously hampered by the growing problem of drug resistance in *P. falciparum* parasite and the DDT-resistance in mosquito vectors (Sharma, 1987; Morel *et al.*, 2002; Hartl, 2004). DDT made the idea of global eradication of mosquitoes/malaria seem possible, but has since been sidelined (Hartl, 2004). It is for these reasons that other methods, like the malaria vaccine, transgenic mosquitoes, use of fish in the control of the vector, both insecticides treated and non-treated mosquito bed nets and use of bacteria, as alternatives have been considered as valid and necessary complements to control malaria (Sharma, 1987). Although these new trends in the control of malaria are welcome and seem to be feasible, some of them will be too expensive for adoption by third world

countries, especially for countries in the sub-tropical regions of Africa because of their poor economies. Correct use of the existing insecticides and chemotherapeutic control especially in the light of drug resistance development which may out pace new drug discovery and development, is absolute (Bloland, 2001).

Chemical vector control involves the use of indoor residual spraying with insecticides like dichlorodiphenyltrichloroethane (DDT) and pyrethroid (Pampana, 1969; Hartl, 2004). This method mostly targets the adult mosquitoes which are killed on contact. Another method utilised under chemical control is fumigation in which several chemicals and herbs – plant leaves are used (Sharma, 1987; Hartl, 2004).

Physical indirect methods of controlling malaria at household level target the vector, by preventing the growth of mosquito larvae in breeding sites where mosquitoes lay their eggs (Pampana, 1969). These are simple methods achieved by getting rid of stagnant water, cutting of long grass, application of a thin layer of oil on the surface of stagnant water and ponds which prevent laying of eggs and / or kills the mosquito larvae by suffocating them (Sharma, 1987; Service, 1989). In endemic countries, houses should be designed with fly-screen wire mesh on windows to prevent mosquitoes from entering the houses. Sleeping under mosquito nets is encouraged in the control of malaria as it helps to reduce the chances and the number of mosquito bites a host might be subjected to, thereby reducing the transmission of the disease (Pampana, 1969). Although this method of controlling malaria has been in use for quite some time, it is only recently that this method has been recognized to be effective (Sharma, 1987; Service, 1989). It is now being encouraged worldwide,

especially in the malaria endemic regions. This method has further been improved by making nets that are treated with insecticides or Insecticide Treated Nets (ITNs). Insecticide Treated Nets kill mosquitoes on contact, but these insecticides have no effect on humans at the recommended dosage used. There is an increased advocacy for the use of ITNs during pregnancy to reduce the overall risk of morbidity and mortality among pregnant women and their infants. Surveys conducted in most African countries indicate that net use among women of reproductive age, 15–49 years, (an indication of use by pregnant women) remains very low (WHO/UNICEF, 2003). The WHO recommends a combination of ITNs use and the application of intermittent prophylaxis with S/P during the first, second and third pregnancies (WHO/UNICEF, 2003; WHO, 2004).

There are other novel possibilities in the fight against malaria which target the mosquito vector. These include biological control (bio-control) methods through the use of naturally occurring processes, or the purposeful use of certain organisms to control and/or manage the mosquito larvae. The biopesticide approach involves application of a bio-control agent when required (often repeatedly), in the same way as the chemical insecticide is used. The most common biological control of the malaria vector involves the use of larvivorous fish which feed on mosquito larvae (Sharma, 1987; Service, 1989). The two well-known larvivorous fish are *Poecilia reticulate* and *Gambusia affinis*. Larvivorous fish have been widely used by public health and mosquito control agencies through out the world to help reduce mosquito breeding. The adaptability and hardness of larvivorous fish and their high biotic

potential has made them to be valuable biological control agents in malaria control (Sharma, 1987).

Other examples of biological control agents include the use of bacteria such as *Bacillus thuringiensis, Agrobacterium* and *Radiobacter* species. Commercially available *Bacillus thuringiensis var isrealensis* (Bti H14) agent is expensive but may soon become affordable since it can now be cheaply propagated grown using coconut media. *Bacillus thuringiensis var isrealensis* is a rod-shaped gram-positive, facultative anaerobic, spore forming bacterium. The Bti H14 subspecies is a known bio-pesticide that controls the larval stage of the mosquito (Knowles, 1994). It is introduced into ponds carrying mosquito larvae, the bacilli are ingested along with the algae on which the fish feed. The bacterium destroys the mid-gut of the larvae, consequently killing them. This bacterium based biological control strategy is yet to be proven for practical application in the field. Recent reports are, however, promising (Knowles, 1994).

2.3.1 Future control methods

2.3.1.1 Transgenic mosquitoes

A transgenic mosquito is one which has been genetically engineered so that it can no longer transmit the *Plasmodium* species (Coates, 2000; Clarke, 2002). The technique used in genetic transformation in insects relies on microinjecting small DNA molecules called plasmids into the eggs of the mosquito species concerned. The plasmids are produced in the bacterial cells using recombinant DNA techniques. Plasmids contain mobile DNA sequences called transposable elements or jumping

genes (Coates, 2000). The transposable elements or transposons (Clarke, 2002) jump from the plasmid DNA molecules and insert themselves into mosquito chromosomes at random locations. When the plasmid is introduced in a mosquito nucleus, it will be expressed in the germ-line cell of the sperm or ova resulting in offspring which will be genetically transformed (Clarke, 2002).

There are several ways that have been suggested as targets into which the wild mosquito could be transformed genetically. It is well known that not all forms of mosquitoes can transmit the malaria parasite. Mosquitoes which cannot transmit *Plasmodium* parasites are called refractory strains (Coates, 2000; Clarke, 2002). Advances into understanding what makes the insects refractory are providing potential target genes that can be transformed into a vector mosquito strain to prevent the development and transmission of malaria parasite through manipulation that direct the *Anopheles gambiae*'s immune system against *P. falciparum* (Coates, 2000; Clarke, 2002). Molecules of immunity such as antibodies from vertebrate system, through a variety of molecular methods, the binding domains of the antibodies can be identified at the DNA base sequence level and then incorporated into the mosquito genome (Coates, 2000). The relevant genomes of the parasite, the host and the vector have since been sequenced and are now well known (Pearson, 2002).

Other generic approaches are being considered, such as genetic manipulations that will render the offspring of the genetically engineered insects sterile (Clarke, 2002). Production of sterile male mosquito to lower the mosquito population is particularly of interest. Creation of any of the above forms of transgenic mosquitoes will have to

be done in the laboratory and released into the wild, hoping they will be able to replace the wild type mosquitoes (Coates, 2000). However, the question still remains as to how the transgenic mosquito would be released into the wild. Findings indicate that countless millions of mosquitoes should be mass-reared and released in a particular place and time to give them hope of replacing the wild. The scale and cost of such an exercise could make transgenic mosquitoes a practically impossible undertaking (Coates, 2000). In theory, after several generations, all wild mosquitoes would have inherited the transformed gene. Natural mutant mosquitoes disappear under field conditions and, moreover, wild-type mosquitoes' genetic diversity already gives them the evolutionary edge over inbred laboratory reared mosquitoes. Natural selection would make it difficult for transgenic mosquito to replace the wild type. The ability to compete for food, find mates and avoid predators could be compromised (Clarke, 2002). Perhaps a solution to this could be to continually cross the laboratory reared strains with vigorous wild type mosquitoes and select for those that retain the malaria resistance gene in the laboratory before releasing them in the wild. The genetic modification itself may also reduce their vigour. However, introducing new genes would free the transgenic mosquitoes from the burden of carrying *P. falciparum*, as there is evidence that the parasite has a detrimental effect on the *Anopheles gambiae*'s vigour (Clarke, 2002).

Another concern is whether the modified introduced gene would remain stable for a long time (Clarke, 2002). The *Plasmodium* parasite may develop resistance to the modification and this would have a profound negative impact on public health. A temporary halt in *Plasmodium* transmission could result in people losing their natural

immunity, rendering the disease even more devastating once the non-refractory parasite returns, especially in endemic areas (Clarke, 2002). A speculative fear is that, as the transposons jumps around the mosquito genome, it could also disrupt the other genes, leading to unpredictable results which may give the mosquitoes the ability to transmit other diseases (Clarke, 2002).

Field application of the transgenic mosquito will therefore require careful assessment before the approach could become a reality (Clarke, 2003). Whatever the final outcome successful use of a transgenic mosquito in human malaria control will be a notable advance in the ability and efforts to combat this devastating disease (Clarke, 2003; Clarke, 2002).

2.3.1.2 Malaria vaccine

A vaccine is a substance that causes the immune system to develop responses that protect against a specific disease. An ideal malaria vaccine is one that would prevent the infection at the first instance and if this is not possible, should decrease the intensity of infection and should be successful in preventing malaria transmission. The vaccine should be safe, easy to use, confer life-long protection against all forms of the disease and easy to store (Mitchell, 1989a; Dubovsky, 2001). An ideal malaria vaccine should stimulate antibody and T-cell responses (Roitt, 1989) that can respond quickly to the infection and prevent the invader from causing serious clinical disease. Since it is difficult to prevent all malaria infection by priming the immune system to destroy all malaria parasites, vaccine developers have focused on creating a vaccine that limits the ability of the parasite to successfully infect large numbers of

red blood cells. This would not prevent the infection but would limit the severity of the disease and help prevent malaria deaths (Dubovsky, 2001).

Based on the life cycle of the parasite, there are three basic malaria vaccine candidates namely the pre-erythrocytic stage, the blood stage and the sexual stage. The efficacy of the vaccines will depend on the antigen(s) selected. It is generally believed that an ultimate vaccine will have components from all developmental stages of the malaria parasite (Dubovsky, 2001; Walgate, 2001).

The pre-erythrocytic stage is the most ideal disease prevention stage as it targets the sporozoites of the parasites while they are still intravascular or intra-hepatic (Dubovsky, 2001). Irradiated sporozoites, Circumsporozoite Surface Protein (CSP) or peptides and the Liver Stage Antigens-I (LSA-I) have been recognized as the promising antigen components of the vaccine. Such a vaccine would have salient features as being stage specific. The antibodies elicited would block infection of the liver but large doses of the vaccine would be required for it to be effective. If such a vaccine were to be 100% effective, the host would not develop malaria. This type of vaccine might be useful for people travelling to malaria endemic areas. If less than 100% effective and allows a single sporozoite to infect the liver, the host would either develop full blown malaria or less severe malaria due to the reduced number of sporozoites that reach the liver (Dubovsky, 2001; Walgate, 2001).

A blood stage vaccine or disease reducing vaccine targets the merozoites or contains blood stage antigens and would mimic the immunity that develops in people living in

endemic areas (Dubovsky, 2001). The merozoite antigens identified for the blood vaccine are Erythrocyte Binding Antigens (EBA), Merozoite Surface Antigens-I, and -II (MSA-I and MSA-II), Ring Infected Erythrocyte Surface Antigen (RESA), Serine Repeat Antigen (SERA), Rhoptry Associated Protein (RAP), Histidine Rich Protein (HRP) and Apical Membrane Antigen-I (APM-I). This group of vaccine antigen candidates would be specific for species and life cycle stage of the parasite and would prevent invasion of the erythrocyte by the parasite (Walgate, 2001). The host would still develop less severe clinical disease because these vaccines can not prevent infection (Dubovsky, 2001). Such a vaccine would be most appropriate for use in affected children and adults living in endemic countries because it would allow development of some degree of natural immunity.

