STUDIES ON DRUG RESISTANT PLASMODIUM FALCIPARUM MALARIA IN YEMEN

Dr. REEM A K MUBJER

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Dr. REEM ABDUL- KADER MUBJER

B.Med. University of Aden, Yemen

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Declaration

I declare that the work presented in this thesis is all my own and that it has not been submitted for any other degree.

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Reem A K Mubjer (2006)

Dedication

Father, this is for you. In my mind my success was always to be yours. I miss you.

ABSTRACT

Malaria, mainly due to *Plasmodium falciparum,* continues to be a top priority health problem in the Republic of Yemen. Sixty percent of the total population in Yemen are at risk of malaria with an estimate of 3 million malaria cases annually and a mortality rate of 1% of the cases, mainly among children below the age of five and pregnant women. Chloroquine is the first-line drug for acute uncomplicated falciparum malaria in Yemen, sulfadoxine/pyrimethamine is the second-line treatment and mefloquine is the third-line. Effective treatment and prevention of falciparum malaria is being limited by the development of drug-resistant *P. falciparum* parasites. However, till 2002 the degree and the extent of chloroquine resistance was not known in Yemen and there was no system for monitoring antimalarial drug resistance in the country.

As a response to the urgent need to establish a practical and sustainable system for continuous monitoring of therapeutic efficacy of antimalarial drugs and the urgent need to test the efficacy of chloroquine, the first-line treatment, this study was conducted in 2002 in collaboration with the Ministry of Public Health in Yemen and WHO/EMRO. The study involved a 14-day in-vivo chloroquine sensitivity test based on a standard WHO protocol and was conducted from October/2002 to January/2003 in one of the sentinel sites in Yemen (Al-Musaimeer district, Lahj governorate) where malaria is seasonal with the peak transmission season between October-April.

In-vivo chloroquine treatment failure was detected in 61% of the 122 cases that completed the 14-day follow-up. True treatment failure (recrudescence detected by PCR of *msp*-2) was attributed to 92% of the treatment failure accounting for PCR corrected treatment failure of 56%. Among treatment failures, late parasitological failures (LPF) were the most prevalent (40%) followed by early treatment failures (ETF) (38%), while the least prevalent (22%) were late clinical failures (LCF). Age <10 years was an independent predictor of chloroquine treatment failure (*OR=8.7, 95% CI=3.6-21.01*), while fever (axillary temperature ≥37.5°C) at presentation and age <5 years were independent predictors of early treatment failure compared to late treatment failure (*OR=5.7, 95% CI=1.96-16.58* and *OR=3.42, 95% CI=1.04-11.21*) for temperature and age respectively. Children <10 years were significantly at higher risk of being late clinical failures, compared to late parasitological failures, than older children and adults (x^2 *p-value* < *.003*). These findings indicated a high acquired immunity in old children and adults reflecting high transmission intensity in the area.

The prevalence of the *pfcrt*-T76 was 98% in 112 amplified pre-treatment samples. The *pfcrt*-T76 mutation was found in the pre-treatment samples of all in-vivo treatment failure cases. However, it was also found in 95.5% of pre-treatment samples of cases who adequately responded to chloroquine, therefore, there was no association between the presence of either the mutant T76 or the wild *pfcrt*-K76 allele and the treatment outcome (*Fisher exact p-value=0.152*). Clearance of parasites carrying the T76 mutation after taking chloroquine was significantly higher in children >10 years and adults than in children <10 years (*OR=0.04, 95% CI= 0.01-0.12*, x^2 *p-value=0.000*). The presence of *pfcrt*-T76 was poorly predictive of in-vivo chloroquine resistance (*PPV= 61.8%, 95% CI= 52.7-70.9*). This was mainly due the prevalent acquired immunity and the clearance of resistant parasites by older

children and adults. Therefore, the genotype failure index GFI was considered as a more reliable predictor of in-vivo treatment failure in this area. Controlled for age, the GFI was 1.2 in children less than 10 years and 2.8 in older children and adults. The prevalence of *dhfr* Arg-59 mutation in 99 amplified samples was 5%, indicating that the triple *dhfr* mutations are already present in the area, which can affect the efficacy of sulfadoxine/pyrimethamine and its combination with other drugs, such as artimisinins, as a replacement therapy. The *dhps* Glu-540, and the *pfcrt*-S163R which is implicated in the restoration of chloroquine sensitivity to resistant parasites, could not be detected in the tested samples (119 and 42 samples respectively).

Using *msp-2* marker alone, 21 different *P. falciparum* genotypes could be detected in 89 pre and post-treatment infections with size variation ranged from 300 to1100 base pairs. The distribution of the alleles of the two families of *msp*-2 (FC27 and IC/3D7) was similar in pre-treatment samples (48% for FC27 and 52% for IC/3D7). In post-treatment samples, the IC/3D7 family alleles were slightly more prevalent (58%). Multiple clone infection per sample (multiplicity) was detected in 56% of pre-treatment samples and in 50% of post-treatment samples. In pre-treatment samples, the mean multiplicity was 1.92 (SEM= .163, range 1-6 genotypes/sample), while in post-treatment samples, the mean multiplicity was 1.63 (SEM= .111, range 1-3 genotypes/sample). The difference in the mean of multiplicity when comparing pre-treatment and post-treatment samples was statistically significant (*Wilcoxon Test, p-value=0.028*). There was a trend of decreasing multiplicity with increasing age in those who are above 5 years. The lowest multiplicity was observed in adults ≥15 years and in children < 5 years. The study suggested the study site as a high transmission area and highlighted the importance of reconsidering the previous WHO classification of Yemen as a low to moderate intensity transmission area as this has important implications on vector control activities in the area.

Sequencing the full length of the *pfcrt* gene of Yemeni parasites confirmed the presence of the *pfcrt*-T76 mutation and revealed that a Yemeni chloroquine resistant *P. falciparum* parasite carries the old world (Asian and African) chloroquine resistant haplotype CVIETSESI at positions 72,73,74,75,76,220,271, 326 and 371 suggesting that chloroquine resistant parasites might be imported from Africa to Yemen..

This study provided the drug policy makers with base-line information about the current situation regarding malaria drug resistance in Yemen. It established a practical and sustainable system for continuous monitoring of therapeutic efficacy of antimalarial drugs. It provided an evidence-based need for the replacement of chloroquine, as a first-line drug for treatment of uncomplicated, *P. falciparum* malaria in Yemen and introduced the PCR technique as a tool for monitoring antimalarial drug resistance in surveillance activities in Yemen.

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ABBREVIATIONS

µl	Microlitre
µM	Micromolar
ACPR	Adequate Clinical and Parasitological Response
ACT	Artimisinin-based Combination Therapy
AL	Arthemeter-lumefantrine
AM	Amantadine
AQ	Amodiaquine
AS	Artesunate
ATP	Adenosine triphosphate
bp	Base pair
cDNA	Complementary DNA
cg2	Candidate gene 2
CIN	Cinchonine
CIND	Cinchonidine
CQ	Chloroquine
CQ^{+2}	Protonated forms of chloroquine
CQR	Chloroquine resistant/resistance
CQS	Chloroquine sensitive
CT	Combination Therapy
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DDT	Dichlorodiphenyl-trichloroethane
dGTP	2'-deoxyguanosine 5'-triphosphate
DHFR	Dihydrofolate reductase
DHPS	Dihydropetroate synthase
DME	Drug/metabolite effluxer
DMT	Drug/metabolite transporter
DNA	Deoxyribonuclic acid
dTTP	2'-deoxythymidine 5'-triphosphate
DV	Digestive vacuole
EDTA	Ethylenediaminetetra-acetic acid
EIR	Entomological Inoculation Rate

EMR	Eastern Mediterranean Region
EMRO	Eastern Mediterranean Region Office
ETF	Early Treatment Failure
FPIX	Ferriprotoporphyrin nine
GFI	Genotype failure index
GLURP	Glutamate-Rich Protein
GRI	Genotype resistance index
HF	Halofantrine
HIV	Human Immunodeficiency Virus
HQ	Head Quarter
IC_{50}	50% inhibitory concentration
IEC	Information, education and communication
ITN	Insecticides Treated Nets
kb	Kilobase
kDa	Kilo Dalton
KND	Kassena-Nankana District (in northern Ghana)
Lap Dap	Chlorproguanil-Dapson
LCF	Late Clinical Failure
LPF	Late Parasitological Failure
LTF	Late Treatment Failure
M/W	Mutant/Wild
$MgCl_2$	Magnesium Chloride
mM	Millimolar
MoPH	Ministry of Public Health
MQ	Mefloquine
mRNA	Messenger ribonucleic acid
MSP1	Merozoite Surface Protein 1
MSP2	Merozoite Surface protein 2
MS-PCR	Mutation Specific Polymerase Chain Reaction
ng	Nanogram
nM	Nanomolar
NMCP	National Malaria Control Program
OR	Odds Ratio
PCR	Polymerase Chain Reaction

PfCRT	Plasmodium falciparum Chloroquine Resistance Transporter
PfMDR1	Plasmodium falciparum multidrug resistance 1
Pgh-1	P-glycoprotein homologus1
PNG	Papua New Guinea
PPV	Positive Predictive Value
NPV	Negative Predictive Value
QD	Quinidine
QN	Quinine
RBC	Red Blood Cell
RBM	Roll Back Malaria
RFLP	Restriction Fragment Length Polymorphism
rpm	Round per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standard error of mean
SP	Sulfadoxine/ Pyrimethamine
SPR	Slide Positivity Rate
SR	Spleen Rate
TF	Treatment Failure
TMD	Transmembrane domain
U	Unit
UTL	Useful Therapeutic Life
UV	Ultraviolet
VP	Verapamil
WHO	World Health Organization

GLOSSARY

Some terms used in this thesis are defined below

Allele
One of two or more alternative forms of a gene.

Amplified Fragment Length Polymorphism (RFLP)
A method for detecting large numbers of polymorphisms in the DNA of different strains of an organism, in which restriction enzyme digestion of DNA is carried out and a subset of DNA fragments selected for PCR amplification and visualisation.

Base pair
A hydrogen-bonded structure formed between two complementary nucleotides.

Clone
A group of genetically identical organisms derived from a single cell by asexual reproduction.

Codon
A triplet of nucleotides that code for a single amino acid.

Diploid
Having two copies of each chromosome.

Entomological Inoculation Rate (EIR)
The sporozoite rate (percentage of female mosquitoes with sporozoites) multiplied by the average number of mosquitoes of that species biting a person per unit of time.

Gamete
A reproductive cell, usually carrying the haploid chromosome complement, that can fuse with a second gamete to produce a new cell during sexual reproduction.

Gel electrophoresis
Electrophoresis performed in a gel matrix so that molecules of similar electrical charge can be separated on the basis of size.

Genotype
A description of the genetic composition of an organism.

Genotype Failure Index (GFI)
The ratio between the prevalence of *pfcrt*-T76 mutation and the prevalence of clinical (in-vivo) chloroquine resistance

Genotype Resistance Index (GRI)
The ratio between the prevalence of *pfcrt*-T76 mutation and the prevalence of parasitological chloroquine resistance.

Exon
One of the coding regions of a discontinuous gene.

Haploid
Refers to a cell that contains a single copy of each chromosome.

Haplotype
A set of single alleles of genes which are inherited through the germ-line; a contraction of the phrase "haploid genotype". It can also apply to groups of linked alleles on specific chromosome.

Holoendemicity
Areas with perennial, intense malaria transmission resulting in a considerable degree of immunity outside early childhood. Spleen rate >75% in children aged 2-9 years, but low in adults. Parasite rate > 75% among infants aged 0-11 months.

Hyperendemicity
Areas with intense, seasonal malaria transmission where the immunity is insufficient in all age groups. Spleen rate >50% in children aged 2-9 years and > 25% in adults. Parasite rate >50% among children aged 2-9 years.

Hypoendemicity
Areas with littel malaria transmission and the effects, during an average year, upon the general population are unimportant. Spleen rate not <10% in children aged 2-9 years and > 25% in adults. Parasite rate <10% in children aged 2-9 years but may be higher for part of the year.

Intron
In a discontinuous gene, one of the segments that does not contain biological information.

Linkage disequilibrium
The occurance of alleles of two genes together in a population at a frequency higher than expected under conditions of random mating and absence of selection.

Mesoendemicity
Areas with wide geographical variations in malaria transmission risk in rural communities of subtropical zones. Spleen rate 11%-50% in children aged 2-9 years. Parasite rate 11%->50% among children aged 2-9 years.

Mieosis
The series of events, involving two cell divisions, by which diploid cells are converted to haploid cells.

Mitosis
The series of events that results in division of a single cell into two daughter cells.

Negative Predictive Value
The proportion of individuals with a negative test result who do not have the disease.

Odds
The ratio of the probabilities of two complimentary events, typically the probability of having disease divided by the probability of not having the disease.

Odds ratio [OR]
The ratio of two odds (e.g. the odds of disease in individuals exposed and unexposed to a factor.

Outcrossing
The mating of individuals who are not closely related.

Parasite count
The number of parasites seen on an average in a number of high power magnification fields (such as 100), or in relation to the number of red blood cells. Usually the parasite count is given in relation to 1 µl of blood after a suitable conversion. The parasite count may be also calculated in relation to the number (400-500) of white blood cells seen in 100 fields, when the number of these cells per microlitre is known.

Parasite rate
The prevalence of peripheral blood-stage infections among a community.

Phenotype
The observable characteristics displayed by a cell or organism.

Point mutation
A mutation that results from a single nucleotide alteration in a DNA molecule.

Polymorphism
A variable DNA sequence, one that can exist in a number of different, although related forms.

Primer
A short oligonucleotide that is attached to a single stranded DNA molecule in order to provide a site at which DNA replication can begin.

Positive Predictive Value
The proportion of individuals with positive diagnostic test result who have the disease.

Recombination
A physical process that can lead to exchange of polynucleotides between two DNA molecules and which can result in the progeny of a genetic cross possessing combinations of alleles not displayed by either parent.

Recrudescence
The process that results from exacerbations of persistent, undetectable parasitaemias in the absence of an exo-erythrocytic cycle. It is a characteristic of *P. falciparum* and *P. malariae*

Reinfection
The re-inoculation of infective sporozoites by the female anopheline mosquitoes after establishment of treatment.

Relapse
The process that results from the reactivation of hypnozoites (dormant liver stages) of the parasite in the liver. It occurs in *P. vivax* and *P. ovale.*

Sensitivity
The proportion of individuals with the disease who are correctly diagnosed by the test.

Sequestration
The ability of *P. falciparum* to cause the infected red blood cells to adhere to the lining of small blood vessels leading to obstruction of tissue perfusion, a pathological characteristic of *P. falciparum* only.

Specificity
The proportion of individuals without the disease who are correctly identified by a diagnostic test.

Spleen rate
The proportion of enlarged spleens in the indigenous population.

Transition
A point mutation that results in a purine being replaced by another purine or a pyrimidine by a pyrimidine.

Transversion
A point mutation that results in a purine being replaced by a pyrimidine or vice versa.

Wild-type
Refers to a gene, cell or organism that displays the typical phenotype and/or genotype for the species and is therefore adopted as a standard.

Zygote

The cell that results from fusion of gametes during meiosis.

CHAPTER 1

GENERAL INTRODUCTION

1.1 MALARIA OVERVIEW

Malaria is a parasitic infection transmitted by the bite of an infected female anopheline mosquito. It is estimated that about ten percent of the global population are suffering from malaria in any given year (at least 350 million clinical cases worldwide), that is five times as many as the combined cases of tuberculosis, AIDS, measles and leprosy. More than a million people die of malaria each year, most of them children under five. In Africa, malaria kills a child every 30 seconds (WHO, RBM. 2004)

Malaria is primarily a disease of the tropics, however, isolated locally transmitted cases still occur in North America, and there are 12,000 malaria cases per year in Western Europe. Malaria endemic countries are caught in a vicious circle of disease and poverty. Malaria slows a country's economic growth, discourages foreign investment and tourism, and depletes human resources (WHO, RBM. 2004)

The situation is complicated due to the development of different challenges to malaria control and management, which has lead to resurgence of the disease. These challenges include mainly the emergence of drug-resistant strains of the parasite, the appearance of insecticide- resistant mosquitoes, environmental changes and population growth and migration.

1.2 MALARIA AS A DISEASE

Species of malaria parasites that can commonly infect humans under natural conditions: *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae.* In 2004 a large focus of human naturally acquired malaria infection with unexpected species, *Plasmodium knowlesi*, misdiagnosed by microscopy mainly as *P. malariae*, was reported in Malaysian Borneo (Singh *et al.*, 2004). *P. knowlesi* was identified in 1931 in a long-tailed macaque monkeys, *Macaca fascicularis,* it is lethal to rhesus monkeys and in 1932 was shown to be infectious to human by inoculation of infected blood (Singh *et al.*, 2004). The first human natural infection with *P. knowlesi* was reported in 1965 in USA in a man who visited peninsular Malaysia (reviewed by Singh *et al.*, 2004). There was also a report in 1971 of what presumed to be a natural infection of a man by *P. knowlesi* in peninsular Malaysia after based on a travel history in the jungle and serological tests (Fong *et al.*, 1971). Monkey-to-human and

human-to-human transmission of *P. knowlesi* by mosquitoes (*Anopheles luecosphyrus*) can occur under experimental conditions (Singh *et al.*, 2004).

Plasmodium falciparum is the agent of severe, potentially fatal malaria. *Plasmodium vivax* and *P. ovale* have dormant liver stage parasites "hypnozoites" (see **section 1.4**), which can reactivate "relapse" and cause malaria several months or years after the infecting mosquito bite. *Plasmodium malariae* produces long-lasting infections and if left untreated can persist asymptomatically in the human host for years, even a lifetime.