The sexual stage or transmission blocking vaccine targets the gametes, the microgametocyte and macrogametocyte. This vaccine would work by blocking the sexual stage of the life cycle in the mosquitoes and thus preventing sporozoite production and consequent infection of a new host by the mosquitoes (Kaslow *et al.*, 1988; Mitchell, 1989a; Dubovsky, 2001). The antigens identified for this transmission blocking vaccine are simply known as *pf*s 25, 48/45K and *pf*s 230. Antibodies against these antigens prevent either fertilization or maturation of gametocytes, zygotes and ookinetes. Therefore, transmission blocking vaccines will prevent transmission of *P. falciparum* by mosquitoes when they feed on the new host's blood (Mendis, 1990; Dubovsky, 2001). Such a vaccine would be suitable for use in endemic areas (Kaslow *et al.*, 1988) where the majority of people enjoy some natural immunity, but not for visitors to such areas. It is generally believed that a

cocktail or combined vaccine would be a possible ultimate fourth type of vaccine, which will have all components indicated above to target the multiple stages of the malaria parasite's life cycle (Dubovsky, 2001).

The sporozoite model in which irradiated sporozoites were delivered via mosquito bites to volunteers point to the feasibility of developing a malaria vaccine. When vaccinated volunteers were subsequently challenged with naturally sporozoite infected mosquitoes, the volunteers were protected from developing malaria (Dubovsky, 2001). A research spanning from 1920 to the 1990s has shown that up to 95% of people exposed over several months to irradiated mosquitoes carrying malaria parasites developed protection against infection with wild type sporozoites and that such protection could last for about 10 months (Walgate, 2001). These findings show that evoking an immune response that would protect people from the disease is possible. The challenge is to define the exact segments of the immune response that are required and the specific antigen components which generate such a protective immune response. This knowledge is necessary to develop ways of manufacturing the vaccines and safely presenting these antigens to the host's immune system (Mitchell, 1989b; Dubovsky, 2001).

With the completion of the sequencing of the genome of the host, vector and parasite (Pearson, 2002), work is now shifting to proteomics with emphasis on the vaccine candidates and genes that encode them. There are efforts to develop DNA vaccines, which when injected into the host cell can make the protein components of the vaccine. DNA vaccines involve immunizing the host with the immunogen (antigen)

expressing plasmid containing a cloned gene of an immunogen. This allows the host to produce introduced gene products in sufficient quantities, resulting in the induction of both humeral and cellular responses (Walgate, 2001).

Optimism to breaking through the vaccine development also stems from the advent of new analytical tools, like genomics, and proteomics. Proteomics is a systematic, exhaustive approach to the analysis and identification of proteins (Steen and Mann, 2004). Because of these new developments, for the first time there is a possibility of identifying the real targets of irradiated *Plasmodium* sporozoite immunity or naturally acquired immunity to malaria. Using the published genome sequences, hundreds of new *Plasmodium* antigen candidates have been identified for malaria vaccines (Walgate, 2001).

Various factors make malaria vaccine development difficult and challenging (Dubovsky, 2001; Walgate, 2001). The size and genetic complexity, including mutations that occur within the parasite's DNA, mean that each malaria infection presents several thousands of antigens to the human immune system. The parasite changes through several complex life cycle stages, even while it is in the human host, presenting different subset of molecules or multiple antigens, which are specific to species and life cycle stages (Brown and Brown, 1965; Mitchell, 1989a; Dubovsky, 2001; Walgate, 2001). Further, it is possible to have multiple malaria infections of not only different species but also of different strains of species in a person at the same time (Dubovsky, 2001). The parasite has evolved a series of ingenious ways or strategies through which it can confuse the host immune response. *Plasmodium* can

thus hide and misdirect the human immune system, thereby avoiding the host immune response. The difficulty of evaluating and the complexity of conducting clinical and field trials is another challenge to the development of the malaria vaccine (Dubovsky, 2001; Walgate, 2001).

2.3.2 Chemotherapeutic control of malaria

There are only a limited number of drugs which can be used to treat or prevent malaria. The most widely used antimalarial drugs are quinine and its derivatives and antifolate combination drugs (Bloland, 2001). The sites of action of the antimalarial drugs in *Plasmodium* cells are shown in Figure 1.4. Control of malaria has for some time been centred on chemotherapy with chloroquine as the first-line drug of choice (Cowman and Foote, 1990; Hautvast *et al.*, 1998; Biemba *et al.*, 2000). First-line antimalarial treatment drugs are drugs used in the initial treatment of uncomplicated malaria, its signs or symptoms, usually on the basis of empirical evidence for its efficacy. While second-line antimalarial treatment drugs are drugs used for the treatment of uncomplicated malaria where the first-line drug fails. More recently chloroquine has been replaced with fansidar® in most of the malaria endemic areas. Treatment failures experienced with almost all the known antimalarial drugs has exacerbated the effective control of malaria resulting in high mortality (Cowman and Foote, 1990).

2.3.2.1 Quinine

Quinine (Figure 1.5) was the earliest antimalarial drug used in malaria control and has been used for close to 350 years in one form or another. It was originally

discovered in South America where it is found concentrated in the bark of the (Cinchona) tree (Cowman and Foote, 1990). Cinchona bark was originally used as an antipyretic agent by the local people. Quinine and its dextroisomer quinidine are to date the drug of last resort for the treatment of complicated malaria, including the cerebral form. Reports of isolated cases of resistance to quinine have been reported (Cowman and Foote, 1990).

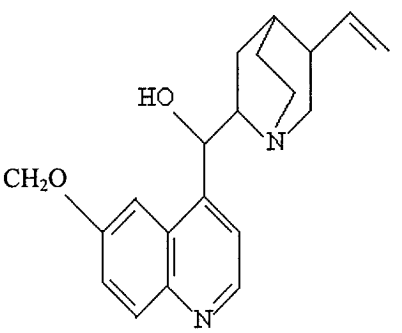

Figure 1.5 Chemical structure of quinine (Source: Delfino *et al.*, 2002)

2.3.2.2 Quinine related compounds

Amodiaquine is a relatively widely available compound closely related to chloroquine. Other quinine-related compounds in common use include primaquine (specifically used for eliminating the exoerythrocytic forms of *P. vivax* and *P. ovale* that cause relapses) and mefloquine, a quinolinemethanol derivative of quinine.

2.3.2.3 Chloroquine

Chloroquine (Figure 1.6) is a 4-aminoquinoline derivative of quinine. It was first synthesized in 1934 and was brought into wide spread use by the Americans (Cowman and Foote, 1990). Chloroquine has since been the most widely used

antimalarial drug in the world. Historically, chloroquine has been the drug of choice in the treatment of non-severe or uncomplicated malaria as well as in chemoprophylaxis. Its wide spread use has been attributed to its being cheap to produce and chemically stable, with low incidences of adverse side effects when used at the recommended doses. Chloroquine is active against the blood stages of all *Plasmodium* species that infect man (Cowman and Foote, 1990). Unfortunately, of late, chloroquine's widespread use has resulted in increased resistance, rendering it ineffective against *P. falciparum* in many areas of the world (Newton and White, 1999).

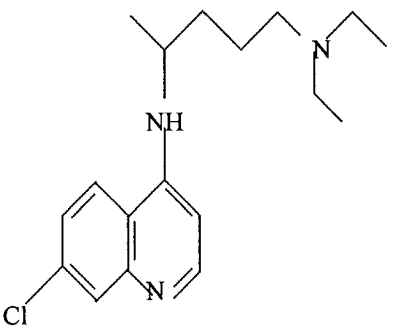

Figure 1.6 Chemical structure of chloroquine (Source: Delfino *et al.*, 2002)

2.3.2.4 Antifolate drugs combinations

Antifolate drugs are various combinations of dihydrofolate-reductase and dihydropteroate synthase inhibitors like proguanil, chlorproguanil, pyrimethamine, trimethoprim and sulfa drugs such as dapsone, sulfalene, sulfamethoxazole sulfadoxine, respectively (Bloland, 2001; Daily, 2006). Although these drugs have antimalarial activity when used alone, parasite resistance against them can develop rapidly (WHO, 2001a). However, when used in combination, they produce a synergistic effect on the *Plasmodium* parasite and can be effective even in the

presence of resistance to the individual components. Unfortunately, resistance is common to these drugs as either individual drugs or combination drugs (Cowman and Foote, 1990). Typical antifolate drug combinations include sulfadoxine and pyrimethamine (Fansidar®) whose chemical structures are given in Figure 1.7, sulfalene and pyrimethamine (Metakelfin®), and sulfamethoxazole and trimethoprim (Co-trimoxazole). A new antifolate combination drug is currently being tested in Africa. This new drug is a combination of chlorproguanil and dapsone, also known as Lap-Dap and has a much more potent synergistic effect on malaria than existing combination drugs such as fansidar® (WHO, 2001a). Benefits of Lap-Dap include a greater cure rate, even in areas currently experiencing some level of fansidar® resistance, a lower likelihood of resistance developing because of a more advantageous pharmacokinetic and pharmacodynamic profile, and its low cost estimated at less than US$ 1 per adult treatment course (Bloland, 2001).

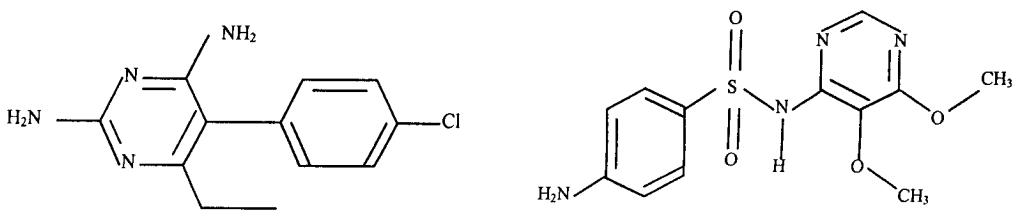

Figure 1.7 Chemical structures of two antifolates making up fansidar® (sulfadoxine/ pyrimethamine) (Source: Delfino *et al.*, 2002)

2.3.2.5 Antibiotics

Tetracycline and its derivatives such as doxycycline have a very potent antimalarial action and have an identical spectrum of action (Bloland, 2001; WHO, 2001a). The two drugs can be used for both treatment and prophylaxis. In areas where response to

quinine has deteriorated, tetracyclines are often used in combination with quinine to improve the cure rates (Bloland, 2001; WHO, 2001b). Clindamycin has also been used but offers only limited advantage when compared to other available antimalarial drugs. Parasitological response to clindamycin is slow (WHO, 2001a) and recrudescence rates are high. Its efficacy among non-immune individuals has not been fully established (Bloland, 2001).

2.3.2.6 Artemisinin compounds

The Artemisinin compounds such as artesunate, artemether, arteether are among the newest and most effective of all antimalarial drugs (WHO, 1987; Woodrow *et al.*, 2005; Daily, 2006). These drugs are sesquiterpine lactone compounds and have been synthesized from the plant *Artemisia annua.* Artemisinin is of Chinese-Vietnamese origin, derived from the herb *Artemisia annua L.*, locally known as "qing hao", a sweet wormwood or annual wormwood which belongs to the family of Asteraccae (Woodrow *et al.*, 2005). These compounds are used for treatment of severe malaria and have shown very rapid parasite clearance times and faster fever resolution when compared with quinine. Combination therapy of artemisinin compound with the other conventional antimalarial drugs, such as one with a long half-life like mefloquine, has been found to be capable of inhibiting the intensification of drug resistance development and of decreasing malaria transmission levels in South-East Asia due to their gametocidal properties (WHO, 1987; Bloland, 2001; Daily, 2006).