Symptoms of malaria appear a week or two after being bitten by an infected mosquito and they include fever, shivering, headache, nausea, vomiting, muscle aches and fatigue. These can rapidly progress to include organ failure, delirium, convulsions and often leading to death. The *P. falciparum* exerts its pathogenic effect through its ability to cause the infected RBCs to adhere to the lining of small blood vessels (sequestration) leading to obstruction of tissue perfusion. Reducing the deformability of uninfected RBCs is an additional mechanism for reducing tissue perfusion in severe malaria (Dondorp *et al.*, 2000). As described above, the clinical outcome of an infection may range from asymptomatic infection to severe disease or death depending on many parasite, host, geographic and social factors **Figure 1.1**

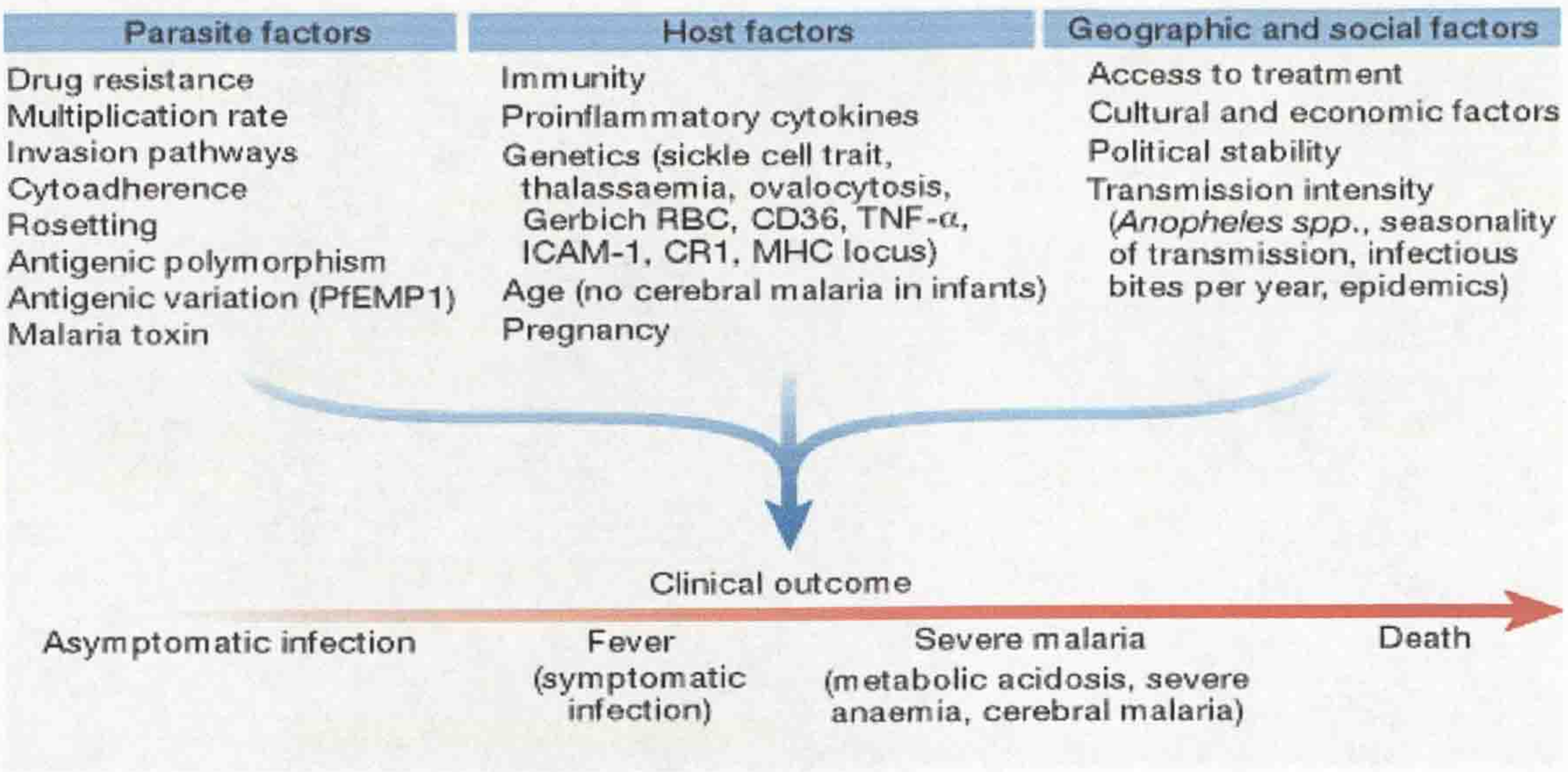


Figure 1.1 The clinical outcome of an African child infected with malaria (source: Miller *et al*, 2002)

1.3 GEOGRAPHICAL DISTRIBUTION OF MALARIA

Malaria is believed to be a disease of poverty and underdevelopment, it is generally endemic in the tropics extending to the subtropics. The changing global pattern of malaria transmission from 1946 to 1994 shows an increased concentration of the disease burden in the tropics with most cases occurring in Africa (Sachs and Malaney, 2002); see **Figure 1.2)**. The distribution of the disease varies greatly from country to country and within the countries themselves depending on climate factors such as temperature, humidity and rainfalls. Currently the disease is endemic in 91 countries, with small pockets of transmission in a further eight.

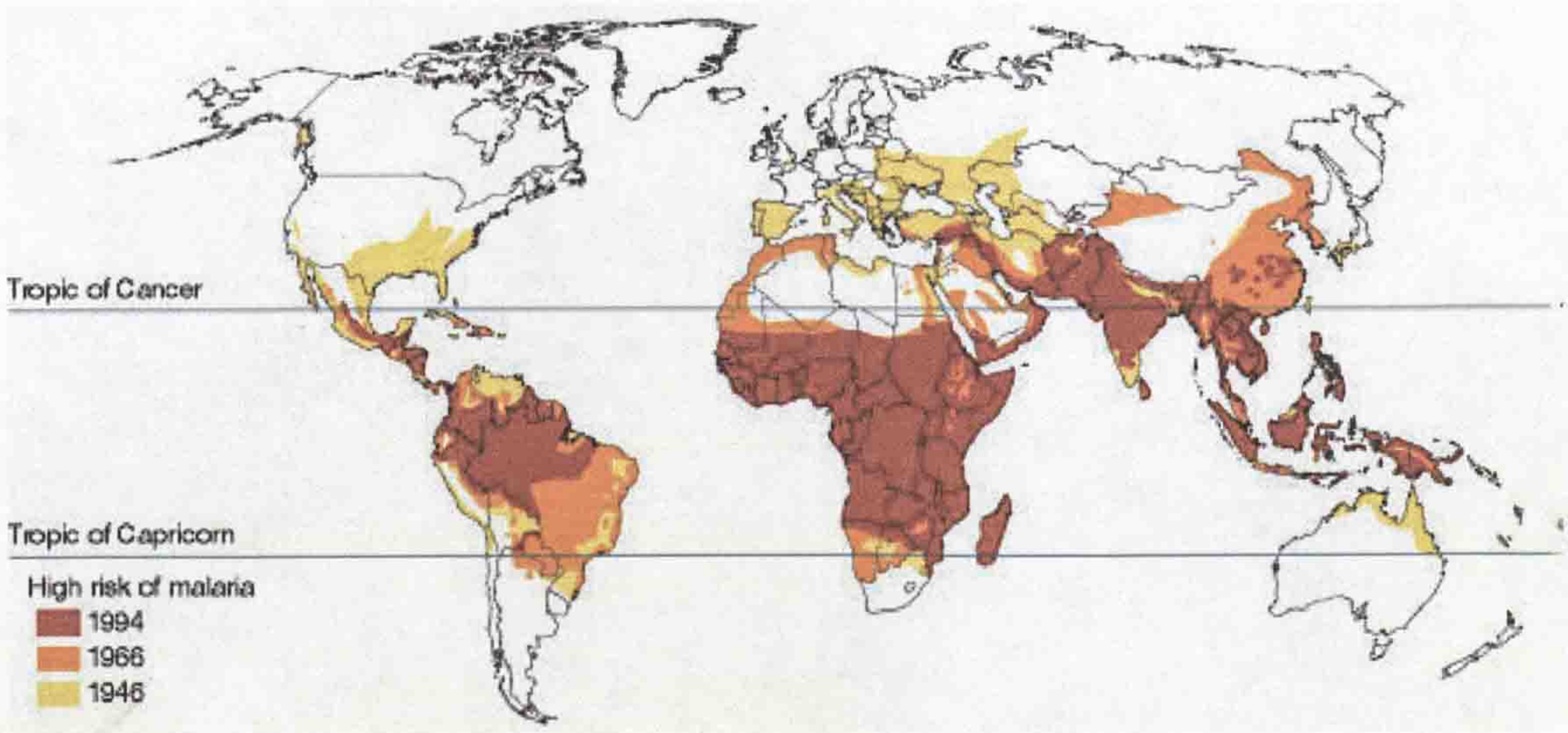


Figure 1.2 Geographical distribution of malaria. (Source: Sachs and Malaney, 2002).

1.4 PARASITE LIFE CYCLE

The malaria parasite life cycle (**Figure 1. 3**) involves two hosts; the anopheline mosquito in which sexual development occurs by meiosis and the human where the mitotic divisions of the life cycle take place (White, 2004). During a blood meal, a malaria-infected female anopheline mosquito inoculates sporozoites into the human host, less than ten sporozoite parasites are inoculated by an infected mosquito in order to establish malaria infection (Rosenberg *et al.*, 1990; Ponnudurai *et al.*, 1991). Sporozoites infect liver cells and mature into schizonts, which rupture and release merozoites. In *P. vivax* and *P. ovale* a dormant stage (hypnozoites) can persist in the liver and cause relapses by invading the bloodstream days or weeks later. The duration of this initial replication in the liver (exo-erythrocytic schizogony) in *P. falciparum* is 5.5-7 days (Warrell and Gilles, 2002) after which each infected hepatocyte liberates merozoites to the blood stream. Liberated merozoites invade erythrocytes and undergo 48-hour asexual multiplication (erythrocytic schizogony). The ring stage trophozoites mature into schizonts, which rupture releasing merozoites. Blood stage parasites are responsible for the clinical manifestations of the disease. Some parasites differentiate into sexual erythrocytic stages (gametocytes).

Male and female gametocytes (microgametocytes and macrogametocytes respectively) are ingested by the anopheline mosquito during a blood meal. Multiplication of the parasites in the mosquito is known as the sporogonic cycle and in *P. falciparum* it lasts for 9-10 days (Warrell and Gilles, 2002). While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes, which is the only diploid stage in the parasite life cycle. The zygote undergoes meiosis during which reassortment and recombination can occur in a normal Mendelian pattern (Walliker *et al.*, 1987). Zygotes in turn become motile and elongated (ookinetes), which invade the mid-gut wall of the mosquito where they develop into oocysts containing haploid meiotic products. The oocysts grow, rupture, and release the sporozoites, which make their way to the mosquito's salivary glands. Inoculation of the haploid sporozoites into a new human host perpetuates the malaria life cycle.

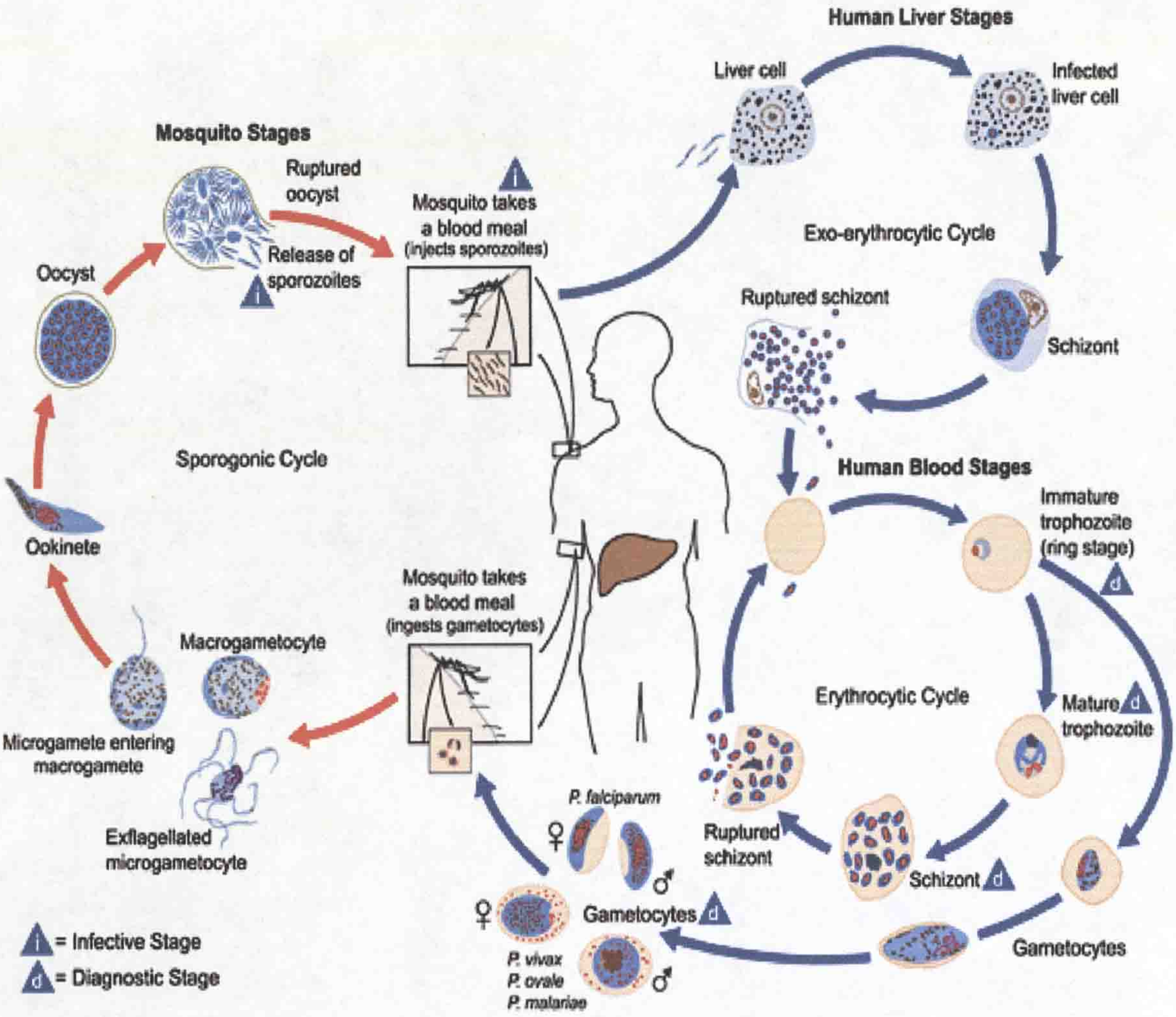


Figure 1.3 The life cycle of malaria parasite in the mosquito and in the human host. (Source: National Centre for Infectious Diseases, Division of Parasitic Diseases, 2004)

1.5 MALARIA PREVENTION AND CONTROL

Malaria eradication was found to be not achievable in a number of countries due to many economic, technical, programmatic, organizational, political and other reasons. The only reasonable course of action against malaria is malaria control, which may be defined as follows:

" A malaria control programme is an organized effort to institute, carry out and evaluate such antimalarial measures as are appropriate for achieving the greatest possible improvement of the health situation of a population living in given

epidemiological and socio-economic conditions and subjected to the burden of this disease or exposed to the risk of its resurgence" (WHO, 1979).

The measures for the prevention of malaria in individuals and for large-scale control of the disease can be divided according to the classification proposed by Russell (1952) and reviewed in Warrell and Gilles, (2002) as follows:

1. Preventing mosquitoes from feeding on humans (human-vector contact).
2. Preventing or reducing the breeding of mosquitoes by eliminating water collections or by altering the environment.
3. Destroying the larvae the mosquitoes.
4. Destroying (reducing the longevity of) adult mosquitoes.
5. Eliminating the malaria parasite in the human host.

This classification regards malaria as a parasitic infection that must be eliminated and not as a disease that must be controlled. Therefore, for malaria control, three additional measures should be added (Warrell and Gilles, 2002):

1. Preventing and reducing mortality from malaria, especially in high risk-groups.
2. Reducing malaria morbidity.
3. Reducing malaria transmission (alter the epidemiological equilibrium).

Provision of early diagnosis and treatment is the first strategic goal of malaria control programmes. Control activities may be directed at an individual level to reduce human-mosquito contact through the use of mosquito repellents or insecticide treated mosquito nets, insecticide-treated curtains, protective clothing, pyrethroid aerosols and anti-mosquito fumigants, house screening, and house siting (selection of suitable site for new housing).

Activities may also be targeted against the vector either to reduce the breeding sites by permanent or temporary environmental alterations or to reduce vector densities by using chemical and biological larvicides (e.g. use of larvivorous fish). Insecticide out-door space spraying aiming at reducing vector densities and indoor insecticide residual spraying aiming at reducing the longevity of vector population are important

measures directed against adult mosquitoes. The appearance of insecticide-resistant mosquitoes is one of the challenges that face malaria control programmes.

Chemotherapy is directed against the parasite in their different life-cycle stages. Development of malaria vaccine, social participation, health education and health systems are important contributors to malaria control.

1.6 ANTIMALARIAL CHEMOTHERAPY

Early diagnosis and treatment of acute malaria is the first strategic goal in every malaria control programme. Antimalarial drugs have selective actions on the different phases of the parasite life cycle (**Table 1.1**). Causal prophylactic drugs prevent the establishment of the parasite in the liver and blood schizontocidal drugs attacks the parasite in the red blood cell, preventing or terminating the clinical attack. Tissue schizontocides act on pre-erythrocytic forms in the liver. Gametocytocidal drugs destroy the sexual forms of the parasite in the blood. Some of these drugs are hypnozoitocidal (kill the dormant hypnozoites in the liver that are responsible for relapses in *P. vivax* and *P. ovale*). Sporontocidal drugs inhibit the development of the oocysts in the stomach wall of the mosquito that has fed on the human gametocyte carrier so that the mosquito cannot transmit the infection.

Blood schizontocidal drugs	Quinine, mefloquine, halofantrine, chloroquine, amodiaquine, atovaquone, artimisinins, pyronaridine, tetracycline, clindamycin and tafenoquine
Tissue schizontocidal drugs	Primaquine, tafenoquine, proguanil, tetracycline (*P.falciparum*), ? pyrimethamine
Gametocytocidal drugs	Primaquine, tafenoquine (all species), quinine, mefloquine, chloroquine, amodiaquine (*P. vivax, P. ovale, P.malariae* only)
Hypnozoitocidal drugs	Primaquine, tafenoquine
Sporontcidal drugs	Proguanil, pyrimethamine, atovaquone

Table 1.1 Antimalarial drugs according to their action on different parasite stages.

1.6.1 Development Of Antimalarial Drugs

1.6.1.1 Quinoline and related antimalarials

In the seventeenth century the Spanish Jesuit missionaries in South America learned of a medicinal bark from indigenous Indian tribes. With this bark, the Countess of Chinchón, the wife of the Viceroy of Peru, was cured of her fever. The bark from the tree was then called *Peruvian bark* and the tree was named Cinchona after the countess. It was in the nineteenth century when the basic Chincona alkaloids were isolated (quinine, QN; quinidine, QD; cinchonine, CIN; and cinchonidine, CIND) (Boyd, 1949). Quinine (**Figure 1.4**) is still one of the most effective antimalarial drugs available today.

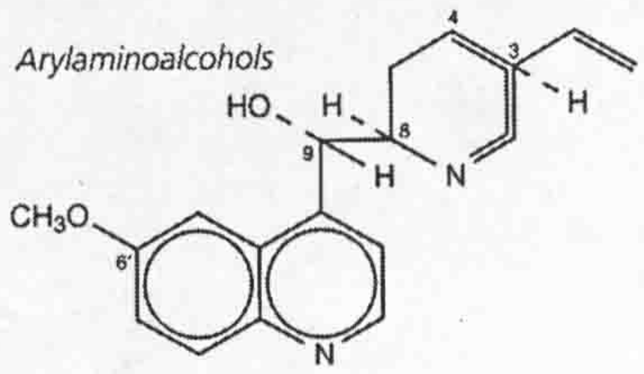
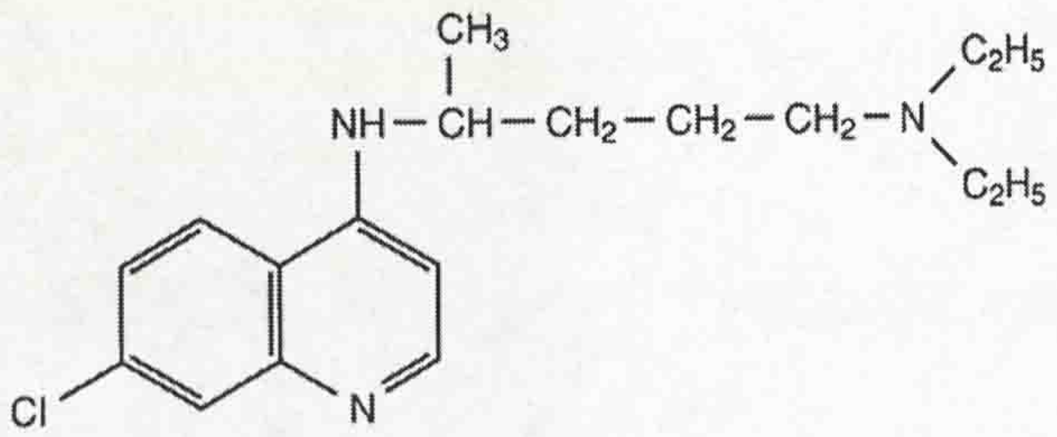


Figure 1. 4 Chemical structure of quinine

However, toxicity associated with quinine (such as tinnitus) and a trice daily administration of the drug over 7 days can lead to poor compliance. The problems associated with obtaining raw QN have led to attempts to synthesize it artificially, which were only totally successful 40 years later (Uskokovic *et al.*, 1970). The opportunities presented by its structural elucidation led to the development of the fully synthetic 4-Amioquinoline antimalrials- notably chloroquine and later amoidaquine (O'Neill *et al.*, 1998) The development of 4-Aminoquinoline is basically due to the observation that the synthetic methylene blue had some antimalarial activity. Substitution of the methyl side chains with basic groups was found to enhance the antimalarial activity, which indicated the crucial role of the basic chemical side chain in the antimalarial activity. This led German scientists at Bayer laboratories of *IG Farben* to synthesize the first "truly" synthetic antimalarial, pamaquine, which was used widely throughout the world but was then withdrawn due to its toxicity and the less toxic analog primaquine was developed along with the acridine derivative quinacrine (known also as mepacrine), which was used until 1940 (O'Neill *et al.,* 1998).

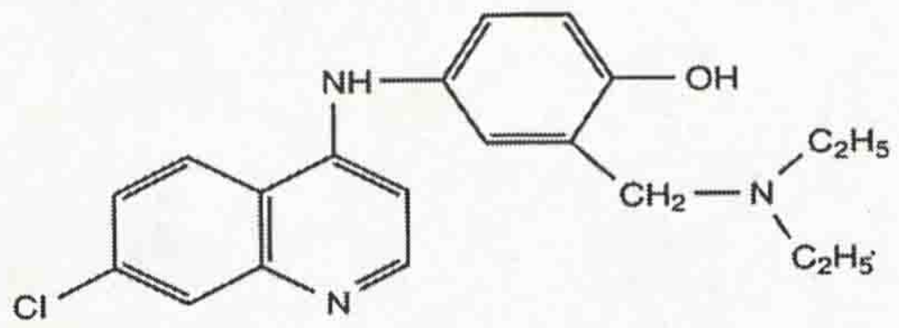
The 4-Aminoquinoline, Resochin, was synthesized by German scientists at Bayer laboratories. Resochin was thought to be toxic for clinical use, which led to its withdrawal for nearly 10 years. During the Second World War, Allied troops captured a supply of the related drug Sontaquine. Further research into the safety of Resochin and Sontaquine showed that Resochin is safe at therapeutic concentrations so it was renamed chloroquine (CQ) (**Figure 1.5**) and enter clinical trials in 1943. (Coatney, 1963).



Chloroquine

Figure 1. 5 Chemical structure of chloroquine

Because of its high effectiveness and safety, CQ was indicated as the drug of choice for treatment and prophylaxis against malaria. This led to the unwise use of the drug worldwide. Massive use of CQ was initiated during the 1960s in an attempt to eradicate malaria and parasite populations were exposed to an intense selection pressure for survival in the presence of CQ helping in the development and spread of CQ resistance in all areas where CQ is used (Peters, 1987) and prompting further research for the development of new antimalarials. As a result, another 4-amioquinoline amodiaquine AQ (**Figure1.6**) was discovered.

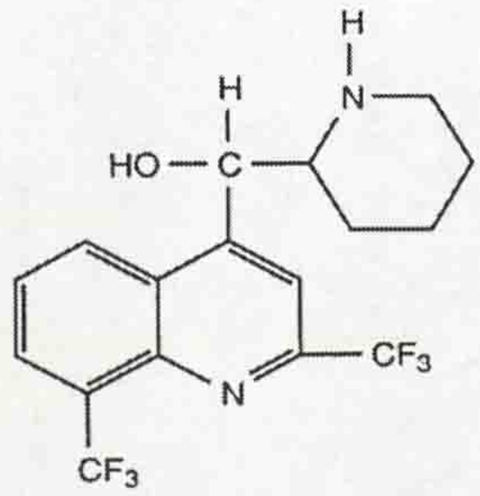


Amodiaquine

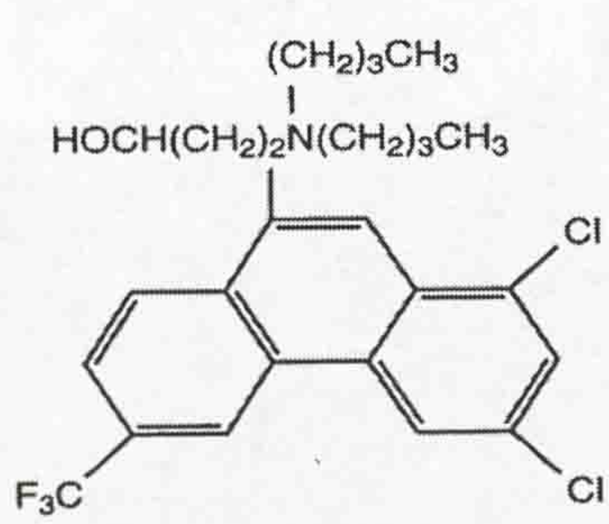
Figure 1.6 Chemical structure of amodiaquine

Amodiaquine is more effective than chloroquine but it is also more expensive (Peters, 1987), however, it has been used as a first-line treatment in areas of prevalent CQ-resistance and as a second-line treatment for cases of CQ treatment failure (Childs *et al.*, 1989). Unfortunately, a number of reports of resistance to AQ have been documented (Campbell *et al.*, 1983; Childs *et al.*, 1989; Glew *et al.*, 1974; Hall *et al.*, 1975). However, further studies showed that AQ was still effective in the treatment of CQ-resistant infections in Thailand (Pinichpongse *et al.*, 1982) and Kenya (Watkins *et al.*, 1984).

A newer generation of quinoline related antimalarials that emerged as a response to the increasing problem of drug resistance include the development of mefloquine (MQ; a quinoline methanol) and halofantrine (HF; a phenanthrene methanol) (**Figure 1.7**). MQ is used for the prophylaxis and treatment of uncomplicated multidrug resistant falciparum malaria. However, clinical resistance is now common in parts of Southeast Asia (Warrell and Gilles, 2002). Halofantrine is also active against CQ-resistant strains of *P. falciparum,* but resistance can develop rapidly (Boudreau *et al.*, 1982), which together with the associated serious cardiotoxicity, has limited its clinical use.



Mefloquine

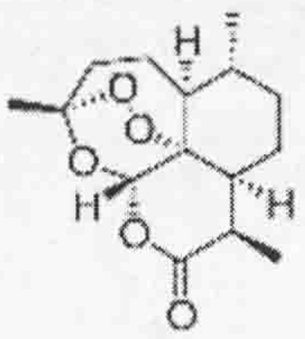


Halofantrine

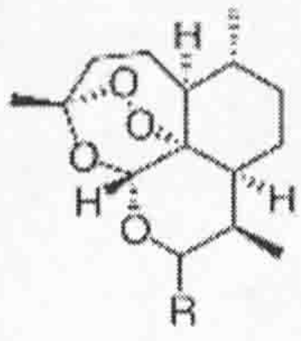
Figure1.7 Chemical structure of mefloquine and halofantrine.

1.6.1.2 Artimisinins

In China, the anti-fever properties of Qinghao plant (*Artemisia annua L*) were described many decades ago. Artemisinin, the active ingredient of Qinghao, was isolated by Chinese scientists in 1972 (Klayman, 1985). It is today a very potent and effective antimalarial drug in multidrug resistant falciparum malaria areas, especially in combination with other medicines. Artemether, arteether, and artesunate (**Figure 1.8**) are derivatives in clinical use (Hien and White, 1993), they are all metabolised to dihydroartemisinin, which is the main active agent in the body.



Artemisinin

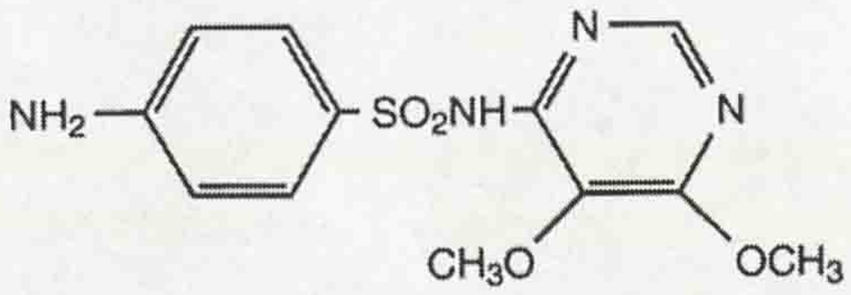


Dihydroartemisinin, R = β + α OH
Artemether R = β–OMe
Artesunate, R = α-OC(O)$CH_2CH_2CO_2Na$

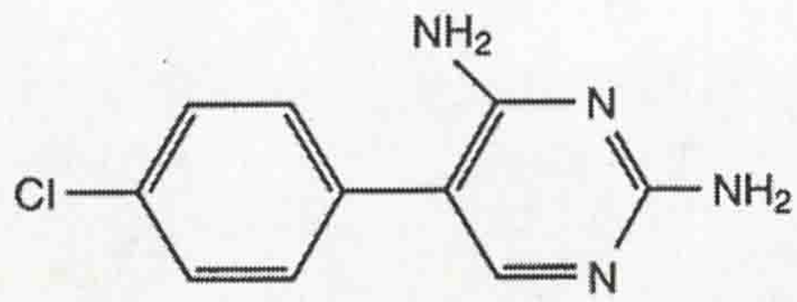
Figure 1.8 Chemical structures of artimisinin and its derivatives

1.6.1.3 Antifolates

This class of antimalarials is not derived from plants; it rather emerged due to knowledge of cell biology and synthetic medicinal chemistry. The most significant antifolate used to treat malaria is the combination of 2,4 diaminopyrimidine pyrimethamine, an inhibitor of dihydrofolate reductase (DHFR), and sulfadoxine, a sulphonamide that interferes with the action of dihydropetroate synthase (DHPS), another enzyme in the folate pathway.



Sulfadoxine



Pyrimethamine

Figure 1. 9 Chemical structures of sulfadoxine and pyrimethamine

Both components of sulfadoxine/ pyrimethamine (SP; Fansidar, **Figure 1.9**) act as synergists with each other, enhancing their activity and reducing the chance for resistance development. However, resistance to SP developed very rapidly when it was used extensively, which limits its use in malaria treatment and necessitates the search for other viable options for treating malaria.

Other compounds including atovaquone, proguanil and some antibiotics (e.g. tetracycline, doxycycline and clindamycin) are only used in combination with other drugs.

1.6.2 Combination Therapy In Malaria

Due to the increasing problem of antimalarial drug resistance, which poses great challenges on malaria control programmes, WHO technical consultation on antimalarial combination therapy was held in Geneva on 4-5 April 2001 and resulted in the strong recommendation for the use of combination antimalarial drug therapy in Africa.

Combination therapy (CT) with antimalarial drugs is "the simultaneous use of two or more blood schizonticidal drugs with independent modes of action and different biochemical targets in the parasite" (WHO, 2001a). According to this definition, multiple-drug therapies that include a non-antimalarial drug to enhance the antimalarial effect of a blood schzonticidal drug are not considered combination therapy. Similarly, some antimalarial drugs that fit the criteria of synergistic fixed-dose combinations, such as sulphadoxine-pyrimethamine (SP), are operationally considered as single products in that neither of the individual components would be given alone for antimalarial therapy (WHO, 2001a).

1.6.2.1 Rationale of antimalarial combination therapy use

- Increasing resistance to all known antimalarials (except artimisinin and its derivatives) leading to an increase of malaria morbidity and mortality. Appropriate measures for rational use of the few antimalrials available to us are urgently needed while waiting for new compounds to be developed.
- Combining two drugs with independent mechanism of action can improve the treatment cure rate and delay the emergence of drug resistance because

mutations that confer resistance to each drug will only rarely co-exist in the same parasite (White, 1999b).

- The potential ability of the combinations to delay the development of resistance against each component is associated with the optimal compatible half-life of the combination components, mode of action (synergistic or additive), and gametocytocidal activity (WHO, 2001a).
- Drugs used in combination should have compatible pharmacokinetics and pharmacodynamics, no adverse pharmacological interaction and no additional toxicity (White and Olliaro, 1996).
- Combination therapy must be implemented early (before the partner drug has been used for monotherapy) to achieve optimal extension of the useful therapeutic life UTL (Watkins *et al.*, 2005)

1.6.2.2 Criteria for selection of combination therapy

The major criteria guiding the choice of the appropriate combination therapy are:

1. Therapeutic efficacy of the combination
2. Safety, especially amongst high-risk groups
3. Potential for widespread use of the combination including home management
4. Potential for compliance
5. Cost effectiveness
6. Potential to delay or prevent development of resistance
7. Availability and production capacity

1.6.2.3 Artimisinin-based combination therapy (ACT)

The advantages of artimisinin-based combination therapy (ACT) are due to the following:

- Its rapid ability to reduce parasite biomass and to resolve clinical symptoms
- Its effectiveness against multi-drug resistant *P. falciparum* and against gametocytes.
- Possible delay or slowing of spread of resistance to the partner drug.
- No documented parasite resistance as yet.
- Few reported adverse clinical effects.

However, potential prohibitive factors for the use of ACT are mainly associated with potential misuse of artimisinins risking their value in treating severe malaria, the problems of adherence to co-administered (non-fixed) combinations and the higher cost of artimisinins.

Of artimisinin derivatives, artesunate (AS) has the most documented clinical information. There are different options for artimisinin-based combination therapy, however, as stated above the extension of the useful therapeutic life achieved by the combination is determined by the previous use of the partner drug (pre-existing level of resistance against the partner). This explains the advantage of SP/AQ over SP/AS observed in east Africa (Watkins *et al.*, 2005). Because artimisinins are natural products, there is a concern that their supply may be limited with time due to the widespread use of artimisinin-based combination therapy.

1.6.2.4 Non- artimisinin-based combination therapy

Due to the widespread *P. falciparum* CQ resistance, CQ based combinations (e.g. CQ plus SP) are not recommended for use. AQ+SP has been shown to be more effective than SP alone or SP+AS in Uganda (Dorsey *et al.*, 2002). In some countries in west and central Africa a change to SP+AQ could be a more cost effective option than a change to SP monotherpy. However, data on safety of AQ for widespread unsupervised repeated treatment of malaria and its use during pregnancy is required.

Atovaquone-proguanil (Malarone™, GlaxoWellcome) is a fixed dose combination, which is highly efficacious against CQ and MQ resistant *P. falciparum* (WHO, 2001). The efficacy and safety data for Malaron™ are encouraging, however, its high cost and restricted availability limit its potential as a suitable option for combination therapy. Malaron™ is not recommended for use in young children with a body weight of less than 11 kg, pregnant women and in breast-feeding women (WHO, 2001a), though a study by (McGready *et al.*, 2003) has shown its safety and effectiveness for both pregnant women and foetuses when used in combination with artesunate, however, this should be confirmed by other studies.