2.3.2.7 Other antimalarials

Halofantrine, a phenanthrene-methanol compound, has activity against the erythrocytic stages of the malaria parasite. The drug is especially recommended for use in areas with multiple drug-resistant *P. falciparum* (Bloland, 2001; WHO, 2001a). Recent studies have shown that the drug can produce fatal cardiac conduction abnormalities which have limited its usefulness (Bloland, 2001). Atovaquone, a hydroxynapthoquinone, is currently being used widely for the treatment of opportunistic infections in patients with suppressed immune system associated with AIDS. Although atovaquone is effective against chloroquine-resistant *P. falciparum* resistance develops rapidly when used alone. It is for this reason that atovaquone is usually used in combination with proguanil (Bloland, 2001; WHO, 2001a; Daily, 2006). Pyronaridine and Lumefantrine, a fluoromethanol compound, originally synthesized in China are currently undergoing field trials. Pyronaridine was reportedly 100% effective in a trial in Cameroon, but was only between 63% and 88% effective in Thailand (Bloland, 2001).

The WHO has recommended the use of artemisinin-based combination therapies (ACT) in the treatment of malaria as a response to the increasing levels of antimalarial drug resistance and to forestall further development of antimalarial drug resistance (Daily, 2006; WHO 2006). Artemisinin-based combination therapies have been recommended for the treatment of uncomplicated malaria caused by *P. falciparum*, including multi-drug resistant strains. One such ACT is coartem® (WHO 2001a; WHO 2006). Coartem® is a co-formulation of artemether and lumefantrine and is so far the most viable artemisinin combination treatment due to its efficacy,

safety, tolerance profile and fixed-dose formulation which is likely to bring about patient compliance to treatment regimen. However, the combination is not recommended for use in pregnant and breastfeeding mothers (WHO, 2001b).

2.4 Selectivity of drugs

Drugs work by selectively killing the parasite with or without side effects on the host. There are three categories of selectivity of drugs or selective toxicity. They exploit differences in drug accumulation, intermediary metabolism and structure of drug target site or difference in cell structure (Albert, 1975; Hayes and Wolf, 1990).

Some organisms accumulate drugs in their cells more than others. This is utilized in selective toxicity of drugs in the treatment of diseases caused by parasites where parasites accumulate the drug more than does the host (Albert, 1975; Hayes and Wolf, 1990). For example, trypanosomes readily concentrate organic arsenicals than do the host cells (Hayes and Wolf, 1990; Ouellette 2001).

Differences in biochemistry utilize the differences in intermediary metabolites or differences in biochemical pathways where one pathway may exist in one and not in the other species (Albert, 1975). Species differences in the pathways of intermediary metabolism can be exemplified by sulphonamide drugs. These anti-bacterial and antimalarial agents, which include sulfanilamide and sulfadianine, are relatively harmless to mammals. These drugs inhibit the enzyme dihydropteroate synthetase, which later converts dihydropteroic acid to the nucleotide precursor dihydrofolic

acid. Mammalian cells do not possess the target enzyme dihydropteroate synthetase, but rather uses preformed folates in their diet to synthesize dihydrofolic acid, they are resistant to toxic effects of sulphonamides (Ouellete, 2001; Sharma, 2005). Most pathogenic bacteria with the exception of *Streptococcus feacalis*, synthesize their own dihydrofolic acid as they lack the folate permease (Hayes and Wolf, 1990).

Selective toxicity that is due to differences in cell structure is exemplified by the lack of toxicity of the β-lactum antibiotics to mammalian cells, i.e. bacteria have a cell wall whereas mammalian cells does not (Albert, 1975; Hayes and Wolf, 1990). The differential inhibition by various drugs of protein synthesis in prokaryotes and eukaryotes is another example of a cytological difference resulting in drug selectivity. The bacteria 70S ribosome comprises a 30S and 50S sub-unit while the mammalian 80S ribosome comprises 40S and 60S subunits. Erythromycin and chloramphenicol bind the 50S sub-unit whereas aminoglycoside antibiotics, such as streptomycin, inhibit protein synthesis by binding to the 30S sub-unit (Voet and Voet, 1990). None of these antibiotics binds to the eukaryotic ribosome (Albert, 1975; Voet and Voet, 1990; Stryer, 1995). Conversely, cycloheximide and emetine, which have a high affinity for eukaryotic ribosomes and selectively inhibit protein synthesis in mammalian cells, do not prevent protein synthesis in prokaryotes (Hayes and Wolf, 1990).

2.5 Drug resistance

Drug resistance is the ability of the parasite species to survive and/or multiply despite the exposure and absorption of a drug given in doses equal to or higher than those usually recommended but within the limit of tolerance by the host (Pampana, 1969; Bloland, 2001; Farooq and Mahajan, 2004). It is the ability of the parasite to tolerate a drug concentration that would normally kill the normal members of the species (WHO, 1965; Goldstein *et al.*, 1968; Bloland, 2001). There are two types of drug resistance.

Intrinsic resistance or *de novo* resistance describes a situation where an organism or cell, possesses a characteristic mechanism which allows all normal members of the species to tolerate a particular chemical environment or drug (Pampana, 1969; Hayes and Wolf, 1990). The mechanism responsible for resistance is inherent or is an integral property of the species that has arisen through the process of evolution. Intrinsic resistance allows an organism or cell to survive repeated encounters of harmful chemicals in the environment. The nature and level of exposure to the chemical involved determines the type of resistance mechanism which evolves. The variations in the sensitivity of cells to chemicals and their level of intrinsic resistance presumably reflect the selection pressure endured in the course of evolution. The distribution of the drug resistance trait in a given population will reflect both the duration of its emergence and the selective advantage it conferred before exposure to the drug. Intrinsic resistance does not imply that all members of the given species possess similar resistance trait. Nonetheless, before exposure to the drug, the

distribution of the protective mechanism in most cases of intrinsic resistance will already be widespread within the wild type population (Hayes and Wolf, 1990).

Acquired drug resistance is where a resistant strain or cell emerges from a population that was previously drug sensitive (Goldstein *et al.*, 1968: Hayes and Wolf, 1990). The resistance may not be towards the selective agent or chemical only, but it may also be observed against other chemicals. The biological mechanism responsible for resistance is either absent from the population or is not expressed in the majority of the population prior to drug exposure. Acquired drug resistance can arise by several different mechanisms (Goldstein *et al.*, 1968). However, mutation and selection for protective genes play a key role in this process. Three types of genetic change are identified. These are mutation and amplification of specific genes directly involved in a protective pathway, mutations in genes regulating stress response processes and lead to the altered expression of large numbers of proteins, and gene transfer. These types of changes are not mutually exclusive, because examinations of the multiple changes that are frequently observed in resistant tumour cell lines suggest a simultaneous operation of several mechanisms (Hayes and Wolf, 1990).

2.6 Biochemical basis of drug resistance

Drug resistance can result from a lot of different biochemical mechanisms (Goldstein *et al.*, 1968). These consist of reduced drug delivery, decreased drug uptake, reduced metabolic activation of the drug, sequestration of the drug to prevent drug interaction with target site, drug efflux and increase in intracellular concentration of target sites. Others are structural alteration in the target site, duplication of the functions of the

target site and increased repair of damaged target site (Albert, 1975; Gutteridge and Coombs, 1977; Hayes and Wolf, 1990). Resistance to a particular drug can be achieved by one or more mechanisms. At most instances, particularly in the case of changes in either drug detoxification or drug transport, protection against more than one chemical is frequently observed. This can result in cross-resistance to structurally related or structurally unrelated compounds and this is referred to as multi-drug resistance, or pleotropic drug resistance (Hayes and Wolf, 1990).

Reduced drug delivery is a consequence of various factors, some of which are essentially independent of the target cell (Goldstein *et al.*, 1968). Drug delivery in multi-cellular or complex organisms is more complicated than in a single celled organism. Blood circulation is an important factor in the delivery of the drug to the target tissues in mammals. For example, the blood-brain barrier makes it difficult to target the brain in cancer chemotherapy, and therefore the brain is referred to as a sanctuary site for tumour cells (Hayes and Wolf, 1990). The half-life of drugs in plasma also influences the delivery of drugs to target cells (Bloland, 2001). Drug half-life is also dependent on a number of features, mainly on their rate of metabolism, most often in cells other than the target cells. Such factors as hormonal status, bacterial and viral infection influence the activity of drug metabolizing enzymes. Previous exposure to drug therapy or other inducing agents can exert a profound effect on the availability of the drug because the majority of detoxification enzymes are also induced by xenobiotics (Hayes and Wolf, 1990).

The general mechanism of resistance involves a defective transport of drugs across the cell membrane. The importance of a defective transport of drugs is considerably dependent on both the lipophilicity of the drug and the structure of the cell membrane (Goldstein *et al.*, 1968; Gutteridge and Coombs, 1977; Hayes and Wolf, 1990). Several distinct mechanisms are involved in the uptake of a particular drug (Ouellette, 2001). Passively absorbed drugs and those that are actively transported into cells are affected by this type of resistance mechanism. Physicochemical properties of the membrane determine the passive uptake or permeability of the drug (Goldstein *et al.*, 1968). For example, Gram-positive bacteria are more susceptible to many hydrophobic antibiotics than Gram-negative bacteria due to the increased drug diffusion rate across the outer membrane. The asymmetric lipid bi-layer membrane structure of the Gram-negative bacteria, which is comprised of only lipopolysaccharides in the outer leaflet and glycerophospholipids in the inner leaflet, makes the outer membrane less permeable to hydrophobic drugs. The water-filled diffusion channels formed by the porin proteins allow transportation of hydrophilic compounds across the outer membrane of Gram-negative bacteria. Active transport mechanisms in resistance also exist as exemplify by the uptake of nitrogen mustard via the choline transport system (Hayes and Wolf, 1990).

Proteins associated with drug efflux have been identified to play an important role in resistance to certain compounds (Goldstein *et al.*, 1968). Enterobacteriaceace provides an example of a number of such proteins encoded by plasmids, which act as membrane-located, energy-dependent efflux pumps, which confer resistance to tetracycline (Albert, 1975). Human P-glycoprotein, a trans-membrane energy-

dependent efflux pump, can similarly confer resistance to a number of anticancer drugs. Increased drug efflux leads to decreased drug accumulation by the cells (Hayes and Wolf, 1990). The acquired drug resistance to chloroquine exemplifies this type of mechanism by *P. falciparum* (Hayes and Wolf, 1990; Ouellette, 2001).

The intracellular concentration of the drug can be reduced by enzymes that are able to metabolize the drug (Goldstein *et al.*, 1968; Albert, 1975; Ouellette, 2001). Certain drugs (pro-drugs) require to be activated through metabolism by these enzymes before they can exert their chemotherapeutic effects, for example the conversion of proguanil to cycloguanil (Goldstein *et al.*, 1968; Albert, 1975). The potentiation or reduction in the toxicity of chemicals depends on the expression of drug metabolizing enzymes, as changes in both the activation and deactivation (Gutteridge and Coombs, 1977) pathways are important variables that can confer drug resistance (Hayes and Wolf, 1990). It involves reduced expression of activating enzymes or the over expression of detoxification enzymes (Hayes and Wolf, 1990). This mechanism has also been observed in resistance to penicillin by certain strains of Micrococcus like *Staphylococcus aureus* (Goldstein *et al.*, 1968; Albert, 1975).

Drug sequestration can lead to the reduction of the active drug concentration (Goldstein *et al.*, 1968) and this has been attributed to the increased intracellular drug binding and metallothionein protein whose increased expression has been implicated in this mechanism (Hayes and Wolf, 1990). There are 10 metallothionein (MT) genes in man which are divided into two major groups, MT-I and MT-II (Hayes and Wolf, 1990). The expression of human MT-II in human carcinoma cells results in a four-

fold increase in resistance to the anti-tumour agents like melphalan and chlorambucil (Hayes and Wolf, 1990; Ouellette, 2001).