Chlorproguanil-Dapson (Lap Dap) is a fixed-dose synergistic antifolate combination similar to SP that has been developed by Glaxo Smith Kline, Liverpool University,

and WHO/TDR (Lang and Greenwood, 2003). Lap Dap shares similar genetic mechanisms of resistance as SP, however, it has been shown to be more efficacious than SP in semi-immune patients in east Africa (Mutabingwa *et al.*, 2001a). It may be possible that Lap Dap exerts a smaller degree of selection pressure for resistance than SP (Warrell and Gilles, 2002).

Other combinations include quinine plus tetracycline or doxycycline. Tetracycline and doxycycline are contraindicated in pregnant and breast-feeding women and in children less than 8 years. This combined with the adverse effects associated with quinine (e.g. tinnitus) make this combination not suitable for general use by malaria control programmes.

Based on available safety and efficacy data, the following options for combination therapy are currently recommended (WHO, 2001a):

1. Artemether-lumefantrine (Coartem™)
2. Artesunate (3 days) plus amodiaquine
3. Artesunate (3 days) plus SP in areas of high SP efficacy
4. SP plus amodiaquine in areas of high efficacy of both drugs (west Africa).

Mefloquine-based combinations like artesunate plus mefloquine and mefloquine plus sulfadoxine-pyrimethamine (Fansimef™, Roche) are not recommended particularly in high transmission areas because of the concern that the long half-life of MQ may lead to selection of resistant parasites. There are also concerns of increase adverse effects when MQ used unsupervised on a large scale for malaria treatment (WHO, 2001a).

1.7 ANTIMALARIAL DRUG RESISTANCE

Antimalarial drug resistance is a major public health problem that hinders the control of malaria. The earliest anecdotal reports of antimalarial drug resistance were those for Quinine in 1844 and 1910 (reviewed in Talisuna *et al.*, 2004a). In the 1960s, an attempt was made to eradicate malaria by the use of mosquito insecticides, as well as, the massive use of CQ for prophylaxis and treatment. This may have increased the evolution of resistant parasite strains by applying more selection pressure for survival in the presence of CQ (Ursos and Roepe, 2002).

Unfortunately, resistance to all known antimalarial drugs, with the exception of the artemisinin derivatives, has developed to various degrees in several countries (WHO, 2001b).

1.7.1 Emergence And Spread Of Chloroquine Resistance

CQ-resistant strains of malaria parasite started to appear after 10-20 years of its introduction as a main antimalarial chemotherapy. CQ- resistant *P.falciparum* appeared first in the late 1950s and early 1960s in Southeast Asia and south-America and then spread throughout all malaria endemic regions reaching Africa in the 1970s. The currently available information about the various patterns of mutations accompanying K76T from different malarial regions indicates that CQ-resistant forms of PfCRT developed independently from at least five foci (Wellems, 2004) (**Figure1.10**).

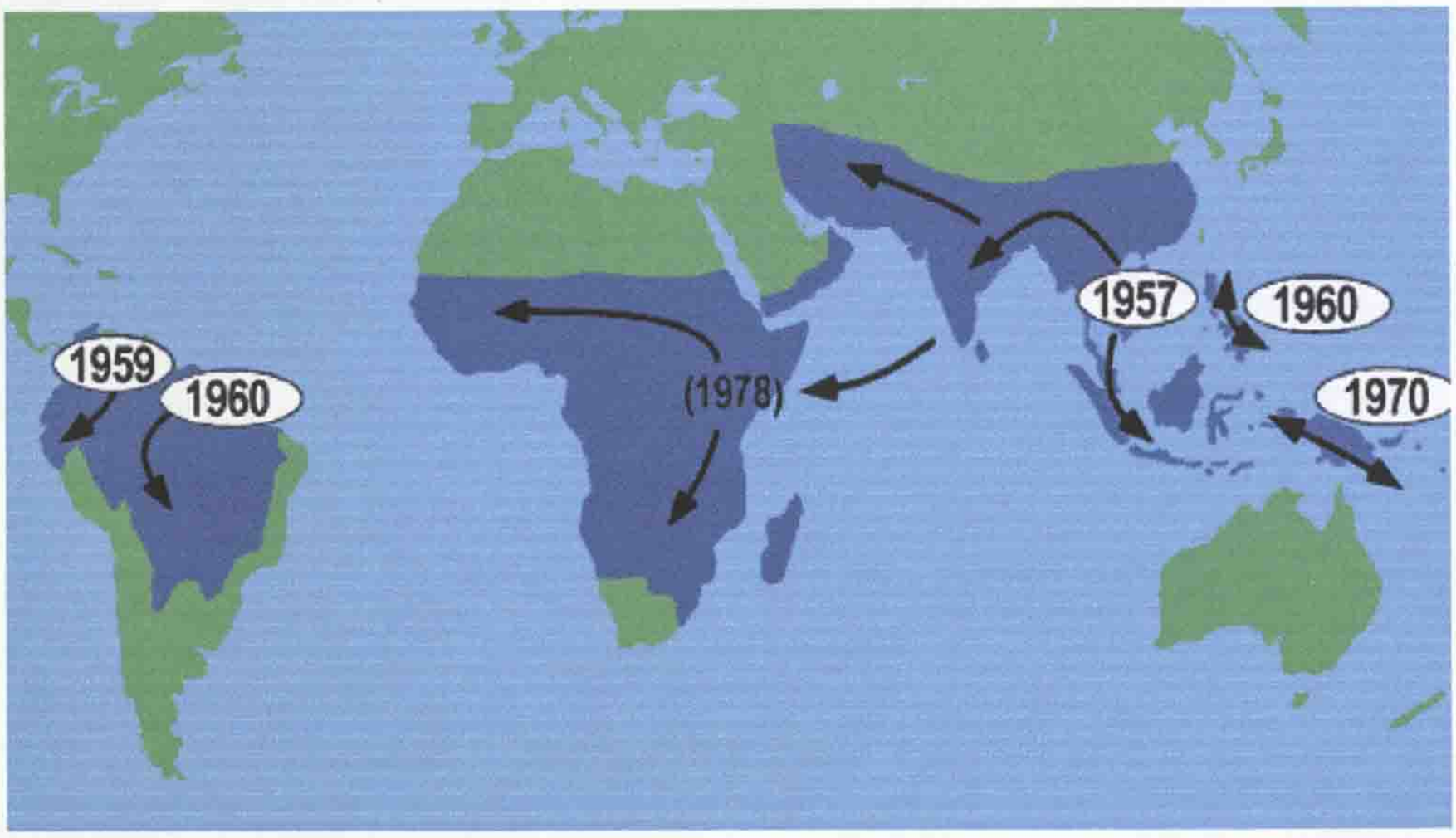


Figure 1.10 Spread of CQ-resistant *P.falciparum* from five known origins. (Source: Wellems, 2004)

The first focus was though to have developed in South-east Asia around the Thai-Cambodian border in 1957 and spread quickly to Thailand. Two other foci were identified in 1960 in South America namely in Venezuela and in the Magdalena Valley, Colombia (reviewed in Talisuna *et al.*, 2004). The report of two confirmed cases of CQ-resistant *P.falciparum* infection from Port Moresby in Papua New Guinea (PNG) in 1976, that are remarkably different from strains from Southeast

Asia, represented the emergence of the fourth independent focus of CQ-resistant *P.falciparum* infection (Mehlotra *et al.*, 2001). The PfCRT molecules from the foci all contain a key K76T amino-acid replacement but have different patterns of accompanying mutations (**Table 1.2**).

In Africa, CQ-resistant *P.falciparum* was first detected in 1978 in non-immune travellers from Kenya and Tanzania (Campbell *et al.*, 1979; Fogh *et al.*, 1979). This was followed quickly by reports from Madagascar (Aronsson *et al.*, 1981), and by reports in semi-immune patients in Tanzania (Kihamia and Gill, 1982; Onori *et al.*, 1982), and in Kenya (Spencer *et al.*, 1983). By 1983 CQ-resistance had reached Sudan, Uganda (Onori and Hempel, 1984), Zambia (Ekue *et al.*, 1983), and Malawi (Fogh *et al.*, 1984). This sweep of resistant *P. falciparum* together with the similarity of parasites of African and Asian origin and their difference from those from South America and PNG (Mehlotra *et al.*, 2001; led to the view that CQ-resistant *P.falciparum* may have spread from Southeast Asia to Africa as a result of population movements. CQ-resistance is now present in nearly all malaria endemic countries (**Figure 1.11**), the only current exceptions are Central America northwest of Panama, Haiti and the Dominican Republic, and the Middle East, where the magnitude of *P. falciparum* resistance is still the subject of investigation (Talisuna *et al.*, 2004).

Parasite origin & type (reference line)	PfCRT position and encoded amino acid														
	72	74	75	76	97	144	148	160	194	220	271	326	333	356	371
CQ-sensitive															
All regions, wild-type (HB3) (Fidock *et al*, 2000a)	C	M	N	K	H	A	L	L	I	A	Q	N	T	I	R
Africa (106/1) (Fidock *et al*, 2000a)	C	I	E	K	H	A	L	L	I	S	E	S	T	I	I
CQ-resistant															
Africa & South-east Asia, Type E1a (Dd2) (Mehlotra *et al,* 2001)	C	I	E	T	H	A	L	L	I	S	E	S	T	T	I
Africa & South-east Asia, Type E1b (FCB, K1) (Johnson *et al*, 2004)	C	I	E	T	H	A	L	L	I	S	E	S	T	I	I
Papua New Guinea, Type P1 (PNG 1935) (Mehlotra *et al*, 2001)	S	M	N	T	H	A	L	L	I	S	Q	D	T	L	R
South America, Type W1a (7G8) (Fidock *et al*, 2000a)	S	M	N	T	H	A	L	L	I	S	Q	D	T	L	R
South America, Type W1b (Euc 1110) (Fidock *et al*, 2000a)	C	M	N	T	H	A	L	L	I	S	Q	D	T	L	R
South America, Type W2 (Jav) (Fidock *et al*, 2000a)	C	M	E	T	Q	A	L	L	I	S	Q	N	T	I	T
Philippines, Type P2a (PH1) (Chen *et al*, 2003)	C	M	N	T	H	T	L	Y	I	A	Q	D	T	I	R
Philippines, Type P2b (PH2) (Chen *et al*, 2003)	S	M	N	T	H	T	L	Y	I	A	Q	D	T	I	R
Indonesian Papua (2300) (Nagesha *et al*., 2003)	C	I	K	T	H	A	L	L	I	S	E	S	T	I	I
Combodia (742) (Durrand *et al*., 2004)	C	I	E	T	H	A	L	L	I	S	E	N	T	I	I
Combodia (734) (Durrand *et al*., 2004)	C	I	D	T	H	F	I	L	T	S	E	N	S	I	R
Combodia (738) (Durrand *et al*., 2004)	C	I	D	T	H	A	I	L	T	S	E	N	S	I	R
Combodia (783) (Durrand *et al*., 2004)	S	I	E	T	H	A	L	L	I	S	E	N	T	T	I

Table 1.2 Allelic variants of *pfcrt* of *P.falciparum* field isolates and laboratory adapted lines from different geographical areas. (Source: Bray *et al.*, 2005). Sequences listed for only those where full length coding sequence was established.

Drug resistance led to a change in antimalarial drug policy in several countries where a change from CQ to other regimens as first-line treatment had been implemented. Sulphadoxine-pyrimethamine (SP), the affordable, relatively safe, and easy to administer drug was chosen to replace CQ. Unfortunately, parasite resistance to SP has developed very quickly in Southeast Asia and then in Africa (Thimasarn *et al.*, 1990; Nwanyanwu *et al.*, 1996). SP resistance has recently reached critical levels in some areas of East and Central Africa, leading to a concern that a public health disaster may be imminent (Barat *et al.*, 1998 ; Bloland and Ettling, 1999; Kamya *et al,* 2002; Mutabingwa *et al.*, 2001a).

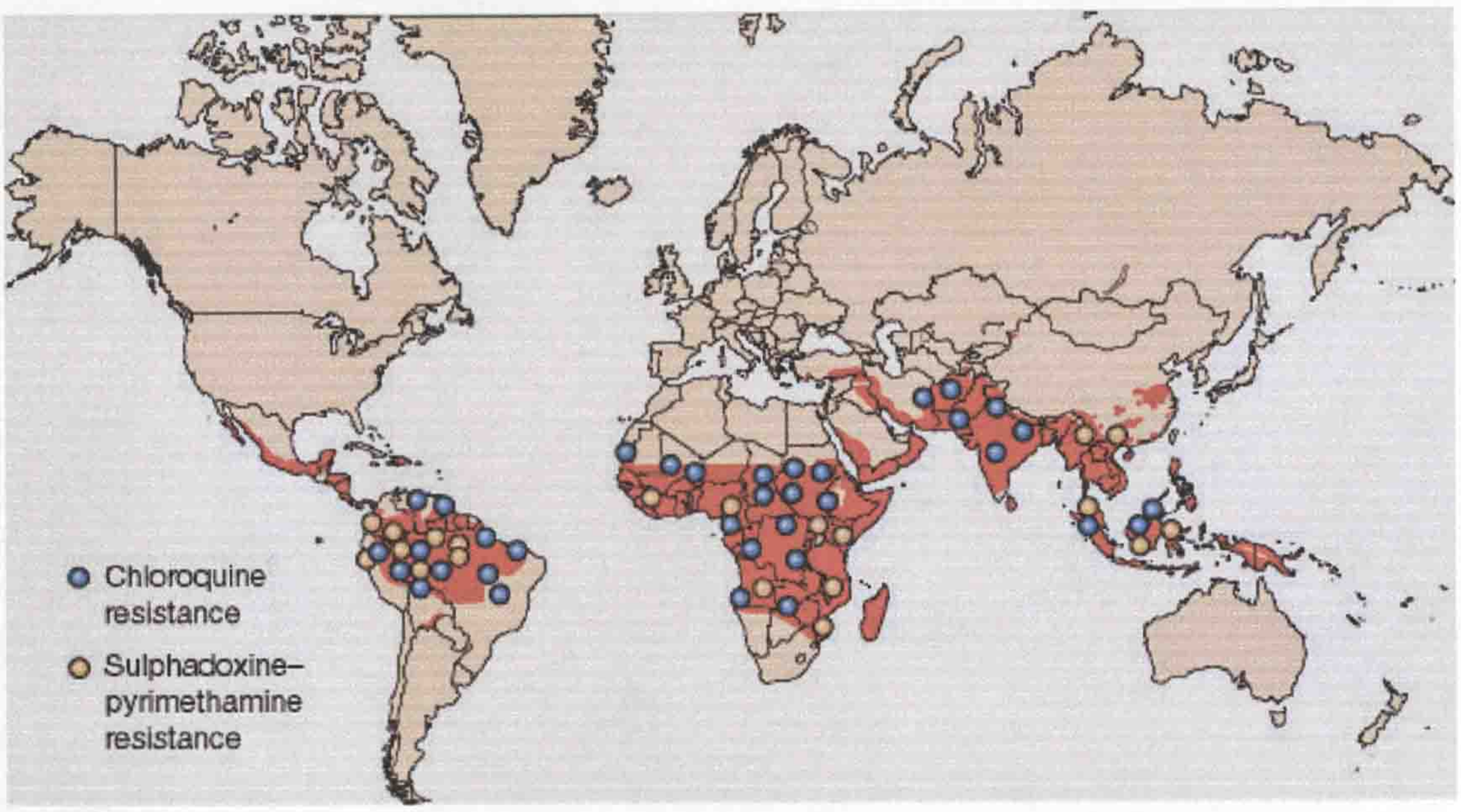


Figure 1.11 The global status of resistance to Chloroquine and sulphadoxine/pyrimethamine according to data from WHO. (Source: Nature 2002,**415**;669-715)

1.7.2 Definition Of Antimalarial Drug Resistance

Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within tolerance of the subject" (WHO, 1973). This definition was later modified to specify that the drug in question must "gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action" (Bruce-Chwatt *et al*, 1986).

A distinction must be made between a failure to clear malarial parasitaemia or resolve clinical disease following a treatment with an anti-malarial drug and true anti-malarial drug resistance. The resistance phenotype is determined by culturing the parasite. It is not necessarily expressed by treatment failure (particularly in subjects who have acquired immune defences during previous malaria episodes). Moreover, while drug resistance can cause treatment failure, not all treatment failures are due to drug resistance. Many factors can contribute to treatment failure (**section 1.7.5**), they may also contribute to the development and intensification of true drug resistance through increasing the likelihood of exposure of parasites to sub-optimal drug levels.

1.7.3 Mechanism Of Antimalarial Drug Resistance:

The mechanisms by which the parasite is chemoresistant to antimalarials generally involve chromosomal mutations. In general, resistance appears to occur through spontaneous mutations that confer reduced sensitivity to a given drug or class of drugs. For some drugs only a single point mutation is required to confer resistance, while for other drugs, multiple mutations appear to be required. If the mutations are not deleterious to the survival or reproduction of the parasite, drug pressure will remove susceptible parasites while resistant parasites survive. Over time, resistance becomes established in the population and can be very stable; persisting long after specific drug pressure is removed. This section will focus on the proposed mechanisms conferring *P. falciparum* resistance to CQ with a special focus on the role of PfCRT in conferring resistance. Before reviewing the proposed mechanisms conferring *P. falciparum* resistance to CQ, it is important to review available data about the mechanism by which CQ exerts its antimalarial activity.

The mechanism of action of CQ has been studied extensively. One of the old theories was based on the observations made by Cohen and Yielding (1965), Macomber *et al.*, (1966); Warhurst, (1969) that antimalarials were able to interact with DNA and RNA synthesis leading to cell death. Later on, Warhurst, (1969) showed that exposure of *P. knowlesi* to chloroquine results in a breakdown of the parasite's ribosomal RNA. These findings suggested that the mode of action of chloroquine was related to inhibition of DNA replication and RNA transcription in the parasite (Peters, 1970). However, this hypothesis was defeated because it was

unable to explain the selective antimalarial toxicity of these compounds (Ginsburg and Krugliak, 1992)

Studies showed that the target for CQ action in *P.falciparum* is its lysosomal food vacuole, where haemoglobin is degraded, (discussed below). Other drug targets are related to the functions of distinct organellar structures like the apicoplast (a plastid organelle originating from a green algal symbiont), and mitochondria with a limited electron transport system (Cohen and Yielding, 1965; **Figure 1.12**).

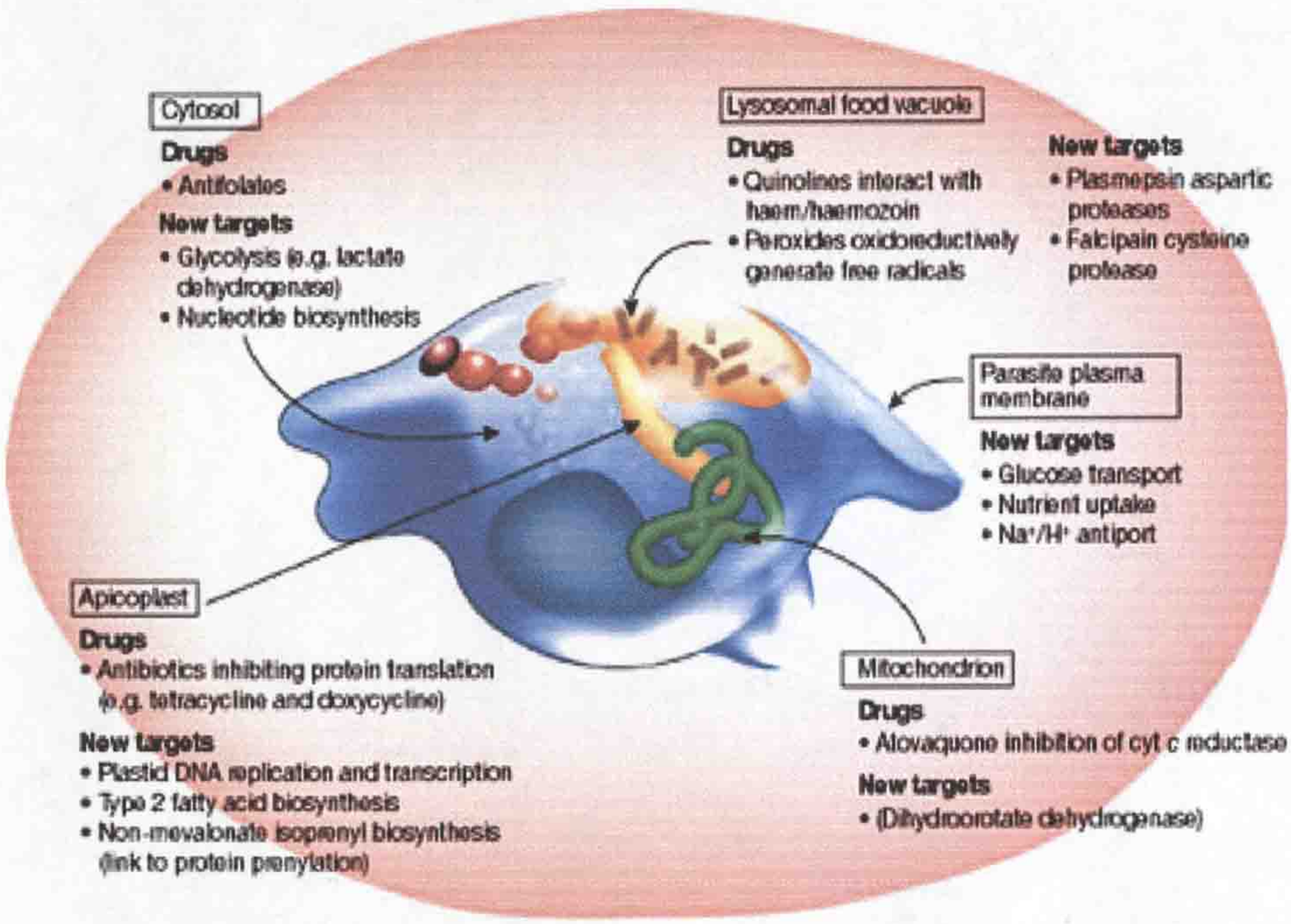


Figure 1.12 Targets of drug action in *P. falciparum*. Diagram of *P. falciparum* trophozoite residing in an erythrocyte showing the main organelles that are associated with drug targets and highlighting sites of action of both current antimalarial drug and new targets that are under investigation (Source: Nature 2002,**415**;669-715).

Digestion of host haemoglobin is essential for the parasite survival. As the parasite matures within its host erythrocyte, it digests a large proportion (up to 80%) of the host cell's haemoglobin (Francis *et al.*, 1997). Haemoglobin is digested inside the acid food vacuole, to provide a source of essential nutrients. Digestion results in ferric haem FPIX (haematin) and a number of small peptides and amino acids, which are essential for parasite growth. FPIX is toxic to the malaria parasite. It promotes

membrane damage via lipid peroxidation and also inhibits a wide range of enzymes (including proteases). To reduce the toxic effects of FPIX, the parasite polymerises liberated free FPIX into an insoluble crystalline substance known as haemozoin (Francis *et al.*, 1997). This brown coloured pigment (malaria pigment) accumulates in the DV and offers one of the most characteristic morphological features of malaria infection. Haemozoin formation is indeed a critical chemical reaction in the DV of the parasite, yet the mechanism is still debated. It was initially suggested that haemozoin was formed enzymatically by an unidentified haem polymerase activity (Slater and Cerami, 1992), but (Dorn *et al.*, 1995) showed that haemozoin formation can be spontaneous and can occur in the absence of protein by an autocatalytic mechanism.

4-Aminoquinolines are thought to exert their antimalarial activity by forming complexes with free FPIX thus preventing the polymerisation of the toxic haematin into haemozoin within the parasite DV.

1.7.4 Molecular Basis For Chloroquine Resistance

CQ-resistance has been linked to a number of mutations in the *P. falciparum* genes. Earlier studies of the mechanism of CQ resistance identified and investigated the role of the *P. falciparum* multidrug resistance (*pfmdr1*) gene, located on chromosome 5 and encoding the P-glycoprotein 1 (Pgh-1). Resistance to CQ was thought to be linked to point mutations, such as the asparagine-to-tyrosine substitution at position 86, and to other probably compensatory mutations at positions 184, 1034, 1042, and 1246 (Foote *et al.*, 1990; Reed *et al.*, 2000).

However, the results of field studies of the association of these mutations with the in vivo and in vitro resistance were not consistent. Subsequent studies identified other mutations in different genes of chromosome 7 (Wellems *et al.*, 1990). A single candidate gene (cg2), a polymorphic gene, was proposed as the one responsible for CQ resistance (Sidhu *et al.*, 2002). However, the observed association was probably due to its close proximity on chromosome 7 to the *pfcrt* gene encoding the *P. falciparum* CQ resistance-related transporter protein (Fidock *et al.*, 2000b) and (Sidhu *et al.*, 2002). Currently it is believed that mutations in the *pfmdr1* modulate the level of in-vitro resistance in the presence of *pfcrt* mutations. However, the role

of *pfmdr1* in determining the therapeutic response following CQ treatment remains unclear.

1.7.4.1 Identification of the genetic determinant of chloroquine resistance in *P. falciparum*

Identification of the major CQ-resistance determinant resulted from the analysis of a genetic cross between the CQR clone (Dd2) from Indochina and the CQS clone (Hb3) from Honduras (Wellems *et al.*, 1990). Analysis of the haploid progeny arising from this cross mapped a single genetic locus, localized to a 36 kb segment on chromosome 7, that segregated with the inheritance of verapamil-reversible CQ resistance (Wellems *et al.*, 1991; Su *et al.*, 1997).

Later on, on the same 36 kb segment, Fidock *et al.*, (2000a) identified a highly interrupted gene, with 13 exons spanning 3.1 kb and encoding a 424 amino acid (48.6 kDa) protein which was localized to the DV membrane of the parasite (Fidock *et al.*, 2000a; Cooper *et al.*, 2002) and was suggested to be either a regulator of transport proteins (Zhang *et al.*, 2002; Nessler *et al.*, 2004) or a chloride channel (Zhang *et al.*, 2002). The protein was termed PfCRT for '*Plasmodium falciparum* Chloroquine Resistant Transporter' due to the 'transporter-like' nature of its predicted secondary structure (Fidock *et al.*, 2000a). Based on the similarities in the Sequence of PfCRT protein and the aqueous chloride channel of *Salmonella typhimurium*. (Warhurst *et al.*, 2002) postulated that PfCRT is a member of the chloride channel (ClC) family. Recent studies concluded that the CRT protein belongs to the drug/metabolite transporter (DMT) superfamily (Martins *et al.*, 2003; Martin and Kirk, 2004; Tran and Saier, 2004).

1.7.4.2 Proposed PfCRT function

The CRT protein has 10 predicted transmembrane domains (TMDs) (**Figure 1.13**) with different functions within the PfCRT protein. The TMDs 4 and 9 are implicated in the binding and translocation of the substrate; TMDs 1, 2, 6 and 7 are implicated in the recognition of, and discrimination between, substrates; TMDs 3 and 8 are implicated in the binding and translocation of the substrate as well as in determining the substrate specificity of the transporter; and TMDs 5 and 10 are implicated in the formation of a homodimer (Martin and Kirk, 2004).

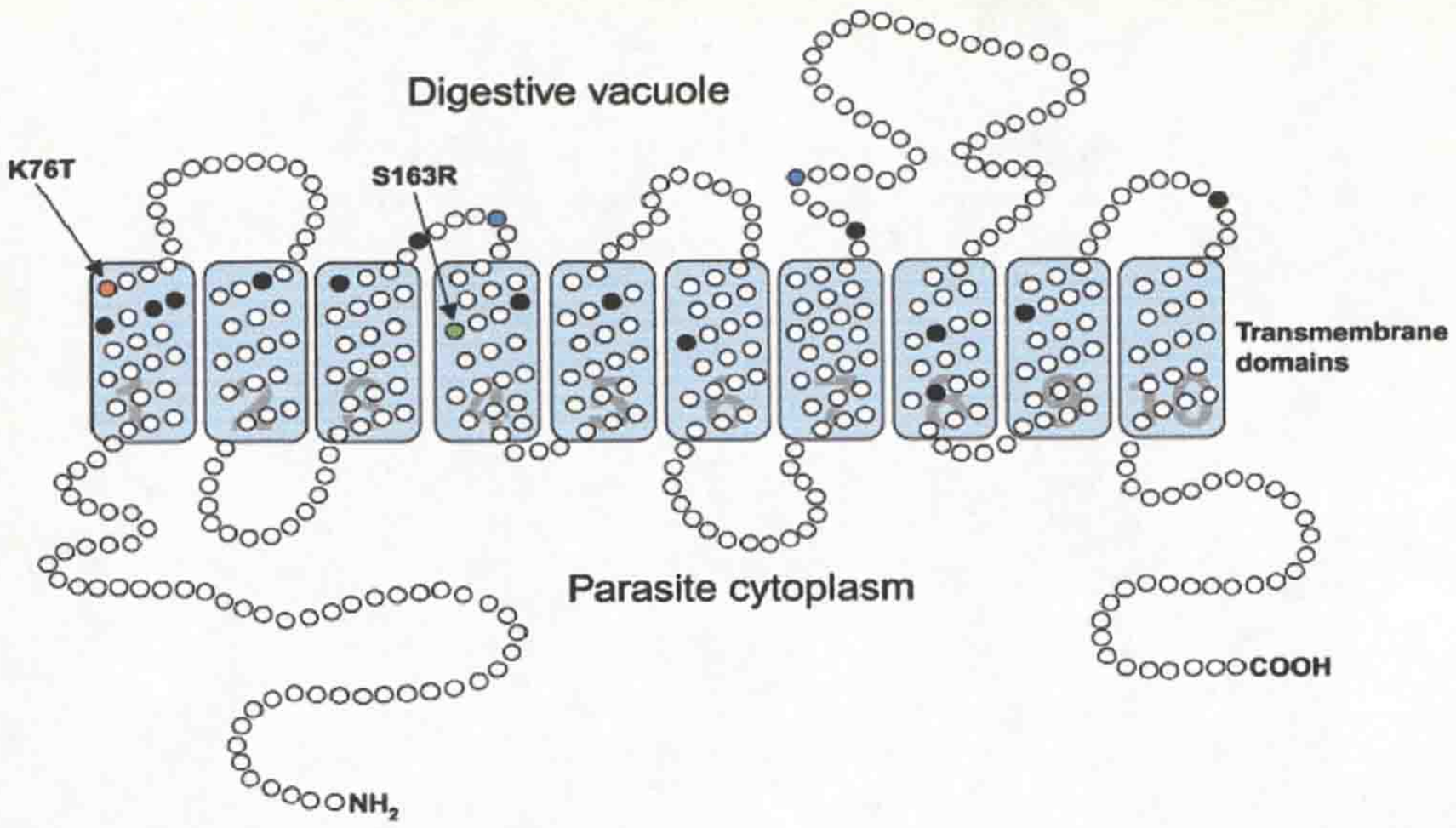


Figure 1.13 Predicted protein structure of PfCRT, highlighting the ten transmembrane α helices. Black circles indicate the positions of mutations frequently identified in the Eastern and Western hemispheres. Red circle represents the critical K67T mutation. The green filled circle indicates the position of the newly identified mutation S163R present in both the amantadine-resistant and halofantrine-resistant lines, while the blue circles represent additional mutations identified in these drug-pressured lines (source: Bray *et al*, 2005).

Due to close similarity to members of the drug/metabolite effluxer (DME) family, especially over the region of the substrate-binding motif in TMDs 4 and 9, the PfCRT is predicted to function as an exporter of 'metabolites' from the DV. In 2004, Martin and Kirk hypothesized that PfCRT is an amino acid/peptide effluxer.

1.7.4.3 Mutations at PfCRT and CQ resistance

Progressive studies of the genetic cross between the CQR clone (Dd2) and the CQS clone (Hb3) suggested that the mechanism of *P. falciparum* resistance to CQ is linked to mutations in the *pfcrt* gene. In the CQ-resistant Dd2 parent, eight point mutations (M74I, N75E, K76T, A220S, Q271E, N326S, I356T and R371I) were found in the predicted protein sequence (PfCRT) encoded by the *pfcrt* gene (Fidock *et al*., 2000a). Seven of them were detected in 15 CQ-resistant parasite lines while the remaining mutation I356T was detected in some lines. All Asian, African and South American CQ-resistant lines shared the K76T and A220S mutations. However, distinct sets of PfCRT mutations were found among nine CQ-resistant lines from

South America suggesting that PfCRT mutations arose separately in association with CQ resistance in South America and Asia/Africa which is consistent with the independent genesis of CQ resistance in these regions (Payne, 1987).

The role of the mutation at position 76 in conferring CQ-resistance is discussed below (**Section 1.7.4.5**), however, It is, also, suggested that mutations at other positions are required to maintain critical functional properties of the molecule in the presence of the K76T change. The concomitant presence of mutation A220S with K76T in CQ-resistant parasites from the different New World and Old World foci suggested its possible role in maintaining PfCRT function in the presence of the K76T mutation. The suggestion that K76T cannot occur in the absence of other PfCRT point mutations may also explain the slow genesis of CQ resistance in the field as well as the difficulties that have been experienced with attempts to select CQ resistance in the laboratory.

1.7.4.4 Evidence for a central role for PfCRT in CQ resistance

Direct evidence for the central role of PfCRT in CQ-resistance came from transfection studies showing that co-expression of mutant forms of PfCRT in the presence of a wild-type background conferred a modest degree of VP-reversible CQ resistance to CQS parasites (Fidock *et al.*, 2000a). Furthermore, replacement of the entire *pfcrt* allele in a CQS parasite with *pfcrt* allele representative of mutant sequences prevalent in CQR parasites from different parts of the world has resulted in recombinant clones having all the criteria of CQ resistance phenotype (increased CQ IC_{50} values, acquisition of verapamil-reversibility, and decreased CQ accumulation (Sidhu *et al.*, 2002).

More recently, Johnson *et al.*, (2004) provided further evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to structurally diverse antimalarial agents. They showed that stepwise selection for resistance to amantadine or halofantrine produced previously unknown *pfcrt* mutations (including S163R, **Figure 1.13**), which were associated with loss of verapamil-reversible CQ resistance. This was accompanied by a restoration of efficient CQ binding to haematin in these selected lines.

Sequencing a Southeast Asian isolate (Pf164) revealed its similarity to K1, However, it was found to contain an additional mutation (S163R). This non-culture-adapted isolate had a CQ IC_{50} value of 21 nM recorded with the WHO microtest, which classify it as CQS. Evidence for the presence of this mutation in a Southeast Asian isolate supports the argument for a broad role for PfCRT in determining levels of susceptibility to structurally diverse antimalarials.

Investigating the implications of this finding for surveillance protocols that use K76T as a marker for chloroquine-resistant *P. falciparum* is now important, particularly in areas where halofantrine or mefloquine have been widely used (Johnson, 2003)

1.7.4.5 The role of position 76 in CQ resistance

Evidence of a central role of position 76 in CQ-resistance came from the study of Fidock et al., (2000a). Sixteen in-vitro CQ-sensitive lines from around the world were tested. Fifteen of them showed the PFCRT sequence of the CQ-sensitive HB3 parent. The one exception, 106/1, was found to encode all of the PfCRT mutations associated with CQ-resistance except the amino acid mutation at position 76, supporting a central role for this residue in CQ resistance. Furthermore, selection of a highly CQ-resistant line that had undergone a single K76I point mutation was obtained from the CQ-sensitive106/1 (Fidock et al., 2000a), providing additional evidence for the central role of position 76 in CQ resistance. Recently, (Lakshmanan *et al.*, 2005) showed that removal of the single PfCRT amino acid change K76T from resistant strains led to wild type levels of CQ susceptibility, increased binding of CQ to ferriprotoporphyrin IX in the DV and loss of verapamil reversibility of CQ and QN resistance.