Structural alteration of the cellular target for the drug leads to reduction in the affinity of the drug for the target enzyme or protein (Goldstein *et al.*, 1968; Hayes and Wolf, 1990). This type of drug resistance, which is usually associated with drugs whose target is well defined, result from point mutations in the structural gene(s) encoding the target. For example, mutation in the dihydrofolate reductase *of P. falciparum* leads to acquired drug resistance to pyrimethamine (Ouellette, 2001). Besides mutations in the genes encoding target proteins, it is possible that mutations or changes in other genes can contribute towards this type of resistance, for example those involved in post-translational modification. Phosphorylation is one such post-translational change that can substantially influence the protein activity. Resistance can also be achieved by increasing the intracellular concentration of the target protein (Gutteridge and Coombs, 1977). In this case although the target site's activity is inhibited, its structure remains unchanged. This means that, the overall activity of the enzyme or protein is maintained through an increase in target site abundance although the turnover number of the enzyme is reduced. This is best exemplified by the increased dihydrofolate reductase concentration or synthesis in methotrexate-resistant cell lines. This type of resistance often result from an increase in copy number of the gene encoding the protein, although, gene amplification is not the exclusive mechanism of protein over expression (Hayes and Wolf, 1990; Ouellette, 2001).

Production of a novel protein to by-pass the blocked step of the metabolic pathway results in resistance to the drug which is the blocking agent (Gutteridge and Coombs, 1977). The new protein which has the same function as the target protein no longer interacts with the drug. This is exemplified by trimethoprim resistance in bacteria where it inhibits dihydrofolate reductase (Goldstein *et al.*, 1968; Hayes and Wolf, 1990; Ouellette, 2001). The organism may secrete an excessive amount of a substance to which the drug is a metabolite antagonist. For instance, *Pneumococci* become resistant to sulfonamides by excessive production of para-aminobenzoic acid (Albert, 1975)

Continued repair of cellular damage is an important mechanism of resistance to alkylating agents and radiation. A number of DNA repair enzymes have been described and in the context of drug resistance, O-alkylguanine-DNA-alkyltransferase has been shown to be responsible for resistance to methylating agents such as N-methyl- N-nitrosourea (Hayes and Wolf, 1990).

2.7 Genetic mechanisms of drug resistance

The biochemical changes described under section 2.6, at molecular level can also result from gene amplification, gene transfer, point mutation, gene deletion, hypo- or hyper-methylation. All these could either occur in the genes directly involved in combating the cytotoxic compounds or could be in genes involved in their regulation and processing or both (Hayes and Wolf, 1990).

Gene amplification is a cellular process characterized by the production of multiple copies of a particular gene or genes to amplify the phenotype that the gene confers on the cell (Goldstein *et al.*, 1968; Voet and Voet, 1990). It is usually an unstable genetic condition that can only be maintained under strong selective pressure such as that conferred by drugs. For example, exposure of cultured animal cells to increased concentration of the dihydropteroate analogy, methotrexate, binds it to dihydropteroate reductase (DHFR) irreversibly and inhibits DNA synthesis. The resultant cells are then induced to have up to a 1000-fold up-regulation of DHFR gene expression and are thus capable of overproducing of the enzyme (Voet and Voet, 1990).

Point mutations are single base substitutions (Voet and Voet, 1990; Verma and Agarwal, 2001). The substitution can either be a purine for a purine or a pyrimidine for a pyrimidine, such substitutions are called transition substitution. The substitution may also involve a purine substituting a pyrimidine and *vice versa* in which case the substitution is called transversion (Verma and Agarwal, 2001). In the case where a new nucleotide alters the codon in a gene, a protein product with different properties from the original protein due to amino acid sequence difference is produced. This is exemplified by the point mutation in the DHFR or DHPS (Ouellette, 2001). However, certain point mutations are known to be silent mutations as they do not alter the properties or protein product as the substitution of a nucleotide in the codon does not alter the amino acid being coded for by the codon (Verma and Agarwal, 2001).

The removal of one or more base pairs in a gene may lead to total loss or absence of that gene. Deletion of one or two or multiple base pairs may lead to the translation of a gene that is frame shifted and consequently a change in the sequence of amino acids being encoded. The mRNA is translated in new groups of three nucleotides (codons) and the resulting protein will be different. Frame shifts often create new stop codons and thus generate nonsense mutations. Gene deletion would probably lead to loss or alteration of the target site or protein for the toxic agent (Verma and Agarwal, 2001).

Gene transfer is the passing on of a gene from one organism to another (Verma and Agarwal, 2001). There are two types of gene transfer horizontal and vertical. Vertical gene transfer is a normal process of reproduction which involves passing on of genes from parent to offspring. However, horizontal gene transfer refers to the transfer of genes or genetic material directly from one organism to another by processes similar to infection. Natural agents exist that can transfer genetic material or genes horizontally between organisms. These are viruses, many of which cause diseases, plasmids which are extra-chromosomal genetic elements that can be from one bacterium to another and transposons, the jumping genes. Many of these agents have been linked to the carrying and spreading of antibiotic and drug resistance. Penicillin resistance in *Streptococcus pneumonia* is a good example (Albert, 1975).

Methylation's essential role is to regulate gene expression (Chan *et al.*, 2005). The degree of methylation of cytosine in DNA is correlated with gene activity. Methylation interferes with the binding of factors to DNA that stimulate

transcription. However, this is not a universal regulatory device in higher eukaryotes. Depending on the species type hypo- or hyper- methylation may cause inhibition or activation of transcription of mRNA for a protein that may be involved in drug resistance. Methylation may therefore lead to either increased target protein concentration in the cell or cessation of its synthesis (Voet and Voet, 1990).

2.8 Biochemical and genetic mechanisms of antimalarial drug resistance

Plasmodium falciparum are haploid species with 14 chromosomes containing about 5300 genes (Warhurst, 2002). The genes involved in *P. falciparum* drug resistance are found on three chromosomes (Warhurst, 2002). Genetic mutations that are currently known or debated to confer antimalarial drug resistance are for only a limited number of drugs. These drugs are primarily the dihydrofolate reductase inhibitors like pyrimethamine, and dihydropteroate synthase inhibitors such as sulfadoxine and chloroquine (Bloland, 2001).

2.8.1 Chloroquine resistance

The food vacuole is a lysosome-like organelle in which the breakdown of haemoglobin and the detoxification of haeme occur (Fidock *et al.*, 2000; Farooq and Mahajan, 2001; Sharma, 2005). Chloroquine concentrates up to several 1000-folds in the food vacuole of *P. falciparum*. Possible mechanisms for this selective accumulation of the chloroquine in the food vacuole are protonation and ion trapping of the chloroquine due to the low pH of the food vacuole (Homewood *et al.*, 1972), active uptake of chloroquine by a parasite transporter(s), and binding of chloroquine to a specific receptor in the food vacuole. The exact contribution of these three

postulated mechanisms is not clear (Ouellette, 2001; Sharma. 2005), but it is generally accepted that chloroquine exerts its toxic effect by interfering with the polymerization of free haeme to haemozoin in the digestive food vacuole of *P. falciparum* during haemoglobin digestion, as shown in figure 2.1 (Ouellette, 2001).

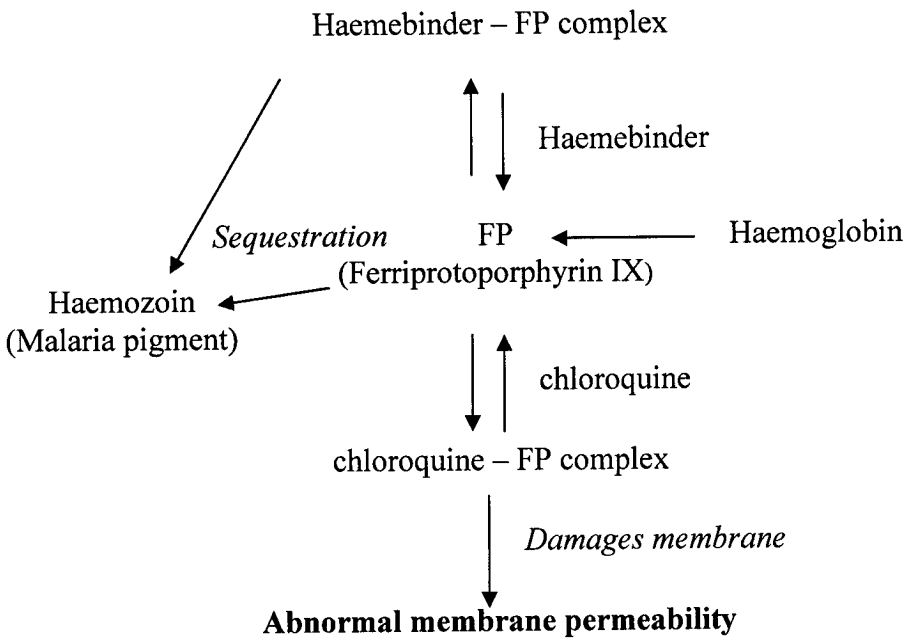

Figure 2.1 A diagrammatic representation of chloroquine action (Source: Eckman, 1977)

Large quantities of haeme are released as a result of haemoglobin digestion in the food vacuole. The free haeme can lyse membranes, leading to the generation of reactive oxygen intermediates, and inhibit many other processes. Haeme can therefore be quite toxic. Haeme is detoxified in the food vacuole via a biocrystallization process in which the haeme is sequestered into large insoluble crystals called haemozoin or the malarial pigment (Figure 2.1). The exact mechanism by which chloroquine inhibits haemozoin formation is not known (Fidock *et al.*, 2000), but chloroquine is believed to bind haeme and thus may prevent haeme from

being incorporated into the haemozoin crystal. *Plasmodium* parasites are killed due to the accumulation of metabolic haeme wastes associated with the digestion of haemoglobin (Chou *et al.*, 1980; Pagola *et al.*, 2000). Chloroquine resistance is therefore, associated with a decrease in the amount of chloroquine that accumulates in the food vacuole, the site of action for chloroquine. The mechanism for this decreased accumulation is still controversial (Farooq and Mahajan, 2000; Ouellette, 2001). Some studies have reported that the decrease in drug accumulation is due to an increase in drug efflux (Fidock *et al.*, 2000) while other studies suggest that diminished levels of chloroquine accumulation is more important (Walliker, 1989). The observation that verapamil and related drugs can reverse the chloroquine resistant phenotype has led to speculation that an ATP dependent transporter plays a role in drug efflux and chloroquine resistance, similar to the multi-drug resistance (MDR) in cancer. A MDR-like transporter, designated *Pf*MDR1, has been identified on the food vacuole membrane (Farooq and Mahajan, 2004). However, no definitive correlations between *Pf*MDR1 and chloroquine resistance could be demonstrated (Sharma, 2005). A genetic cross and mapping study between a chloroquine resistant clone and a chloroquine sensitive clone resulted in the identification of a 36 kb region on chromosome 7 associated with chloroquine resistance (Walliker, 1989; Ouellette, 2001). One of the 10 genes in this 36 kb region encodes a protein with 10 transmembrane domains and resembles a transporter protein similar to chloride channels. The gene has been designated as *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) and the protein is localized to the food vacuole membrane (Fidock *et al.*, 2000). Several mutations in the *pfcrt* show correlations with the chloroquine resistance phenotype and one mutation, a substitution of a

threonine (T or Thr) for a lysine (K or Lys) residue at codon 76 (K76T or *pfcrt* $76_{Lys/Thr}$) of the *pfcrt* shows a perfect correlation with chloroquine resistance (Warhurst, 2003; Sharma, 2005). The mutation *pfcrt* $76_{Lys/Thr}$ has been identified as potentially crucial for development of resistance (Djimde *et al.*, 2001; Warhurst, 2003). Presumably these mutations affect the accumulation of chloroquine in the food vacuole, but the exact mechanism of chloroquine resistance is not known (Fidock *et al.*, 2000). The mutation at *pfcrt* codon 76 is believed to result in the loss of the positive charge responsible for repelling into or maintaining positively charged chloroquine in the lysosome. The removal of this positive charge plays a key role in *pf*CRT's ability to expel chloroquine through an energy dependent efflux mechanism (Wellems, 2004). However, there are speculations that multiple genes are involved in the development of chloroquine resistance (Cowman and Foote, 1990).