The fact that the CQ-resistant line containing the K76I point mutation reported by Fidock *et al.*, (2000a) was obtained from the CQ-sensitive106/1 line that already contained six PfCRT mutations at other positions seen in Southeast Asian and African parasites and the observation that the K76T mutation has not been seen in the absence of mutations at other positions in PfCRT, has led to the suggestion that CQ resistance may involve a progressive accumulation of mutations in the *pfcrt* gene, and the mutation at position 76 seems to be the last in the long process leading to CQ clinical failure (Djimde *et al.*, 2001a; Hastings *et al.*, 2002a). The suggestion

that K76T cannot occur in the absence of other PfCRT point mutations may also explain the slow genesis of CQ resistance in the field as well as the difficulties that have been experienced with attempts to select CQ resistance in the laboratory.

The strong association between the presence of K76T and in-vivo CQ treatment failure was documented in a number of field in-vivo studies. Moreover, K76T was detected in all samples that failed treatment with CQ indicating the absolute selection of K76T by CQ (Djimde *et al*, 2001a). K76T is established as a useful molecular marker for CQ-resistance that can be easily applied in field studies (Djimde *et al.*, 2001b). However, this association between the presence of K76T and in-vivo CQ treatment failure was not perfect in some other studies suggesting the contribution of other factors like immunity and presence of other unknown contributing mutations in *pfcrt* or other genes.

1.7.4.6 Current theories on the mechanism by which PFCRT mutations confer CQ resistance

Due to the location of PfCRT in the parasite DV membrane it is believed that mutations in PfCRT may confer CQ-resistance by reducing the amount of CQ accumulated within the DV of the parasite. (Saliba *et al.*, 1998) have provided direct evidence for reduced accumulation of CQ in the DV of CQR parasites. The exact mechanism leading to low intracellular CQ levels is not clear yet. However, several mechanisms have been proposed, such as altered pH (Druilhe *et al.*, 2001), decreased drug uptake into the parasite food vacuole (Sanchez *et al.*, 1997), or increased drug efflux out of the vacuole (Krogstad *et al.*, 1987).

Three currently accepted theories on the mechanism of CQ-resistance are discussed below. The first deals with the pH of the DV, the second with the efflux of CQ out of the DV and the third is the "charged drug leak" theory.

1.7.4.6.1 CQ resistance and pH of the digestive vacuole

Views about the association between the pH of the parasite digestive vacuole and CQ resistance are still contradictory. An early hypothesis proposed that reduced accumulation of CQ at steady state in CQR parasites is due to elevated DV pH,

(Ginsburg and Stein, 1991; Yayon *et al.,* 1985). Studies comparing the digestive vacuole pH of CQS and CQR parasite lines suggested that CQR parasites might have a more acidic DV than CQS parasites (Dzekunov *et al.,* 2000; Ursos *et al.*, 2000). Inconsistent results were obtained from studies with acridine orange dye. Fluorescence from the parasite DV was observed from CQR compared to CQS parasite lines and estimates of the digestive vacuole pH were made accordingly (Dzekunov *et al.,* 2000; Ursos *et al.*, 2000; Ursos and Roepe, 2002). However, it has been shown that fluorescence from acridine orange is, indeed, localized to the parasite cytoplasm and that acridine orange fluorescence from the DV is due to stacked or aggregated forms of the dye, and does not display a quantifiable relationship to the pH gradient (Bray *et al.,* 2002; Wissing *et al.*, 2002).

Recently, Bennett *et al.*, (2004) found that CQR parasite lines have a more acidic digestive vacuole pH (5.2) compared to CQS lines (5.7), a result that could not be confirmed by studies from other laboratories (Bray *et al.*, 2005). The observation of a 6-fold decrease in the uptake of CQ into CQR isolates, relative to that in CQS isolates (Sanchez *et al.*, 1997; 2003; Bray *et al.*, 1998) is not consistent with the weak base theory which rather suggests an associated increase in the uptake of CQ of up to a 10-fold with an increase of 0.5 in the pH gradient across the DV membrane in CQR parasites (Bray *et al.*, 2005).

Ursos and Roepe, (2002) suggested that a more acidic digestive vacuole pH in CQR isolates may reduce the concentration of FPIX leading to reduction in cellular CQ uptake. However, it was found that the quantity of the FPIX receptor is unchanged in CQR isolates (Sanchez *et al.*, 1997; 2003; Bray *et al.,* 1998).

1.7.4.6.2 CQ resistance and efflux of CQ from the digestive vacuole

Transport of CQ out of the DV is postulated as an alternative explanation for the reduced uptake of CQ by CQR parasites. An energy-dependent efflux process was proposed for CQ resistance (Krogstad *et al.*, 1987; Krogstad *et al.*, 1992). This was recently supported by (Sanchez *et al.*, 2003). However, previous findings that depleting the parasite of ATP (Kirk *et al.*, 1996) has no effect on the accumulation of CQ by CQR parasites (Bray *et al.*, 1996) argues against this model of active efflux. Further evidence against the active efflux theory is the observation of the reduced

CQ accumulation of CQR parasites when the incubation temperature was lowered (Sanchez *et al.*, 1997) as an increase in drug accumulation is expected at low temperature if an active efflux process is operating in the resistant organisms (Gottesman and Pastan, 1993). These data suggest that the reduced CQ uptake observed in CQR lines (Sanchez *et al.*, 2003) might be unrelated to the concentration of ATP and so, might not be caused by of the active efflux of CQ from the DV (Bray *et al.*, 2005).

1.7.4.6.3 The 'charged drug leak' hypothesis

This theory proposed a mechanism of leakage of charged (protonated) forms of CQ (CQ^{+2}) from the DV, down its concentration gradient rather than active extrusion.

On the basis of the previous observation that CQR lines selected for increasing levels of resistance to either amantadine or halofantrine makes the parasite sensitive to CQ Nateghpour *et al.*, (1993), Ritchie *et al.*, (1996), and Johnson *et al.*, (2004) showed that CQ accumulation and access to haematin were restored and the resistance-reversing effect of VP was lost in these parasite lines. Moreover, both lines exhibited novel mutations of PfCRT including one mutation (S163R) that was common to both drug-selected lines. Interestingly, however, both lines retained the *pfcrt* K76T mutation, making these the first fully characterized examples of CQS parasite lines with this mutation (Johnson *et al.*, 2004). This mutation (S163R) is proposed to replace a positive charge in the transporter barrel, thereby compensating for the loss of positive charge associated with the K76T mutation, thus blocking the leak of CQ^{2+} caused by K76T CQ and thereby return the parasites to full CQS status, restoring access of CQ to FPIX and ablating VP synergism (Johnson *et al.*, 2004).

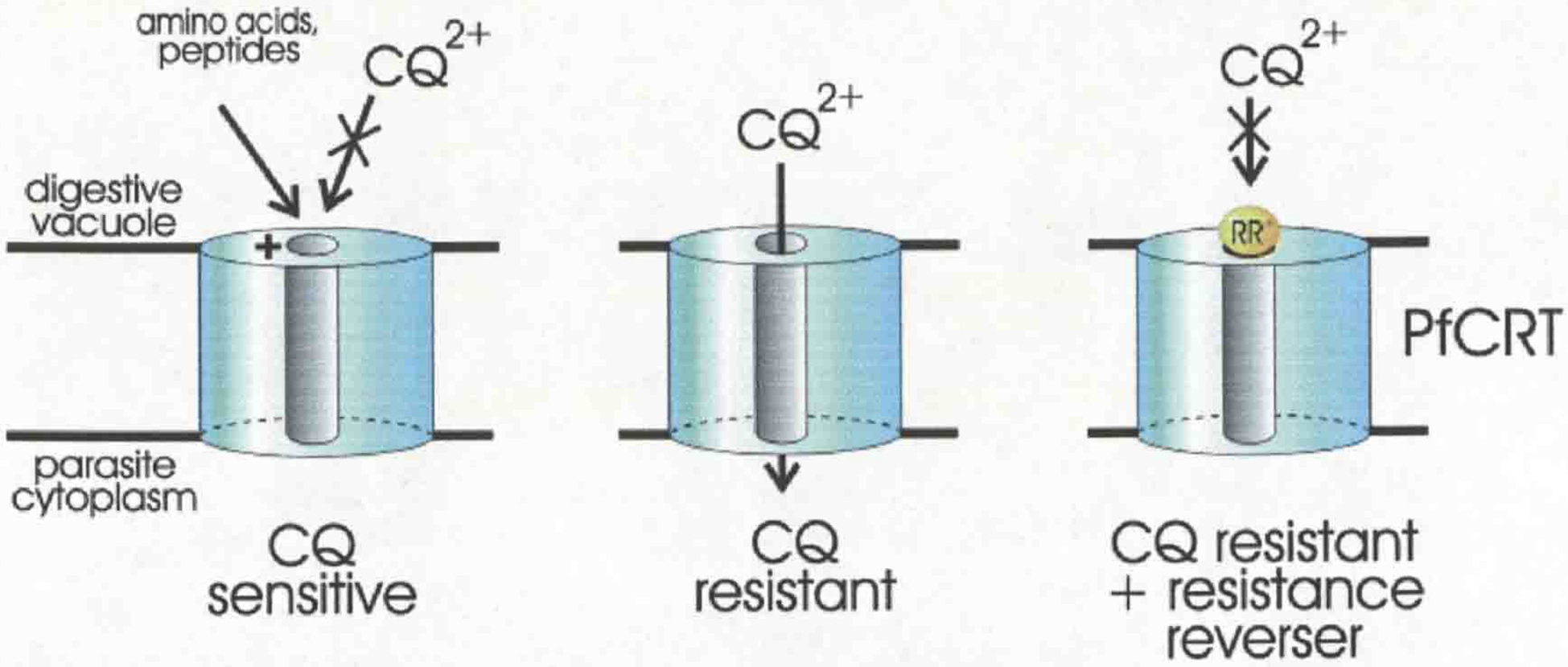


Figure 1.14 The 'charged drug leak' model for the role of PfCRT in CQ resistance. (RR^+)= positively charged resistance-reversing agents (Source: Bray *et al.*, 2005).

According to the position of the S163R mutation within transmembrane domains of PfCRT (**Figure 1.13**) and different roles assigned to the different TMDs in PfCRT function (**section 1.7.4.2**), this model suggests that in the CQS strain harbouring both the K76T and the S163R mutation, the transporter is also predicted to recognize CQ^{2+} as a substrate; however, the presence of a positive charge in the binding motif is likely to destabilize the CQ^{2+}-transporter complex and inhibit the translocation of CQ^{2+}. Thus, while PfCRT of CQS, wild-type parasites is predicted to neither recognize nor translocate CQ^{2+}, PfCRT containing the CQ resistance-conferring K76T mutation is predicted to do both, while PfCRT harbouring both the K76T and S163R mutations is predicted to recognize, but not transport CQ^{2+}.

1.7.5 Determinants For Emergence And Spread Of Antimalarial Drug Resistance

Many factors contribute to the development and spread of resistance. They involve the interaction of drug-use patterns, characteristics of the drug itself, human host factors, parasite characteristics, malaria transmission intensity and migration of humans or vectors.

1.7.5.1 Drug characteristics:

Characteristics of the drug such as drug use, drug quality, drug interactions and drug elimination half-life are important determinants of resistance. Resistance to CQ was observed to develop in areas where long-term CQ was used for either prophylaxis or

treatment (Payne, 1988). Later on, studies showed a positive correlation between the pattern of drug use and in-vitro parasite resistance or the prevalence of mutations linked to resistance (Diourte *et al.*, 1999; Nzila *et al.*, 2000b). Recently, it has been shown that the prevalence of CQ resistance is higher in sites with a high frequency of CQ use (Talisuna *et al.*, 2002). The relationship between drug use and drug resistance is explained by the ability of the drug to clear sensitive parasites leaving the resistant ones (selection), which then multiply and spread rapidly. However, SP resistance was highest in sites with relatively low SP use but with high transmission, suggesting the role of other factors in addition to drug pressure in the spread of drug resistance (Talisuna *et al.*, 2004). Sub-optimal quality of the drug can also lead to plasma subtherpeutic level of the drug exerting drug selection pressure (see below).

The role of drug elimination half-life in the evolution of parasite resistance has recently been reviewed and modelled (Hastings *et al.*, 2002b). Drugs with a long elimination half-life, (such as mefloquine and SP), though they may have multiple therapeutic advantages, they are likely to exert undesirable drug selection pressure when their concentrations drop below a critical threshold that can still prevent reinfection by sensitive parasites but not by resistant ones. This can occur even under conditions of supervised drug administration and optimal dosage (Watkins and Mosobo, 1993)

1.7.5.2 Human-host factors

A number of factors related to the host (man) can affect the evolution of antimalarial drug resistance. Drug absorption, distribution, metabolism and rate of elimination are important factors and are variable among individuals. Poor or erratic absorption, poor metabolism of drugs, e.g. "poor metabolizers" of the biguanide antimalarials chlorproguanil and proguanil due to genetic polymorphism as shown by Watkins *et al.*, (1990); Ward *et al.*, (1989); and Helsby *et al.*, (1991), incorrect dosing, non-compliance with duration of dosing regimen, all can lead to sub-therapeutic concentrations of the drug in plasma helping in the development of drug resistance through a drug selection mechanism.

One of the most important human host determinants for the development of the antimalarial drug resistance is host immunity. Immunity against malaria is acquired

progressively and slowly with age due to repeated exposures to malarial parasite. It is a species specific, as well as, stage specific. A state of complete immunity is never attained since immunity is highly specific and the parasite is highly diverse, so it is referred to as "semi-immunity". Host defence mechanisms have a major antiparasitic effect. Transmission of a resistant infection could be reduced in a semi-immune patient as a result of both asexual-stage immunity (which reduces the multiplication rate and lowers the density at which the infection is controlled) and specific antigametocyte (transmission-blocking) immunity.

1.7.5.3 Parasite characteristics

Single or multiple point mutations in the *Plasmodium* genome may confer resistance in the face of chemotherapy. Chloroquine resistance has been thought to involve greater genetic complexity than pyrimethamine resistance. The emergence and spread of drug resistance will be faster if the number of mutations required to encode resistance is low and their effects on parasite fitness are minimal (White, 1999a). Consistent with this is the rapid development of SP resistance to pyrimethamine that requires (in-vitro) only a single mutation at codon 108 in the *dhfr* gene. Conversely, resistance is slower to emerge if the genetic basis involves more than one gene. The observation that CQ resistance took longer to develop suggests that the genetic basis for CQ resistance involves two or more genes.

Parasite biomass is another important determinant of the evolution and spread of CQ-resistance. Large parasite biomass infections are thought to be very important for the de novo generation of gene mutation (White and Pongtavornpinyo, 2003). Large parasite biomass infections are more common in non-immune individuals, due to failure to clear mutant parasites (White, 1999a).

1.7.5.4 Transmission level

There are contrasting views on whether malaria transmission plays an independent role in the spread of resistance. Babiker *et al.*, (1997) have suggested that intensity of transmission is an important determinant of drug resistance due to its relationship to clone multiplicity. More recently (Talisuna *et al.*, 2005) suggested that malaria transmission intensity is critical determinant of the spread and evolution of drug

resistance. Currently, there are three contrasting theories on the role of malaria transmission.

The first suggests that low transmission intensity increases the spread of resistance (Hastings, 1997; White, 1999b) because monoclonal infections are common and self-fertilization by gametocytes from the same parasite clone (Ogutu *et al.*, 2000) occurs more often, increasing the probability that a combination of resistant mutations will be transmitted to the next generation of parasites (Hastings and D'Alessandro, 2000). Furthermore, due to low population immunity, most infections will progress to clinical disease requiring drug treatment (Rogier *et al.*, 1999), thus increasing the drug pressure (Hastings, 1997; Hastings and D'Alessandro, 2000; White, 1999b).

The second theory suggests that resistant parasites spread faster when transmission is high if intra-host dynamics (crowding effects) exist (Hastings, 1997; Hastings and D'Alessandro, 2000). It assumes that the high transmission that is associated with higher clone multiplicity could facilitate efficient re-colonization by resistant parasites after the elimination of susceptible ones following chemotherapy (Hastings, 1997).

The third theory states that transmission intensity plays no role in the early stages of the evolution of parasite resistance (Hastings, 1997; Hastings *et al.*, 2002b).

The effect of transmission intensity on the spread of drug resistance seems to be complex, and the net result is probably a balance between the effects of sexual recombination, intra-host dynamics, and the number of genes involved. Resistance might spread faster at the extremes of the transmission spectrum if two or more genes are involved, while spread of resistance could be faster in areas of high transmission if only one gene encodes resistance (Hastings, 2003). However, malaria transmission indirectly affects drug use, and a high frequency of drug use could also result in a faster spread in low-transmission areas when only one gene is involved.

1.7.5.5 Migration of humans or vectors

Population or vector movements could introduce new and resistant parasite genotypes that could be rapidly selected according to the amount of drug use. It has

been proposed that the genetic events that confer resistance to CQ are so rare (White and Pongtavornpinyo, 2003) that they might have occurred in few foci around the world and then spread as a result of human or vector movements.

1.7.6 Methods For Monitoring Antimalarial Drug Resistance

In general, four basic methods have been routinely used to study or measure antimalarial drug resistance: *in vivo, in vitro,* molecular characterization, and animal model studies. Additionally, less rigorous methods have been used, such as case reports, case series, or passive surveillance. Each method has its own advantages and disadvantages. The type of information each method yields indicates that these are complementary, rather than competing, sources of information about resistance.

1.7.6.1 In-vivo tests

In vivo tests are traditionally the "gold standard" method for detecting drug resistance (WHO, 1996). The test consists of the treatment of a group of symptomatic and parasitaemic individuals with known doses of drug and the subsequent monitoring of the parasitological and/or clinical response over time.

1.7.6.1.1 Development of in-vivo tests

Initially the classical 28-day extended test and the 7-day test were used and were interpreted according to parasitologic response (using the standard S-RI-RII-RIII classification system) (**Table1.3**). The major limitation of these earlier tests is that the study subjects are typically asymptomatic (often schoolchildren) and the results cannot be easily applied to malaria patients. The protocol was then revised and the simplified 14-day test of therapeutic efficacy was developed in 1996. In the revised protocol, clinical patients are used as study subject and their clinical response is taken into account in the outcome classification (**Table1.3**). In high transmission areas, reinfection is less likely with the 14-day test than with the 28-day test. However, the 14-day test has some limitations:

1) *Limitations related to inclusion criteria*

- Restricting the test to the group with the highest risk (children under 5 years) in areas with intense transmission which may lead to incomplete information due to inability to stratify results according to age.
- The upper limit of parasite density at recruitment 100,000 asexual parasites/µl of blood may result in the underestimation of the true prevalence of treatment failure as studies showed a significant association between higher parasite density and increased risk of treatment failure (Doherty *et al.*, 1999). It also may result in the exclusion of a substantial number of patients who would have been otherwise eligible, so increasing the time for recruitment and consequently the costs and logistics of the test. .
- The use of the lot quality assurance sampling in sample size estimation has often resulted in a small number of patients and point estimations of resistance with large confidence intervals, making meaningful temporal comparisons difficult because of random variability (Talisuna *et al.*, 2004).

2) *Limitations related to classification criteria:*

- Poor concordance between early treatment failure (ETF) and RIII (Plowe *et al.*, 2001).
- Tendency to overestimate ETF (Ringwald and Basco, 1999).
- Tendency to underestimate the true treatment failure rate (particularly the parasitological failure) because the adequate clinical response (ACR) category includes patients on day 14 with parasitaemia but without fever.

In view of these limitations, a recent WHO consultative meeting (WHO, 2001c) revised the study protocol and proposed several modifications. With respect to the inclusion and exclusion criteria, children (younger than 5 years) with clinical malaria are the study subjects in all areas. However, where feasible all ages can be enrolled in sufficient numbers to allow stratification by age (younger than 5 years, 5 years and older). It has been also recommended that in areas of intense transmission, enrolment of patients should be based on measured fever and not on history of fever alone. Similarly, the determination of outcome should also be based on measured temperature only and not on history of fever alone. Finally, the upper limit parasite density has also been revised from 100,000 asexual parasites/µl to 200,000 asexual

parasites/μl in areas with intense transmission and from 30,000 asexual parasites/μl to 100,000 asexual parasites /μl in areas of low transmission.

Regarding the classification of the response to treatment the ACR category in the 1996 protocol is stratified into two classes: adequate clinical and parasitological response (ACPR) and late parasitological failure (LPF). Accordingly, patients with parasitaemia on day 14 are classified as LPF (**Table 1.4**).

The use of the classical method for sample size calculation based on the expected treatment failure and the 95% confidence level with (5 or 10%) precision has been recommended. A minimum of 14 days was recommended for follow-up, however in areas of intense transmission, studies with a longer follow-up period should also include molecular genotyping of blood samples to help distinguishing between recrudescence and new infection.

1.7.6.1.2 Advantages of in vivo tests

- They can be conducted in the field with little equipment and personnel and the results are easy to interpret.
- They reflect the true biological nature of treatment response, which involves a complex interaction between the parasites, the drugs, and the host response.
- They offer the best information on the efficacy of antimalarial treatment under close to actual operational conditions—what can be expected to occur among clinic patients if provider and patient compliance is high (WHO, 2001b).
- Allows investigation of haematological recovery after malaria therapy (Bloland *et al.*, 1993).
- Allows study of some predictors of treatment failure that can be used by the clinicians or health workers before treatment decision has been taken.

1.7.6.1.3 Disadvantages of in vivo tests

- Long periods of follow-up of patients.
- Non-compliance and loss of follow-up.

- Because of the influence of external factors (host immunity, variations of drug absorption and metabolism, and potential misclassification of reinfection as recrudescence), the results of in-vivo tests do not necessarily reflect the true level of pure antimalarial drug resistance.

Parasitologic classification	Treatment response classification
Sensitive Clearance of parasite after treatment without subsequent recrudescence within a defined period	**Adequate clinical response (ACR)** (i) Absence of parasitaemia on day 14, irrespective of fever status, without previously meeting any of the criteria for ETF or LTF (ii) Absence of fever irrespective of parasitaemia status, without previously meeting any of the criteria for ETF or LTF
RI parasitologic failure Initial clearance followed by recrudescence after day 7	**Early treatment failure (ETF)** (i) Danger signs or severe malaria on day 1, 2, or 3 in the presence of parasitaemia (ii) Fever (axillary temperature $\geq 37.5^{o}C$) persists on day 2 and the parasite density is greater than that at enrolment (day 0 parasite density) (iii) Fever and parasitaemia on day 3 (iv) Parasite density on day 3 is $\geq 25\%$ of the day 0 parasite density
RII parasitologic failure Reduction of parasitaemia on day 2 to less than 25% of day 0 parasitaemia, but no complete clearance	
RIII parasitologic failure On day 2, either no reduction of parasitaemia or reduction to a level equal to or greater than 25% of the day 0 parasitaemia	**Late treatment failure (LTF)** (i) Danger signs or severe malaria develop in the presence of parasitaemia on any day from day 4 to day 14 (ii) Fever and parasitaemia on any day from day 4 to day 14, and yet the patient could not be classified as ETF

Table 1.3 Clinical and parasitological classification according to the WHO test protocols.

Early Treatment Failure (ETF):

- Development of danger signs or severe malaria on day 0-3 in the presence of parasitaemia
- Parasitaemia on day 3 with axillary temperature ≥37.5°C
- Parasitaemia on day 2 higher than day 0 count, irrespective of temperature
- Parasitaemia on day 3 ≥25% of count on day 0

Late clinical Failure (LCF):

- Development of danger signs or severe malaria after day 3 in the presence of parasitaemia without previously meeting any of the criteria of ETF
- Presence of parasitaemia with either a measured axillary temperature ≥37.5°C or a history of fever in the last 24 hrs on any day between Day 4 to 14 or 28, without previously meeting any of the criteria of ETF

Late Parasitological Failure (LPF):

- Presence of parasitaemia on any day from day 7 to day14 or 28, and axillary temperature < 37.5°C, without previously meeting any of the criteria of ETF or LCF

Adequate Clinical and Parasitological Response (ACPR):

- Absence of parasitaemia on day 14 or 28, irrespective of axillary temperature, without previously meeting any of the criteria of ETF or LCF or LPF.

Table 1.4 Revised WHO guidelines for assessing the response to treatment (2002)

1.7.6.2 In-vitro tests

In vitro assays are based on the inhibition of the growth and development of malaria parasites by different concentrations of a given drug relative to drug-free controls. The WHO in vitro microtest is based on counting the parasites developing into schizonts (Rieckmann *et al.*, 1978), while the isotopic microtest is based on measurement of the quantity of radiolabeled hypoxanthine, a DNA precursor, incorporated into the parasites (Childs *et al.*, 1988). Drug resistance is identified when parasite growth occurs above a threshold concentration. This threshold is usually defined as the concentration (in nanomoles per litre) at which 50% of parasite growth (geometric mean IC_{50}) (WHO microtest) or the incorporation of 50% hypoxanthine is inhibited (IC_{50}) compared to the drug-free control wells.

There are newer colorimetric tests that can test low parasitaemias taken directly from patients in the field: the parasite lactate dehydrogenase enzymatic assay (Makler and Hinrichs, 1993; Makler *et al.*, 1993), the parasite lactate dehydrogenase double-site

enzyme-linked immunodetection assay (Druilhe *et al.*, 2001) and the histidine-rich protein II assay (Noedl *et al.*, 2002). However, because of the limited amount of data published, it is still unclear whether these new tests will replace the traditional (micro and isotopic) tests.

1.7.6.2.1 Advantages of in-vitro tests:

- They reflect "pure" anti-malarial drug resistance.
- No need for follow-up of the patients and results can be obtained within few days
- They yield quantitative results and identify the phenotype of the parasite independently of the immune and physio-pathological conditions of the host
- Multiple tests can be performed on isolates.
- Several drugs can be assessed simultaneously, and experimental, new drugs can be tested.

1.7.6.2.2 Disadvantages of in-vitro tests

- The correlation of in-vitro response with clinical response in patients is neither clear nor consistent and appears to depend on the level of acquired immunity within the population being tested.
- They provide little information on the efficacy of the pro-drugs, such as Proguanil and chlorproguanil, which require host conversion into active metabolite because metabolism varies considerably among individuals.
- The accuracy of the inhibitory concentrations for a given sample is influenced by several factors such as the in-vitro test conditions, the presence of mixed resistant and sensitive parasite populations in the same sample, and humoral factors from the donor that can interfere with parasite maturation (Wellems and Plowe, 2001).
- Parasites isolated from patients who have taken medication a few days before the test usually do not grow in-vitro.
- There is no consensus about the determination of the threshold IC_{50} that distinguish susceptible from resistant parasites, and there are currently no fully validated cut-off points for assessing in vitro resistance (Ringwald and Basco, 1999)

- In-vitro tests are technologically more demanding, they require highly skilled personnel and laboratory equipment. They are relatively expensive.

1.7.6.3 Molecular techniques:

Molecular tests use Polymerase Chain Reaction (PCR) to indicate the presence of mutations encoding biological resistance to anti-malarial drugs. Theoretically, the frequency of the occurrence of specific gene mutations within a sample of parasites from a given area could provide an indication of the frequency of drug resistance in that area analogous to information derived from *in-vitro* methods (WHO, 2001b).

1.7.6.3.1 The use of molecular markers to detect and predict anti malarial drug resistance

Despite the difficulties encountered in demonstrating the correlation between molecular markers and clinical treatment outcomes, molecular markers that can predict resistance to SP (Kublin *et al.*, 2002) and chloroquine (Djimde *et al.*, 2001a) are now available with reasonable reliability. In the case of chloroquine, *pfcrt* 76T provides a single marker for chloroquine resistance. For SP resistance, there is growing evidence that the *dhfr* triple mutations with or without the *dhps* mutations can predict SP treatment failure. The triple (*dhfr* Asn-108, Ile-51, and Arg-59) mutant genotype was associated with SP treatment failure (Mutabingwa *et al.*, 2001a); (Nzila *et al.*, 2000a). However, a study in Malawi observed that the quintuple mutant genotype (*dhfr* Asn-108, Ile-51, and Arg-59 plus *dhps* Gly-437 and Glu-540) was more strongly associated with SP treatment failure than was the *dhfr* triple mutant (Kublin *et al.*, 2002). Two mutations alone, *dhfr* Arg-59 and *dhps* Glu-540, were found to predict the presence of all five (quintple) *dhfr* and *dhps* mutations accurately (Kublin *et al.*, 2002; Kyabayinze *et al.*, 2003).

Prevalence rates of molecular markers for resistance have been found to be higher than the prevalence of in-vivo drug resistance in nearly all studies. Djimde *et al.*, (2001a) have addressed this problem in Mali by calculating ratios between rates of the chloroquine-resistant *pfcrt* genotype and therapeutic and parasitological outcomes. A genotype resistance index (GRI) and genotype failure index (GFI), derived by computing the ratio between the age-adjusted frequency of the *pfcrt*

mutation at position 76 and the prevalence of parasitological and clinical CQ resistance, respectively, have been proposed for the surveillance of CQ resistance. After controlling for age, both GRIs and GFIs ranged from 1.6 to 2.8 at all study sites over the three-year period (Djimde *et al.*, 2001b), indicating that the prevalence of clinical or parasitologic failure was two to three times lower than the prevalence of the gene mutation. In Uganda, a strong positive correlation between the *pfcrt* codon 76 mutant/wild (M/W) ratio and the late stages of high-grade CQ treatment failure (i.e., ETF and RIII parasitologic failure) has been observed (Talisuna *et al.*, 2002). Such a ratio could be useful in estimating high-grade CQ resistance in cases where the prevalence of the *pfcrt* mutation is already high. M/W ratio might be a more robust index because it is not affected by parasite clone multiplicity (Talisuna *et al.*, 2002). Furthermore, the *dhfr* codon 59 M/W ratio, but not the prevalence of infections with the *dhfr* codon 59 mutation, was positively correlated with SP treatment failure in Ugandan sentinel sites (Talisuna *et al.*, 2003)

As a complementary tool, and not as a replacement for in vivo tests, they could be used for large-scale mapping and estimation of parasite resistance. In vivo tests could then be conducted in sites chosen on the basis of the molecular data to estimate more precisely the actual prevalence of resistance (Talisuna *et al.*, 2004a).

1.7.6.3.2 The use of molecular markers to distinguish recrudescence from reinfection in P. falciparum.

Decisions regarding drug policy rely largely on in-vivo studies. In high transmission areas new infections are common after chemotherapy and can be misclassified as treatment failure due to the inability of the in-vivo tests (microscopy) to differentiate parasites resulting from initial infection (recrudescence) from those due to new infections. Molecular genotyping is used to help distinguishing recrudescence (that results from exacerbations of persistent, undetectable parasitaemias in the absence of an exo-erythrocytic cycle) from reinfection (Snounou and Beck 1998). PCR techniques using polymorphic markers such as the merozoite surface proteins (MSP1 and MSP2), glutamate-rich protein (GLURP), or microsatellite markers (Leclerc *et al.*, 2002) and based on the assumption that genetically different parasites at

recruitment and at follow-up indicate a new infection (Ranford-Cartwright *et al.*, 1997) are employed. The diversity of parasite population and multiplicity of infection could also be obtained as a secondary outcome measurement. MSP2 was found to have the highest degree of polymorphism and the analysis of this single gene effectively distinguished recrudescence from reinfection (Snounou *et al.*, 1999; Cattamanchi *et al.*, 2003; Happi *et al.*, 2004). PCR analysis showed that reinfection can occur between day 7 and day 14 after chemotherapy with CQ in one study and SP in another (Irion *et al.*, 1998; Magesa *et al.*, 2001).

Unfortunately PCR techniques have some limitations (Snounou and Beck 1998). Some limitations are inherent to the PCR method like sensitivity and resolution while others are imposed by biological characteristics of the parasite's life cycle, which include sequestration and asynchrony, presence of gametocytes that can give false positive results overestimating the rate of recrudescence, daily differences in the diversity of an infection. Host immunity and the pharmacokinetics of the drug are also important factors to be taken in consideration. Furthermore, for isolates where both new and recrudescent genotypes are present, there is no agreed standard on whether such mixed infections should be classified as new, recrudescent, or undetermined genotypes. However, if sufficient number of consecutive samples are analysed, and the efficiency and resolution power of the PCR are improved meaningful results can be obtained with reasonable confidence.

1.7.6.3.3 Advantages of molecular techniques

- Easy sample collection and storage.
- Only small amount of genetic material is needed, as opposed to live parasites.
- The PCR is a rapid, sensitive test and large numbers of tests can be conducted in a relatively short period of time.
- No need for follow-up.
- No interference of host or environmental factors.
- Screening an infected population for the presence of specific mutations could detect the arrival of resistance and, with regular surveillance, could be used to monitor its prevalence.

1.7.6.3.4 Disadvantages of molecular techniques

- Need of sophisticated equipments and training.
- Confirmation of the association between given mutations and actual drug resistance may be difficult, specially when resistance involves more than one gene locus and multiple mutations.
- Correlation between the prevalence of the molecular marker and in-vivo treatment failure is not consistent in some situations due to effect of some host factors mainly immunity

1.7.6.4 Case reports and passive detection of treatment failure

Less important methods for monitoring anti-malarial drug resistance include case reports or case series of spontaneously reported treatment failures. However, they may be biased because denominators are unknown and rates of resistance cannot be calculated (WHO, 2001b). Passive detection of treatment failure, also, includes treated patients following usual treatment guidelines and told to come back to the clinic or hospital if symptoms persist or return. Those cases, which return, are considered to represent the population of treatment failure. However, data are seriously biased because compliance with treatment regimens cannot be ensured and no attempt to locate and determine the outcome of patients who do not return on their own (WHO, 2001b).

1.7.7 Role Of Immunity In Treatment Response

There is a complex interaction between chemotherapy, drug resistance and immunity (Bjorkman, 1988). Different degrees of immunity will result in different outcome of chemotherapy. Hence parasites which are partly resistant to a treatment may still be radically cured if the parasite clearance is supported enough by the immune system. It was found that the ability to clear resistant parasite is strongly dependent on age, suggesting that host immunity plays a critical role in the clearance of *P. falciparum* infections (Djimde *et al.*, 2003). The failure to clear parasites by the nonimmune individuals is supported by the higher prevalence of infections with mutations linked to CQ resistance or higher CQ treatment failure observed in African children compared to adults (Djimde *et al.*, 2001a; Dorsey *et al.*, 2000; Talisuna *et al.*, 2002).

(Cravo *et al.*, 2001) showed that drug resistant parasites might behave as sensitive ones in the presence of partial immunity. They found that, in rodents, partial immunity in the presence of drugs (CQ and MQ) can reduce the growth of the drug resistant clone more significantly than in the absence of the drugs. These findings have important implications for vaccine development and antimalarial drug policy. In semi-immune populations, suboptimal vaccines may have value when combined with antimalarial chemotherapy to clear resistant parasites and control disease level (Cravo *et al.*, 2001).

1.7.8 Predictors Of Chloroquine Treatment Failure

Despite the spread of chloroquine resistance in most of the tropical countries, chloroquine continues to be used as a first-line drug for uncomplicated malaria. Arguing against the abandonment of chloroquine as a first-line drug are observations that many patients do respond symptomatically to chloroquine despite failure to clear parasites from the blood (Brandling-Bennett *et al.*, 1988; Khoromana *et al.*, 1986), the appreciation that chloroquine resistance rate may vary considerably within a country (Bloland *et al.*, 1998), a lack of ideal alternatives to chloroquine, and the realization that increased use of other agents will lead to increased resistance to these drugs. Indeed, all available chloroquine replacements are currently limited by resistance, high cost and/or toxicity.