2.8.2 Antifolate (sulfadoxine/pyrimethamine) resistance

The antifolates inhibit the essential metabolic pathway synthesizing tetrahydrofolate (THF) as illustrated in figure 2.2, which acts as a methyl group carrier for the thymidylate synthase reaction in DNA synthesis. While vertebrates use preformed folate from the diet, microorganisms and plants synthesize folates *de novo* (Ouellette, 2001). Folate metabolism is the target of several antimalarials as well as drugs used against other pathogens. Reduced folates serve as co-factors in many one-carbon transfer reactions involved in the biosynthesis of amino acids and nucleotides. Due to its high rate of replication, the malaria parasite has a high demand for nucleotides as precursors for DNA synthesis, and thus is particularly sensitive to antifolates. The two primary targets of antifolate metabolism are the *de novo* biosynthesis of folates

and dihydrofolate reductase (DHFR), an enzyme coded by the gene, *dhfr*, found on chromosome 4 (Ouellette, 2001).

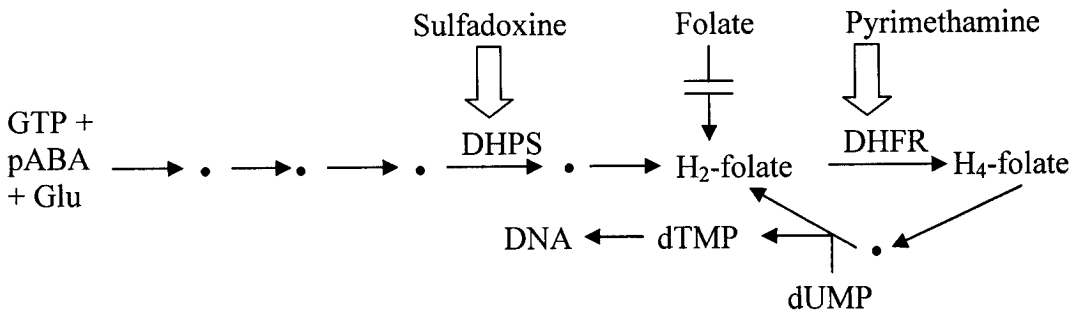

Figure 2.2 Malaria parasite folate biosynthetic pathway. (The two primary targets of antimalarial drugs which target folate metabolism are denoted with the boxed arrows) (Source: Ouellette, 2001; Wiser, 2003; Sharma *et al.*, 2005).

The malaria parasite synthesises folates *de novo* whereas the human host must obtain preformed folates because it cannot synthesise them (Ouellette, 2001). In nature, parasites that lack the ability to utilise host folate and are exquisitely sensitive to sulphonamides inhibition (Sims *et al.*, 1999; Watkins *et al.*, 1999). The inability of the parasite to utilize exogenous folates makes folate biosynthesis a good drug target. Folate is synthesized from three basic building blocks, guanosine triphosphate (GTP), para-aminobenzoic acid (pABA), and glutamate (Glu), in a pathway involving 5 enzymes (Ouellette, 2001; Delfino *et al.*, 2002). One of these enzymes, dihydropteroate synthase (DHPS) coded for by the gene *dhps*, found on chromosome 8, is inhibited by sulfa-based drugs. Sulfadoxine, one of the two components of the drug commercially known as fansidar®, and dapsone are two common antimalarials that target DHPS. The sulfa drugs are structural analogs of pABA and are converted into non-metabolizable adducts by DHPS (Sharma, 2005). This leads to a depletion

of the folate pool and thereby reduces the amount of thymidylate available for DNA synthesis (Schellenberg and Coatney 1961; Gutteridge and Trigg, 1971).

Dihydrofolate reductase is a ubiquitous enzyme that participates in the recycling of folates by reducing dihydrofolate to tetrahydrofolate. The tetrahydrofolate is then oxidized back to dihydrofolate as it participates in biosynthetic reactions such as thymidylate synthase. Inhibiting DHFR will prevent the formation of thymidylate and lead to an arrest in DNA synthesis and subsequent parasite death. Pyrimethamine, the second component of the drug fansidar®, and proguanil are the two most common DHFR inhibitors used as antimalarials. These drugs inhibit DHFR from the parasite to a greater degree than the host enzyme and thus show a selective toxicity towards the parasite (Cowman and Foote, 1990).

Most often, inhibitors of DHPS and DHFR are used in combination for a synergistic effect and to slow the development of drug resistance (Bloland, 2001; Ouellette, 2001: Sharma, 2005). Specific point mutations in the genes coding for these enzymes lead to a lower affinity for the drugs (Ouellette, 2001; Sharma, 2005). Point mutations at Dihydrofolate Reductase (DHFR) and Dihydropteroate Synthetase (DHPS) enzyme genes of *P. falciparum*, *dhfr* and *dhps* have been associated with emergence of drug resistance to pyrimethamine and sulfadoxine, respectively (Ouellette, 2001; Farooq and Mahajan, 2004; Sharma, 2005). Thus, *P. falciparum* must first gain mutation on both genes in order to develop resistance to combined antifolates e.g. sulfadoxine / pyrimethamine combination (Jelinek, *et al.*, 2002).

Of the mutations that are associated with resistance to antifolates, the mutation at codon 108 of the DHFR gene involving amino acid change from aspartic acid to serine (DHFR $108_{Ser/Asp}$) has been identified as crucial mutation for resistance to group I antifolates, while mutations involving amino acid change from isoleucine (Ile) to aspartic acid at codons 51 and from arginine to cysteine at codon 59 of the DHFR (DHFR $51_{Asp/Ile}$ and DHFR $59_{Cys/Arg}$) have a supporting function in enhancing the effect (Ouellette, 2001; Kublin *et al.*, 2002). The point mutation which occurs at codon 108 of the *dhfr* of *P. falciparum* results in the amino acid residue change to asparagine (Asn). The change results from a change in the second base in the codon specifying serine, that is from AGC to AAC. The effect of this change in amino acid residue on the enzyme leading to pyrimethamine resistance has been explained through the use of molecular modelling as the tertiary structure of *P. falciparum* DHFR has not been determined (Delfino *et al.*, 2002; Warhurst, 2002). The modelling has led to the determination of the structure and functional properties of the enzyme. According to Delfino *et al.* (2002) one model proposes that the mutation at codon 108 resulting in amino acid residue change from serine to Asn would set residue Asn108 too close to the chlorine atom of pyrimethamine in such a way that there might not be enough space for the drug to bind the enzyme. Another model agrees to this proposal that the mutation could cause some hindrance to the dynamic process of admission and accommodation of the inhibitors to the active site. A third model that included several mutations in its analysis proposes that the change from serine to Asn at codon 108 acts by displacing the drug from its original position, due to a steric clash between Asn108 and chlorine atom of pyrimethamine resulting in a loss of planarity of the bidentate hydrogen bond of the drug with aspartic acid (Asp)

54, thus leading to destabilization of the ternary complex (Delfino *et al.*, 2002). Asparagine is an acidic amino acid with a bulky side chain and is more polar than serine, an aliphatic amino acid (Stryer, 1995). Since the length and bulkiness of the side chains correlate significantly to reduced sensitivity, (Warhurst, 2003) this could also attribute to the reduced sensitivity of the DHFR to pyrimethamine. In models, the methylene carbon of the serine side chain at codon 108 is likely to interact hydrophobically with the chlorine atom of pyrimethamine which is less than 4 angstrom (Å) away (Warhurst, 2003). When the change to Asn 108 takes place, an unfavourably approximation of less than 3 Å is likely between the side chain amino nitrogen of the residue and the phenyl ring and chloride atom of the drug requiring the drug to shift from its optimal position in the active site thereby reducing activity (Warhurst, 2003). Minimal reduction in affinity for substrate is also associated with this mutation (Warhurst, 2003). The change in amino acid from Asn to isoleucine (Ile) at codon 51 involves point mutations in the codon AAT/AAC for Asn to ATT/ATC for Ile. The change involves an acidic amino acid Asn to an aliphatic amino acid Ile (Stryer, 1995). The mutation at codon 59 involves a change from the wild type cysteine/TGT-59 to the mutant arginine (Arg). The change involves cysteine (Cys), a sulphur containing amino acid, to Arg a basic amino acid (Stryer, 1995). The action of these mutations resulting in a change from Asn to isoleucine at *dhfr* codon 51 and from Cys to Arg at *dhfr* codon 59 is not clear and could not be explained from the molecular models. However, these mutations have been linked to the possibility that the residues might have an indirect influence on the steric or electrostatic properties of the binding site that might result in repulsion between the positively charged Arg 59 and the protonated form of the drug, in which form the

drug (pyrimethamine) interacts (Delfino *et al*, 2002). It has also been suggested that the distances between these residues and the drug position in the active site affects the approximation of the drug to the active site, rather than on its accommodation and this results probably from electrostatic effects (Delfino *et al*, 2002).

Point mutation resulting in change of amino acid from alanine (Ala) to glycine (Gly) at codon 437 of DHPS (DHPS $437_{Ala/Gly}$) is viewed as prerequisite for resistance to group II antifolates (Sharma, 2005). The combination DHPS $437_{Ala/Gly}$ and a mutation resulting in a change of amino acid from Lys to glutamic acid (Glu) at DHPS codon 540 (DHPS $540_{Lys/Glu}$) is viewed as the most important mutation associated with resistance to sulfonamides (Ouellette, 2001; Kublin *et al.*, 2002). The point mutation at codon 437 in the *dhps* resulting in sulfadoxine resistance by *P. falciparum* is as a result of the replacement of a cytosine with a guanine in the codon specifying Ala. The change results in the replacement of Ala with Gly in the DHPS (Farooq and Mahajan, 2004; Sharma, 2005). The effect of this change on the enzyme function can not fully be explained as the three dimensional crystal structure or tertiary structure of *P. falciparum* DHPS is not yet known (Triglia *et al.*, 1998). However, the effect of the various mutations on the inhibitor and substrate binding by the enzyme has been worked out by comparison with recently solved DHPS structures of the *Staphyloccous aureus* and *E. coli* homologues (Triglia *et al.*, 1998). The oxygen from the sulfonamide was shown to accept a hydrogen bond from the guanidinium group of Arg 63 in *E. coli* DHPS. Arginine 63 in *E. coli* corresponds to Gly 437 in *P. falciparum*, which is the determinant mutation found in almost all sulfadoxine resistant parasites (Triglia *et al.*, 1998). This suggest that residue 437 in

P. falciparum DHPS is very close to or involved in both substrate and inhibitor binding (Triglia *et al.*, 1998). Glycine has a small side chain, which is hydrogen, compared to a larger side chain, a methyl, on alanine but both are aliphatic amino acids (Stryer, 1995). The introduced small side chain could possibly affect the orientation of the drug in the active site bringing about a reduction in its binding or access to the active site (Triglia *et al.*, 1998). The mutation at codon 540 of the *dhps* involves a change in the codon AAA specifying lysine in the wild type parasites to GAA specifying Glu in the mutant parasites. The mutation involves a change from a basic amino acid, Lys, to an acidic amino acid, Glu (Stryer, 1995). The mutation is known to have a large effect on both the reduced affinity for binding sulfadoxine and the level of resistance in live parasites. However, this residue is not close to any amino acid residue implicated in substrate or inhibitor binding (Triglia *et al.*, 1998).

2.9 Health implications of *Plasmodium* drug resistance

Diseases caused by resistant organisms, such as *P. falciparum,* fail to respond to treatment, resulting in prolonged illness and greater risk of death. Resistant infections therefore become fatal and increase the mortality rates in all age groups affected (Plowe, 2003). Drug resistance leads to treatment failures, longer periods of illness, and an increased number of infected people moving in the communities (Bloland, 2001), thus exposing the general population to the risks of contracting a resistant strain and the morbidity due to malaria becomes very high (WHO, 2002a). Due to increased morbidity or prolonged illness, the cost of caring for the sick also goes up. Equally affected is the economic aspect in the sense that if workers are affected they stay away from work for longer periods of time and this leads to decreased

productivity (Pampana, 1969). When a person is infected with parasites which are resistant to first-line antimalarial drugs, treatment has to be switched to second or even third-line drugs usually with longer treatment regimens. The scenario is nearly always more expensive and sometimes even more toxic (WHO, 2002a). In many countries, the high cost of such replacement drugs is economically prohibitive (Plowe, 2003), resulting in disease conditions which can no longer be easily treated due to resistance problem involving virtually all the easily available drugs (Farooq and Mahajan, 2004). Drug resistance problem has been exacerbated by the limited number of solutions in terms of drug discovery, as there are very few new drugs on the horizon for use against drug resistant malaria infections (Ouellette, 2001; Fidock *et al.*, 2004). Even if the pharmaceutical industry were to step up the efforts to develop new replacement drugs, current trends seem to suggest that malaria being a third world disease, there is generally likely to be reluctance to invest in new antimalarial drug development (Fidock *et al.*, 2004).

2.10 Induction of drug resistance in the Laboratory

Experimental drug resistance induction in the laboratory can be achieved by giving prolonged sub-curative doses or levels of the drug to the parasite (Goldstein *et al.*, 1968; Chitambo and Arakawa, 1992). While certain parasites die from sub-curative doses, others survive. This means that the stress level of the parasites is gradually reduced. The surviving parasites are further exposed to an increased dose of the drug and surviving parasites end up acquiring resistance to the drug applied (Chitambo and Arakawa, 1992). The parasites used in the laboratory for the induction of drug resistance are isogenic parasites from the same origin.

2.11 Drug resistance in the field

In the field, drug resistance can cause treatment failure, but not all treatment failures observed are due to drug resistance (Bloland, 2001). Therefore, there is distinct difference between failure to clear malarial parasitaemia or resolve clinical disease after treatment with an antimalarial drug and true antimalarial drug resistance. The accessibility of the drug in question, to the parasite or the infected red blood cell for the duration of the time necessary for its normal action must also be taken into consideration. Incorrect dosing, misdiagnosis, non-compliance with duration of dosing regimen, poor drug quality, drug interactions, and erratic absorption can all contribute to treatment failure (Pampana, 1969; Bloland, 2001). While causing treatment failure in the individual, these factors may also contribute to the development and intensification of true drug resistance through increasing the likelihood of exposure of *Plasmodium* parasites to suboptimal antimalarial drug levels (Bloland, 2001).

2.11.1 Causes and the development of drug resistance

Numerous factors can, singly or in combination contribute to the advent, spread and intensification of drug resistance. The relative contribution to induction of parasite resistance is however, unknown (Bloland, 2001). Factors associated with sub-curative or sub-therapeutic or sub-optimal level of the drugs in blood plasma contribute more to the development and intensification of drug resistance.

2.11.2 Factors inducing drug resistance in *Plasmodium falciparum*

The response of sensitive *Plasmodium* parasites to antimalarial drugs *in-vitro* and the pharmacokinetic profiles of common antimalarial drugs indicate that there is always a residuum of parasites that are able to survive treatment (Wernsdorfer, 1991). Selection of mutants by the drugs themselves appears to be an important mechanism. Under normal circumstances, the residuum parasites are removed by the immune system, non-specifically, in non-immune individuals (Bloland, 2001; Cravo *et al*, 2001). Therefore, any factors that compromise the effectiveness of an individual's immune system affect the clearing of residuum parasites post-treatment and, thus, facilitate development and intensification of resistant strains. In an environment where sub-curative levels of the antimalarial drug are rampant, parasites develop resistance through biological natural selection variation and mutation. This allows the resistant strains to attain numerical superiority through intra-specific competition imposed by continued use of the drug for the elimination of non-resistant strains, initially in the minority, in the surviving population (Bloland, 2001).

Drug pressure has been implicated in the emergence of drug resistant malaria (WHO, 1987; Basco *et al*, 1998). Drug pressure can be induced through uncontrolled and irresponsible prophylaxis and therapeutic treatments which tend to favour the elimination of highly drug sensitive strains of the parasites, in effect selecting the less sensitive ones which undergo spontaneous mutations to further reduce the sensitivity of the parasites to the drugs in use. Development and intensification of drug resistance is exacerbated by programmes utilizing mass drug administration (Payne, 1988). Studies carried out in urban, peri-urban and rural areas, have

suggested that resistance rates are higher in urban and peri-urban areas, where access to and the use of drugs is greater, than in rural communities (Bloland, 2001).

Poor compliance or inappropriate dosing regimens can contribute to inadequate drug intake (WHO, 2002a). The treatment regimen is often based on age rather than weight in children. This has been shown to produce systematic under dosing among children (WHO, 2001a). The use of presumptive treatment for malaria has also the potential for facilitating resistance by greatly increasing the number of people who are treated unnecessarily and thus helping to exert selective pressure on the circulating parasite population. In certain cases, treatment failure may result from concurrent treatment with other drugs which may increase the chances of drug resistance development (Bloland, 2001; WHO, 2002a).

Poor drug quality has been implicated in antimalarial treatment failures and probable drug resistance development (Bloland, 2001). Poor manufacturing practices, intentional counterfeiting, or deterioration due to inadequate or poor handling and storage, can render the active ingredients of drugs become inactive and insufficient in quantity (Bloland, 2001; WHO, 2002a). Such drugs expose the parasites to lower concentrations than those required for their elimination resulting in the selection and development of resistant strains (Bloland, 2001).

2.12 Identification of drug resistance in *Plasmodium*

Detection of drug resistance or drug tolerance in individual patients is made difficult in many settings by non availability and poor quality of microscopy (Bloland, 2001).

In Africa, presumptive diagnosis and treatment for malaria are used, hence, detection of treatment failures also tends to be presumptive (Bloland, 2001; Plowe, 2003). The persistence or re-appearance of clinical symptoms in a patient recently receiving malaria treatment is often assumed to be due to treatment failure, even where such persistence or re-appearance of clinical symptoms may be due to re-infection. Because of the non-specific nature of clinical signs and symptoms associated with malaria, this can lead to a false negative or positive treatment efficacy result. Presence of parasitaemia in a supposedly fully treated malaria patient may indicate treatment failure, but is not necessarily due to drug resistance (Bloland, 2001).

There are three basic methods which are routinely used to detect antimalarial drug resistance. These are *in-vivo*, *in-vitro* and molecular characterization (Bloland, 2001; Plowe, 2003). Other less rigorous methods include case reports, case series, or passive surveillance. Careful consideration of the type of information each of these latter methods yields indicate, however, that these are merely complementary, rather than competing sources of information about drug resistance (Bloland, 2001).

2.12.1 *In-vivo* test

2.12.1.1 Drug efficacy test

In-vivo drug efficacy tests involve treating a group of symptomatic and parasitaemic individuals with known drug doses and then monitoring the parasitological and/or clinical response over time (Bloland, 2001; Farooq and Mahajan, 2004). The *in-vivo* drug efficacy tests have since been modified by the WHO to make them more convenient and easier than the original tests which lasted 28 days to the one lasting

14 days (Bloland, 2001; Plowe, 2003; Farooq and Mahajan, 2004). The modified *in-vivo* test does not require seclusion of the patients as it is assumed that the reappearance of parasites within 14 days of treatment would be more likely due to recrudescence than reinfection (Bloland, 2001; Farooq and Mahajan, 2004). This method also emphasizes on clinical response besides the parasitological response. Later modifications have further combined parasitological and clinical indicators to varying extents. These modifications are based on adequate clinical response, early and late treatment failure (WHO, 2002b; Farooq and Mahajan, 2004).

The *in-vivo* test most closely reflects actual clinical or epidemiological situations, that is, the therapeutic response to the drug of currently circulating parasites infecting the actual population in which the drug will be used. This approach offers the best information on the efficacy of antimalarial treatment among clinical patients especially where medical provider and patient compliance is high (Bloland, 2001). The results of *in-vivo* tests are affected by such external factors as host immunity, variation of the drug pharmacokinetics and potential misclassification of re-infections as recrudescence. The results, therefore, are not a true reflection of the level of pure antimalarial drug resistance (Bloland, 2001; Plowe, 2003). Unfortunately, differences in sample size, enrolment criteria, exclusion criteria, length and intensity of the follow-up, loss-to-follow-up rates and interpretation and reporting of results are evident in most reported *in-vivo* trials. This makes these tests not standardized even when they are considered to be standardized tests. These differences can be so dramatic that a comparison of results from one study to

another, with any meaningful level of confidence, is difficult if not impossible (Bloland, 2001).

2.12.1.2 Animal models Tests

This is an *in-vivo* drug efficacy assay conducted in a non-human animal model. It is, therefore, influenced by many extrinsic factors as seen in drug efficacy tests (Bloland, 2001). The host immunity's influence on the tests can be minimized by using specifically selected lab-reared animals or animal-parasite combinations which are unlikely to occur in nature, other host factors would however, still be present. The availability of a suitable animal host in such assays may allow for the testing of parasites which can otherwise not be adapted to *in-vitro* conditions (Bloland, 2001; Farooq and Mahajan, 2004). The requirement of parasites that can grow in, or are adaptable to, non-human primates for investigation, puts this assay at a significant disadvantage However, experimental drugs which have not yet been approved for use in humans are tested using these assays (Bloland, 2001).

2.12.2 *In-vitro* tests

In-vitro drug efficacy assays involve the isolation of parasites from the patients and subjecting them to a controlled experimental environment (Bloland, 2001; Farooq and Mahajan, 2004). The micro-technique is the most frequently used procedure. Parasites obtained from a finger-prick blood sample are exposed to precisely known drug doses on a microtitre plate (Bloland, 2001). The parasites are then monitored for any inhibition of their maturation into schizonts. This assay accurately reflects the relatively true antimalarial drug resistance when compared to other assays (Bloland,

2001; Farooq and Mahajan, 2004) and is convenient as it can be performed on multiple isolates with several drugs simultaneously, including experimental drugs. It also has certain significant disadvantages. There is neither a clear nor consistent correlation of *in-vitro* response with clinical response in patients, as the level of acquired immunity within the population being tested appears to influence the results (Bloland, 2001). Pro-drugs, such as proguanil, cannot be tested, because they are required to be converted into active metabolites by the host. Drugs that require some level of synergism with the host's immune system are also not feasible in this assay (Bloland, 2001). The differential die-off of cultured parasites would make resistant strains of *P. falciparum* less likely to adapt, and therefore the results would be biased towards sensitive strain responses. The requirement for venous blood (Plowe, 2003), makes the study of resistance in the more vulnerable younger age groups almost impossible. Finally, *in-vitro* assays are potentially more difficult to adapt for routine field work as they are technologically more demanding and expensive (Bloland, 2001).

2.12.3 Molecular techniques

Molecular tools use polymerase chain reaction (PCR) technique coupled with restriction enzymes to indicate the presence or absence of mutations that confer biological resistance to antimalarial drugs (Singh, 1997; Bloland, 2001)., The prevalence rates of specific gene mutations within a sample of parasites obtained from patients from a given area could theoretically provide an indication of the frequency and level of drug resistance in that area (Bloland, 2001; Plowe, 2003).

2.12.3.1 Detection of genetic variation

Polymerase chain reaction based assays provide valuable tools for studying parasites genetic diversities (Plowe, 2003). The commonly used PCR-based methods for detecting parasite genetic variation are hybridization, size polymorphism, restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) (Newton and Graham, 1997; Singh, 1997). Information on the nature and extent of genetic diversity of the parasite population in a specific geographical location can be obtained using these methods (Singh, 1997).

Polymerase chain reaction is used to amplify a specific portion of a parasite (Newton and Graham, 1997; Singh, 1997). The PCR product is separated using gel electrophoresis and transferred onto nitrocellulose or nylon membrane. Specific probes for a particular allele or family of alleles for the gene are then used to hybridize to the PCR product on the membrane (Newton and Graham 1997; Singh, 1997). Stringent hybridization conditions are used to ensure that there is specific probe hybridization to the genes belonging to the same allele or family of alleles. This method has been applied to study the genetic variation within the *P. falciparum* population of genes coding for two merozoite surface antigens (MSA), MSA 1 and MSA 2 as candidates for a potential subunit vaccine against malaria (Singh, 1997).

Polymerase chain reaction primers to conserved regions flanking one or more polymorphic regions of the gene are used in PCR size polymorphism (Newton and Graham, 1997; Singh, 1997). Deoxyribonucleic acid polymorphisms can result from either minor variations caused by a single or multiple base substitutions, or presence

of DNA blocks of about two or more than 50 bases long repeated in tandem arrays of blocks (Singh, 1997). The DNA polymorphisms can vary between different strains of the same parasite, resulting in different lengths of PCR products (Singh, 1997). The PCR products are separated by agarose gel electrophoresis, stained with ethidium bromide and viewed under UV illumination (Sambrook *et al.*, 1989). This method has been applied to the study of polymorphic regions of the MSA-1 and MSA-2, which contain variable numbers of tandem repeats and also to the genes encoding the circumsporozoite surface protein (CSP) (Singh, 1997).

Restriction fragment length polymorphism (RFLP) detects minor variations in a gene caused by a single base substitution resulting in either creation or abolition of a restriction site capable of being digested by a restriction endonuclease (Singh, 1997). Restriction endonucleases are bacterial enzymes that cut or digest specific sequences of four to eight bases in double stranded DNA (Voet and Voet, 1990; Singh, 1997). In RFLP, a region of a gene containing a single or multiple base substitutions is amplified using PCR and the PCR product is digested with one or more restriction endonucleases. These digested products are separated by agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light (Sambrook *et al.*, 1989; Newton and Graham, 1997) to determine the particular allelic type through banding pattern (Singh, 1997). The polymorphic marker being studied and the PCR primers used for amplification, determines the choice of the restriction enzyme to be used (Singh, 1997).

The RAPD technique requires no prior knowledge of DNA sequence that is to be amplified, as is the case with hybridization, size polymorphism, and RFLP because only one relatively short oligonucleotide or primer of arbitrarily sequence is utilized in the PCR (Newton and Graham, 1997; Singh, 1997). A short and less specific primer binds to complementary sequences of both DNA strands, resulting in amplification of any intervening regions. The amplification products appear as specific banding patterns for each parasite strain after gel electrophoresis (Singh, 1997). The primer utilized for genetic studies, gives reproducible banding patterns after amplification and is chosen from a number of short primers which are tested individually (Newton and Graham, 1997). An unreliable banding pattern for a particular parasite strain can be obtained due to contaminating DNA since the primers used are capable of binding to any complementary DNA. RAPD has been used for DNA finger printing and genetic variation for many parasites (Newton and Graham, 1997; Singh, 1997).

2.12.3.2 Advantages of molecular techniques

Small amounts of genetic material are required instead of live parasites. The test is independent from host and environmental factors and it is easy to conduct large numbers of tests on a large number of samples in a relatively short period of time (Bloland, 2001).

2.12.3.3 Disadvantages of molecular techniques

There is need for sophisticated equipment and well trained personnel. The known gene mutations that correlate with antimalarial drug resistance in *P. falciparum* are

currently known or debated for only a few antimalarial drugs such as pyrimethamine, sulfadoxine and chloroquine (Bloland, 2001; Plowe, 2003) and therefore, only resistance to these drugs can be assessed. It is difficult to confirm the association between given mutations and actual drug resistance. This is especially so when the mode of resistance involves more than one gene and multiple mutations. Resolution of these complexities will make molecular technique extremely valuable surveillance tools for monitoring the occurrence, spread and/or intensification of drug resistance (Bloland, 2001).

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study site

Solwezi urban clinic is located in the middle of Solwezi town. The town is found in northwestern province of Zambia and is about 583 km by road from the capital city, Lusaka. It is located at longitude 26.2° E and latitude 12.2° S. Solwezi lies in the northern part of the country with the highest transmission rates of malaria.

3.2 Methods

3.2.1 Sample collection

Samples were collected from malaria confirmed patients with permission from Solwezi district health management team. All patients were checked by a medical officer and referred to the laboratory technician to have their malaria blood slide test done. Patients found with the parasite in their blood were requested to enrol for the study and sign the consent form (Appendix 1). Infected blood was collected by pricking finger tip with a lancet, soaking it into the 3 MM® Whatman filter paper, until the filter paper was evenly red. All blood-impregnated filter papers were air-dried, placed in an airtight plastic bag, labelled and sealed. Each blood-impregnated filter paper from an individual was put in a separate plastic bag. Care was taken not to touch the filter papers with bare hands, especially the blood spot, to avoid either contamination or degradation of the parasite DNA. The samples were then stored at -20 °C (Schlichtherle *et al.*, 2000). Patient's records taken included name, age, sex, parasite density, date of sample collection, a brief history of malaria disease for the

patient and any antimalarial drugs used in the recent past with appropriate dates on which drugs were taken.

A check was made to find out whether the recruited patients went back to the health centre or clinic with symptoms and signs of malaria 14 days post-treatment. Any patient who returned with a malaria problem within this period was allotted into a relapse and not re-infection group and was designated into the drug treatment failure group suspected to be carrying fansidar® resistant *P. falciparum*. Any antimalarial drug prescribed at the second visit was also recorded. The patients were followed up for a further period of two months.

3.2.2 DNA extraction

Pieces of approximately 4 mm^2 piece of blood-impregnated filter paper were cut with a sterile surgical blade. A new blade was used for each sample. The pieces cut from the middle of the blood spot were placed into 100 µl of methanol for 15 min and then air dried for 1 h by laying the microcentrifuge tubes on their sides on a filter paper. The cut blood impregnated filter papers were then incubated at about 95 °C in 50 µl of freshly sterilized distilled water for 10 min in a covered water bath. During incubation the tubes were subjected to high-speed vortexing three times. Five microlitres of the resulting solutions were then used as DNA templates for amplification (Djimde *et al.*, 2001).

3.2.2.1 DNA amplification

The presence of mutations on codons 437, 540, 108 and 59 associated with sulfadoxine/pyrimethamine resistance was investigated following the methods of Duraisingh *et al.* (1998). A Techgene thermal cycler model FTGENE 5D (Techne Duxford, Cambridge, U.K) was used for DNA amplification. The DNA amplification was done using nested PCR. This involved the use of two pairs of primers, with one pair composed of the forward and reverse primers which were used in the primary PCR to identify the segment of DNA containing the suspected mutation. The second pair of primers was then used in the secondary PCR, with the amplified DNA in primary PCR acting as the template DNA. This allowed amplification of the desired DNA fragment, avoiding non-specific amplifications.

3.2.2.1.1 Amplification of Plasmodium falciparum chloroquine resistance transporter (pfcrt) fragments containing codons 75 and 76

The primers in Table 3.1 (Amplimmun AG, Madulain, Switzerland) were used in the amplification of the DNA segments containing the *pfcrt* codons 75 and 76.

Table 3.1 Primer sequences used for PCR amplification of *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) fragments containing codons 75 and 76

Codon	Nest	Primer name	Primer sequences	Product size
76 & 75	1	76 – A	5' GCGCGCGCATGGCTCACGTTTAGGTGGAG 3'	206 bp
		76 – B	5' GGGCCCGGCGGATGTTACAAAACTATAGTTACC 3'	
76	2	76 – D1	5' TGTGCTCATGTGTTTAAACTT 3'	145 bp
		76 – D2	5' CAAAACTATAGTTACCAATTTTG 3'	
75	2	CRT745-MS	5' TAAGTATTATTTATTTAAGTGTATGTGTGAT 3'	84 bp
		76 – D2	Same as codon 76	

The primary PCR was performed on a total reaction volume of 25 µl containing 5 µl of extracted DNA sample as template, 0.25 µl of 100 µM of each of the forward (76–A) and reverse (76 – B) primers at 1 µM final concentration, 2.5 µl of 2 mM dNTPs mixture at 200 µM final concentration for each, 0.5 µl Taq DNA polymerase (2.5 U), 2.5 µl of 10× PCR buffer (Promega, USA), and 14.0 µl sterile distilled water.

The secondary PCR reaction mixture was the same as the primary mixture except that the DNA template used was 2 µl and 0.2 µl of the primary PCR product for codons 76 and 75, respectively. The primer pairs used were forward (76 – D1) and reverse (76 - D2) and 17 µl of sterile distilled water for codon 76 while for codon 75 the forward (CRT745MS) and reverse (76 – D2) primers and 18.8 µl of sterile distilled water were used. A master mix was constituted depending on the number of sample DNA to be amplified. This was done by multiplying the total number of samples to be amplified with the volume of each reagent for one sample, for all the reagents in a 25 µl reaction mixture. In all reactions, a control was included in which sterile distilled water instead of the template DNA was added to check for contamination and non-specific amplification.

The primary and secondary PCR programs followed are given in Table 3.2.

Table 3.2 Polymerase chain reaction programs for *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) DNA amplification

AMPLIFICATION STEPS	PRIMARY PCR temperature and time	SECONDARY PCR temperature and time
Initial denaturation	94 °C, 3 min	95 °C, 5 min
Number of cycles	45	30
Denaturation	94 °C, 30 s	92 °C, 30 s
Annealling	56 °C, 30 s	42 °C, 30 s
Extension	60 °C, 3 min	65 °C, 20 s
Final extension	65 °C, 3 min	72 °C, 15 min

3.2.2.1.2 DNA amplification for dihydropteroate synthase (dhps) and dihydrofolate reductase (dhfr) mutations

The primers listed in Table 3.3 were used for the amplification of sample DNA as template for analysis of mutations associated with resistance to sulfadoxine/ pyrimethamine. The reaction mixtures were prepared as described before. The primary PCR was performed in a total reaction volume of 50 µl containing 5 µl of 10× PCR buffer, 5 µl of dNTPs mixture (200 µM each), 5 µl template DNA, 0.2 µl (1 U) Taq DNA polymerase (Promega, USA) and 29.1 µl sterile distilled water. For the secondary PCR similar reaction mixtures were used but template DNA was 2 µl of the primary PCR product and 35.1 µl sterile distilled water. For both primary and secondary PCR primers (Table 3.3), the volume of each primer was 0.125 µl (0.25 µM). The primary and secondary PCR programs followed are given in Table 3.4.

Table 3.3 Primers used for PCR amplification of dihydropteroate synthase (dhps) codons 437 and 540, and dihyfolate reductase (dhfr) codons 108 and 59 for sulfadoxine/pyrimethamine (fansidar®) associated resistance

Codon	Nest	Primer name	Primer sequences	Product sizes
437 & 540	1	R2	5' AACCTAAACGTGCTGTTCAA 3'	
		R/	5' AATTGTGTGATTTGTCCACA A 3'	711 bp
	2	K	5' TGCTAGTGTTATAGATATAGGatGAGcATC 3'	
		K/	5' CTATAACGAGGTATTgCATTTAATgCAAGAA 3'	438 bp
108 & 59	1	M1	5' TTTATGATGGAACAAGTCTGC 3'	
		M5	5' AGTATATACATCGCTAACAGA 3'	642 bp
108	2	M3	5' TTTATGATGGAACAAGTCTGCGACGTT 3'	
		F/	5' AAATTCTTGATAAACAACGGAACCTttTA 3'	522 bp
59	2	F	5' GAAATGTAATTCCCTAGATATGgAATATT 3'	
		M4	5' TTAATTTCCCAAGTAAAACTATTAGAgCTTC 3'	326 bp

Table 3.4 Polymerase chain reaction programs for dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*) DNA amplification

AMPLIFICATION STEPS	PRIMARY PCR *temperature and time*	SECONDARY PCR *temperature and time*
Initial denaturation	94 °C, 3 min	94 °C, 2 min
Number of cycles	40	30
Denaturation	95 °C, 1 min	94 °C, 1 min
Annealling	50 °C, 2 min	45 °C, 1 min
Extension	72 °C, 3 min	72 °C, 2 min
Final extension	72 °C, 10 min	72 °C, 10 min

3.2.3 Detection of mutations associated with drug resistance

To test for presence of already described mutations associated with resistance, specific restriction enzymes were used to digest the various segments of amplified DNA. The restriction enzymes used were those whose restriction sites are conferred by the mutation in the mutant parasite DNA while others had restriction sites that are found in the wild type parasite genome.

3.2.3.1 Restriction digestion for *pfcrt* mutation analysis

Restriction digestion was done in a total reaction volume of 20 µl per sample. The 20 µl reaction mixture contained sterile distilled water, a particular type of buffer, bovine serum albumin (BSA) where necessary, and the appropriate enzyme for the particular mutation to be analyzed. The volumes and concentrations of the constituents of the reaction mixture for each enzyme are listed in Table 3.5 and all enzymes were supplied by New England Biolabs, Beverly, MA, USA. A master mix,

with the appropriate enzyme, was prepared according to the number of samples to be analyzed at a particular time. The master mix was constituted by multiplying the number of tubes or samples to be analyzed with the volume of each constituent reagent in a reaction mixture per tube and was mixed by pipetting. Aliquots of 12 µl of the master mix were put into pre-labelled tubes, each tube for one sample. Eight microlitres of the secondary PCR product was added to an appropriately labelled tube containing an aliquot from the enzyme master mix and incubated at the specific enzyme temperature overnight. Restriction enzymes were inactivated, where required, at temperatures and durations after the reaction was complete as indicated in Table 3.5. A Hytec-Yamato incubator and a UniEquip hybridization oven were used for all the incubations.

Table 3.5 Restriction enzyme digestion protocols for mutation analysis in the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfrct*)

Enzyme & Codon number	Sterile dH_2O (µl)	NEB Buffer 1×		BSA 100 µg/ml (µl)	Enzyme 1 U (µl)	Incubation temp. (°C)	Enzyme inactivation	
		Type	Vol. (µl)				temp. (°C)	time (min)
*Apo*I 76	9.7	3	2.0	2.0	0.1	50	80	20
*BspH*I 75	9.9	4	2.0	-	0.1	37	65	20

3.2.3.2 Restriction digestion for *dhfr* and *dhps* mutation analysis

Restriction enzyme digestion was done in a 20 µl reaction volume per sample. The constituents of each reaction mixture and the master mix were prepared as described previously. The volumes and concentrations of the constituents of the reaction mixtures were as in Table 3.6 and aliquots of 15 µl from the master mix were used. Five microlitres of the secondary PCR product was added to an appropriately labelled tube containing the reaction mixture and incubated at appropriate temperatures (Table 3.6) overnight. The reaction mixtures incubated at temperatures above 50 °C were overlaid with 10 µl mineral oil to prevent evaporation. After completion, the restriction enzyme digestions were inactivated, either in a water bath or an incubator for the duration and temperature as indicated in Table 3.6.

3.2.3.3 Separation of bands

The resulting DNA fragments from restriction digestion were fractionated by electrophoresis on 2 – 2.5% agarose gel. The gels were prepared using 1× TBE buffer prepared from a 5× stock solution (Appendix 2).

3.2.3.3.1 Preparation of agarose gels

A beaker two or three times the volume of the gel solution required was used for the preparation of the agarose gels and a small volume of 1× TBE buffer and a magnetic stirrer were added to the beaker at room temperature. A pre-measured amount of agarose powder was added while the solution was being stirred rapidly using the magnetic stirrer. After the powder had completely dissolved, the stirrer was removed and the solution was made up to the required final volume with the TBE to achieve

Table 3.6 Restriction enzyme digestion protocols for mutation analysis of dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhfr*)

Enzyme & Codon number	Sterile dH_2O (µl)	NEB Buffer 1×		BSA 100 µg/ml (µl)	Enzyme 1 U (µl)	Mineral oil (µl)	Incubation temp (°C)	Enzyme inactivation	
		Type	Vol. (µl)					temp. (°C)	time (min)
*Xmn*I 59	12.7	2	2.0	0.2	0.10	-	37	65	20
*Alu*I 108	12.9	2	2.0	-	0.10	-	37	65	20
*Bsr*I 108	12.8	3	2.0	-	0.20	10	65	80	20
*Mwo*I 437	12.8	3	2.0	-	0.20	10 or 0	60	-	-
*Ava*II 437	12.9	4	2.0	-	0.10	-	37	65	20
*Fok*I 540	12.75	4	2.0	-	0.25	-	37	60	20

the required concentration of the agarose solution. The beaker and its contents were weighed and covered leaving a hole for ventilation. The agarose solution was brought to boiling, the heat adjusted to gentle boiling and thereafter maintained at that temperature for a further five minutes. Hot boiling water was added to the agarose solution to adjust to the initial weight and mixed thoroughly. The solution was then cooled to around 50 °C prior to casting of the gels.

While the gel solution was cooling the gel-casting tray was assembled and levelled prior to casting. The agarose solution was poured into the tray to a thickness of 3 to 5 mm. Clean combs were then inserted into the gel with an allowance of about 0.5 mm between the bottom of the casting tray and the comb teeth. The gel was allowed to solidify at room temperature for 30 min after which it was flooded with the running 1× TBE buffer and the combs were gently removed. The gel on the casting tray was put into the electrophoresis chamber and the chamber filled with running buffer up to about 3 to 5 mm above the surface of the gel. Gel wells were flushed out gently with electrophoresis buffer using a Pasteur pipette to remove the loose gel fragments prior to loading the samples (Sambrook *at el.*, 1989).

3.2.3.3.2 Loading of the digested DNA samples

Ten microlitres of each digestion product was mixed with 2 µl of 6× loading dye (Appendix 2) on a parafilm and then 10 µl of the resulting mixture was loaded into the gel wells. Along side the samples the outer lane was loaded with a mixture of 0.5 µl of the DNA size marker mixed, by pipetting, with 1 µl of loading dye and 4 µl of distilled water. Prior to the digestion of the amplified samples with restriction

enzymes, aliquots of the secondary amplicons were first run on the agarose gels to determine if the samples had been amplified and to check that the right size of the bands had been amplified. The DNA marker was used to determine if the correct size of the bands had been obtained from the amplification.

3.2.3.3.3 DNA staining in run agarose gels

Electrophoresis was carried out at 100 volts for about 1 h 30 min until the loading dye had migrated about two thirds of the gels' length. The gels were stained by immersing in a 0.5 µg/ml ethidium bromide solution in 1× TBE buffer for 10 min at room temperature (Sambrook *at el.*, 1989). The stained gels were then washed in distilled water or tap water 3 to 4 times at room temperature, visualized using a UV transilluminator (Light VU^{TM} transilluminator, model TUV-35, Owl Scientific Inc) and pictures of the gels were taken using a Polaroid DS-300 camera.

3.2.3.4 Interpretation of DNA seperated bands

The digested fragments were run alongside the undigested DNA secondary amplicons where the latter served as controls. The bands of digested DNA corresponding to bands of undigested DNA indicated absence of cleavage site for the restriction enzyme used hence showing the absence or presence of mutations depending on the enzyme used (Tables 3.5 and 3.6). On the other hand, bands that migrated different distances and therefore had different sizes compared with the undigested DNA also indicated the presence or absence of mutations, as some enzymes cleave the wild-type and not the mutant type and *vice versa* as indicated in the tables 3.7 and 3.8.

Table 3.7 Interpretation of restriction enzyme digestion of the *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*)

GENE & CODON NUMBER	ENZYME	1° AND 2° PRIMER COMBINATION	1° AND 2° EXPECTED AMPLIFICATION PRODUCT SIZE (in bp)	RESTRICTION DIGEST EXPECTED FRAGMENT SIZE (in bp)	
				WILD TYPE	MUTANT TYPE
pfcrt 75	*BspH*I	76-A & 76B CRT45MS & 76-D2	206 84	53 & 31 Methionine (only if 75 is Wild Asn)	
pfcrt 76	*Apo*I	76-A & 76B 76-D1 & 76-D2	206 145	99 & 46 Wild lysine	

Table 3.8 Interpretation of restriction enzyme digestion of the dihydropteroate synthase gene (*dhps*) and the dihydrofolate reductase gene (*dhfr*) of *Plasmodium falciparum* associated with sulfadoxine and pyrimethamine resistance, respectively

GENE AND CODON NUMBER	ENZYME	1° AND 2° PRIMER COMBINATION	1° AND 2° EXPECTED AMPLIFICATION PRODUCT SIZE (in bp)	RESTRICTION DIGEST EXPECTED FRAGMENT SIZE (in bp)	
				WILD TYPE	MUTANT TYPE
dhfr 59	*Xmn*I	M1-M5; M3-F/	642; 326	189 & 137 (Cys-59; TGT)	163, 137 & 26 (Arg-59; CGT)
dhfr 108	*Alu*I	M1-M5; M3-F/	642; 522	326 &196 (Ser-108; AGC)	
dhfr 108	*Bsr*I	M1-M5; M3–F/	642; 522		332 &190 (Asn-108; AAC)
dhps 437	*Mwo*I	R2-R/; K-K/	711; 438	387 & 51 (Ala -437; GCT)	
dhps 437	*Ava*II	R2-R/; K-K/	711; 438		404 & 34 (Gly-437; GGT)
dhps 540	*Fok*I	R2-R/; K-K/	711; 438	405 & 33 (Lys -540;AAA)	

3.3 Statistical analysis

The computer software Statistical Package for Social Sciences (SPSS) for windows version 11.0 was used to analyze the association between the *in-vivo* analysis and molecular (genotypes of the parasites) analyses of the samples that were collected from the patients that were recruited for the *in-vivo* analysis and were prescribed fansidar®. This analysis was restricted to fansidar® because at the time of sampling it was the first-line drug and chloroquine had been phased out. The Chi-Square test (χ^2) was used to assess the various associations. A probability less than 0.05 was considered to be significant.