Accordingly, in many areas, chloroquine is still in use despite the presence of some resistance to it. One strategy for extending the period of chloroquine use is to consider host-related characteristics predictive of clinical response when making therapeutic decisions. Using this strategy, patients who are predicted to have a low risk of chloroquine failure can continue to receive this drug, while high-risk patients can be given alternative therapies. Although, in rural areas where unsupervised home management is prevailing, this cannot be ensured.

Few studies have considered host-related factors as predictors of antimalarial drug resistance. In a study of asymptomatic children in the Solomon Islands, young age, high parasite density, normal spleen size, malnutrition, and the presence of gametocytes were found to be independent risk factors for chloroquine resistance

(Hess *et al.*, 1996). In another study of symptomatic children and adults in Kampala, Uganda, three easily identifiable host-related factors—young age, high temperature (>38˚C) at presentation and recent chloroquine use were independent predictors of chloroquine treatment failure (Dorsey *et al.*, 2000).

Studies of simple predictors may provide information about a rational method of stratifying patients into those for whom chloroquine remains an appropriate treatment for uncomplicated malaria and those for whom alternative treatment should be used.

1.7.9 Public Health Impact Of Antimalarial Drug Resistance

Each year up to an estimated 3 million people die from malaria and 500 million to 5 billion clinical episodes of malaria warranting anti-malaria treatment occur throughout the world (Breman *et al.*, 2004; Snow *et al.*, 2005). Secondary effects of malaria such as anaemia, low birth weight, growth retardation, undernutrition, neuro-cognitive impairment, co-infection effects through immunosuppression and other co-morbidities such as adverse drug events following treatment should also be taken in consideration when analysing the impact of drug resistant malaria.

The primary impact of anti-malarial resistance is on the clinical and epidemiological manifestations of malaria but drug resistance also has social and economic implications and increases the burden on the health systems.

1.7.9.1 Epidemiological impact

The widespread increase in drug resistance has been shown to increase the transmission and morbidity in low immunity population in areas with low or unstable transmission, it may result in a relatively rapid increase in parasite biomass and malaria transmission resulting in malaria epidemics with serious morbidity and mortality due to generally low immune status in the population (Warsame *et al.*, 1995; Shanks *et al.*, 2000; Lindsay *et al.*, 2000)

1.7.9.2 Clinical impact (morbidity and mortality)

Increasing drug resistance can result in an increase in fever episodes and persistent parasitaemia without haematological recovery (Bloland *et al.*, 1998; Bloland *et al.*,

1993; Ekvall *et al.*, 1998). This can increase the risk of progression to severe and even fatal anaemia. However, treatment of severe malaria is also affected clinically by drug resistance. Declining efficacy of quinine in Thailand resulted in prolonged parasite and fever clearance and coma recovery time but not in an apparent increased mortality (Newton *et al.*, 2003).

The risk of malaria death in children under 5 years of age has been increased many folds after the emergence and spread of chloroquine resistance. For example, in east and southern Africa, the under five malaria mortality has increased from 6.5 per 1000 child-years between 1982 and 1989 to 11.9 per 1000 child-years between 1990 and 1998 (Korenromp *et al.*, 2003). On the contrary, in West Africa, where the spread of chloroquine resistance is "lesser and later" there has been no absolute increase in the malaria mortality over time (Korenromp *et al.*, 2003) supporting that chloroquine resistance is a major cause of *P. falciparum* related mortality. In Mlomp, Senegal, after the emergence and spread of chloroquine resistance, the risk of malaria death in children under 5 years of age increased over 11-fold (Trape *et al.*, 1998; Trape, 2001). Increases in malaria case fatality rates have reported in Kinshasa and Brazzaville, Congo (Greenberg *et al.*, 1989; Carme *et al.*, 1992a), Malawi, Uganda, Tanzania and Nigeria (Khoromana *et al.*, 1986; Talisuna *et al.*, 2004b; Asindi *et al.*, 1993). The extra mortality coincides with the detection and spread of chloroquine-resistant *P. falciparum* in 1980s in sub-Saharan Africa. In Kenya, an inverse relationship was observed between effective anti-malarial drug and case fatality (Zucker *et al.*, 2003).

Other possible factors for increasing the mortality burden of malaria in sub-Saharan Africa include the breakdown of health systems (Korenromp *et al.*, 2003) and control programmes (Sharp *et al.*, 2002). Emergence of HIV/AIDS (Nwanyanwu *et al.*, 1997), as well as, climatic and environmental changes (Mouchet *et al.*, 1998; Loevinsohn, 1994).

1.7.9.3 Drug resistant malaria as an indirect cause for increased morbidity and mortality

A number of indirect negative effects of malaria on health and development are increasingly being recognized such as impaired cognitive functions and increased

susceptibility to other diseases e.g., HIV. This suggests that the disease burden is greater than what has been previously been estimated in World Health Reports.

In Africa it is estimated that every year at least 1300–7800 children will have neurological sequelae following cerebral malaria (Mung'Ala-Odera *et al.*, 2004). In addition, increases in severe episodes of childhood anaemia due to malaria will result in an increased transmission risk of HIV and other blood borne pathogens following blood transfusions (Ekvall, 2003; Obonyo *et al.*, 1998). Recently, malaria chemoprophylaxis was found to reduce crude under-five mortality from 30–50% (Geerligs *et al.*, 2003), which may suggest that malaria may be responsible for half the deaths in highly endemic areas.

1.7.9.4 Impact of drug resistance on health systems

Anti-malarial drug resistance also represents a major challenge for the health services. Increased malaria transmission and therapeutic failures have increased outpatient attendance and proportional malaria admission rates have more than doubled due to severe malaria in many parts of Africa (Trape, 2001; Greenberg *et al.*, 1989; Carme *et al.*, 1992b). Higher costs of new combination treatment regimens as well as prevention of resistance by reducing drug use implies more specific diagnosis. Clearly, a strengthening and a better understanding of how to reinforce case management at primary health care level are critical and urgently required. The increased inpatient load may also deplete available stocks of parenteral fluids and other logistics support including anti-malarials and other drugs.

The impact of drug resistant malaria on the health systems is likely to be particularly manifest in epidemic prone areas with unstable transmission where facilities may not be adapted and resources may not be allocated to respond to the sudden increase in treatment needs for malaria patients especially those with severe manifestations (Kiszewski and Teklehaimanot, 2004).

1.8 MALARIA IN YEMEN

Yemen is one of 14 countries in the WHO Eastern Mediterranean Region (EMR) where malaria is endemic (**Figure1.15**). Malaria is one of the top priority health problems in the Republic of Yemen. Out of a total population of 18 million, about 60% are at risk of malaria. The recorded malaria cases range from 1,500,000 to 1,800,000 annually, with an estimated mortality rate of 1% of the recorded cases, mainly among children below the age of five and pregnant women (Khalifa, 2000).

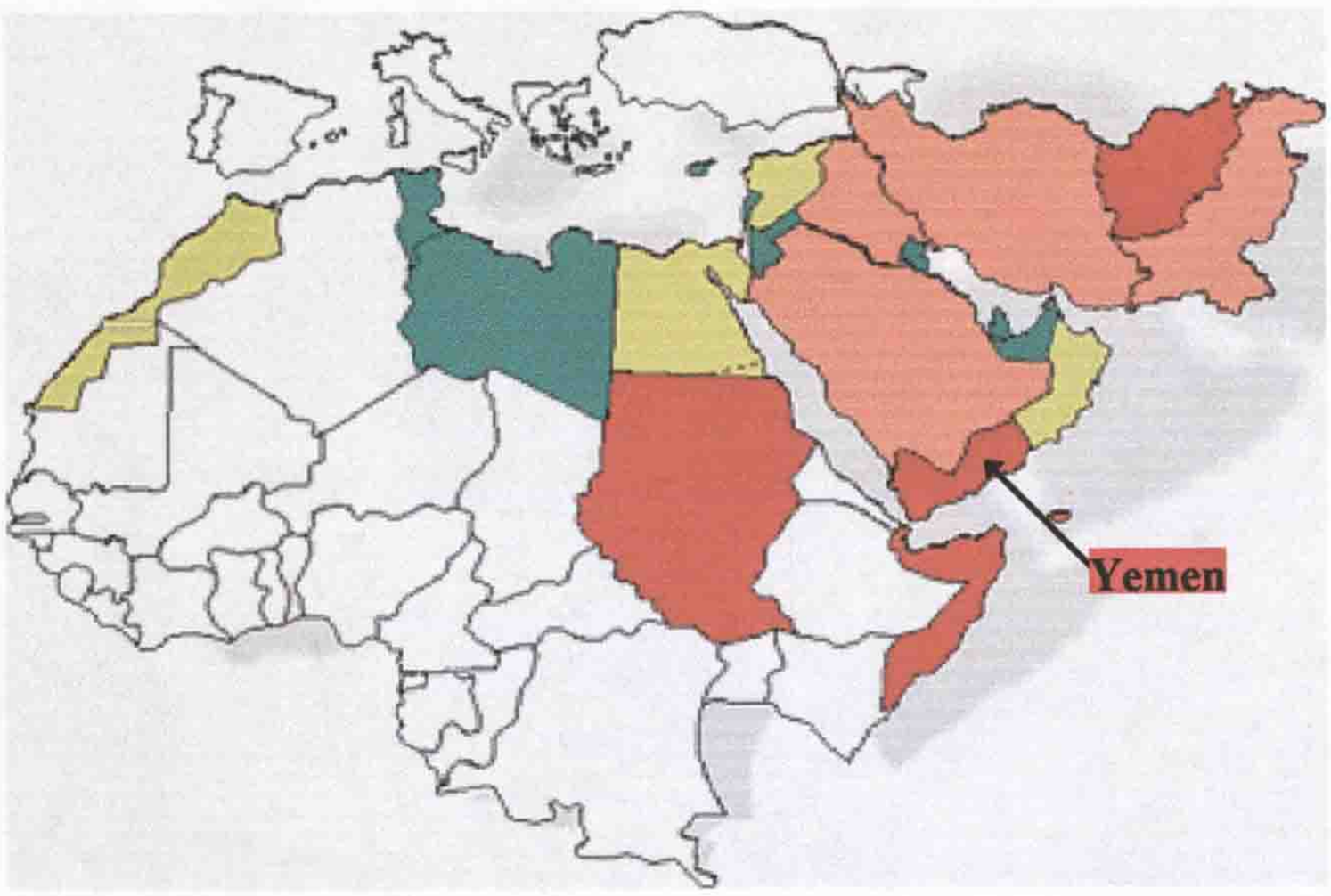


Figure 1.15 Epidemiological categorization of countries in Eastern Mediterranean Region (EMR). Countries in EMR are categorized into four groups according to the epidemiological situation and status in relation to malaria control. Group 1 (green): countries that achieved interruption of malaria transmission, group 2 (yellow): countries where malaria is firmly under control and are targeting the eradication, group 3 (orange): countries with a moderate endemicity and relatively well-established control programmes and group 4 (red): countries with a severe malaria problem and/or threatened by epidemics and complex situations including Yemen (source: *http://www.emro.who.int/rbm/Epidemiology-categorization.htm)*

According to the epidemiological situation and status in relation to malaria control, Yemen is one of five Eastern Mediterranean countries classified as having severe malaria problem (**Figure 1.15**). Within this group the Sudan has the highest estimated number of malaria cases per year (7.5 million), Yemen comes next (3

million) followed by Afghanistan (2.5 million), Somalia (2 million) and Djibouti (800.000) (WHO, EMRO, 2002)

1.8.1 Epidemiology Of Malaria In Yemen

Malaria in Yemen is classified epidemiologically as afrotropical except in the island of Socotra where it belongs to the oriental type (Roll Back Malaria Program, Yemen, 2000). *Plasmodium falciparum* is the predominant species of malaria parasite accounting for about 90-95% and *Anopheles arabiensis* as the predominant vector, other less potent vectors are present namely *An. culicifacies, An. fluviatilis* and *An. sergentii* (Roll Back Malaria Program, Yemen, 2000).

Yemen Republic has been divided into four epidemiological regions (Mount, 1953); (Thuriaux, 1971)

1 The costal plain (Tihama); from sea level to 200m elevation.
2 The foothills and middle altitude; from 200m to 2000m elevation.
3 The central highland plateaux; from 2000m and above elevation.
4 The arid slops; from the plateaux to the desert.

The endemicity is not the same all over the country. It is meso to hyperendemic along the costal belt extending from Yemeni-Omani border in the east to Bab-al-mandab in the west along the Indian ocean and from Bab-al–Mandab to the Yemeni Saudi border in the north along the Red Sea where the season of transmission is mainly during the winter (from October to March). In the foothills and middle altitudes, it is meso or hyperendemic and the transmission is all the year round or following the rainy season that is mainly during summer with epidemics of malaria re-occurring after heavy rain changes. It is almost malaria free in some parts of the country like the mountainous areas and the deserts, but malaria transmission and even malaria epidemics are expected following heavy climatic and rain changes (Khalifa, 2000).

The annual parasite incidence (API) for the whole country is about 30 per thousand reaching to very high rates up to 130 or above in some governorates. The slide positivity rate (SPR) and the spleen rate (SR) reached up to 85% and 100%

respectively in some governorates according to some recent malariometric school surveys conducted in November 1998 (Roll Back Malaria Program, Yemen, 2000).

1.8.2 Anophleine Mosquitoes Of Yemen Republic

Fifteen anopheline species were recorded by different workers from Yemen Republic belong to three zoogeographical regions namely, the Afrotropical (Ethiopian), the Palaearctic and the oriental region. The following relation was suggested for the four geographical zones:

In the coastal plains, *An. arabiensis* and *An. culicifacies* are the known vectors. Their breeding places are practically all man-made habitat. In settlements located close to the foothills along the dry beds of streams, *An. arabiensis* is brought down by the sudden surges of floodwater from the mountains (September-November) and is intensively breeding in the short-lived pools formed in the riverbeds. The construction of an irrigation system in some parts of this area has worsened the malaria situation. In the foothills and middle altitudes, *An. arabiensis* is the major vector. It is responsible for meso-endemic malaria in the valleys of the middle heights, where ecological conditions favour perennial malaria transmission. Malaria is hypo endemic and seasonal in the low foothills area, where most of streams dry up during the winter and spring months. The malaria transmission in the low foothills area and the peak of malaria transmission in the middle heights coincide with the late summer and beginning of autumn. However, *An. arabiensis* is not the only vector in the area, since at altitudes above 1500m, where it is extremely rare, a few small malaria foci were detected. The other anopheline species, which, by analogy with the neighbouring countries, could be considered as vectors are *An. sergenti* and *An. fluviatilis*.

Repeated findings of *An. sergenti* in the highland plateaux with reports of malaria cases and its presence in substantial number during malaria outbreaks strongly indicate its possible involvement in malaria transmission. In the arid slopes from plateaux to Arabian Desert, the malaria prevalence was comparatively higher in the area with *An. arabiensis* than in the area with *An. sergenti*.

1.8.3 History Of Malaria Vector Control In Yemen

Vector control activities were started in 1940 by establishing a Malaria Unit in Abyan governorate, followed by a Malaria Control Programme in Aden governorate (1969) and in Hodeidah governorate (1978). Control measures were mainly based on DDT spraying, and sanitation. In the late 1970s and 1980s effective control measures (DDT spraying, sanitation and larviciding using Temephos in high risk areas) reduced malaria transmission and caused a significant decrease in malaria morbidity and consequent loss of anti-malaria immunity by the population. Clinical cases of malaria remain scanty throughout this period.

However, in the 1990's control over the disease suffered serious setbacks, mostly due to breakdown of organized vector control activities, larviciding programme using Temephos was, only, limited to 5 Wadies (valleys) in Hodeidah governorate, mainly due to financial limitations. During the period 1991-2000 all control activities were practically stopped particularly in rural areas. Malaria and its vectors progressed, invaded new territories and consolidated positions. This caused an increase in malaria incidence and some local epidemics. By late 1990's, malaria in some governorates had almost reached a saturation point and fatalities from the disease had been increased significantly.

The preparatory phase of RBM in Yemen was started in the year 2000. One of the 8 main strategic directions of the RBM was the selective integrated vector control through multiple and sustainable preventive measures. Currently the vector control activities available in Yemen include the larviciding activities with identification of breeding sites in target areas and the use of Temphos (Abate) as the insecticide of choice, indoor residual spraying with Lambda-cyhalothrin (Icon) as the main vector *An. arabiensis* is susceptible to it, space spraying where Fogging and ULV space spraying with Lambda-cyhalothrin and Deltamethrin are carried out in outbreak events.

Introduction of Impregnated bed nets ITNs can be seen as the most effective and sustainable tool against vector born diseases in Yemen. Its introduction requires time and effort to educate and mobilize communities for its acceptance and proper use. Iconnet is used for impregnation. Biological control using the larvivorous fish

Aphanius dispar is under study although it has been implemented on small scales in Tihama areas and Socotra Island.

1.8.4 Malaria Treatment And The Emergence Of Chloroquine-Resistant Malaria In Yemen

Early, correct diagnosis and prompt, proper treatment are essential for any malaria control program. However, the effectiveness of treatment and of efforts to prevent falciparum malaria is being limited by the development of drug-resistant strains of *P. falciparum* that are expected to have been introduced in the country during the last decade through population movement and the influx of refugees from some African countries (Somalis, Ethiopians, Eritrians) due to political conflicts (Roll Back Malaria Program, Yemen, 2000). According to the national anti-malarial drug policy, chloroquine is the first-line drug for acute uncomplicated *falciparum* malaria in Yemen, while sulfadoxine/pyrimethamine is the second-line treatment and mefloquine is the third-line. Intra venous Quinine is used for the treatment of severe and complicated malaria. A number of other drugs are used clinically including halofantrine, coartem and doxycycline.

Between 1986 and 2002 14 *in-vivo* sensitivity studies were conducted in 5 governorates (Hoeida, Lahj, Taiz, Abyan and Hadramawt) and Socotra Island (National Malaria control Program, Yemen, 2002). Out of these studies, 11 were 7-day tests and 3 were 28-day tests. The results of the 28 *in-vivo* test studies in Taiz (1987), Hodeida (1991) and Lahj (1995) were 3% early RI/RII, 17% early RI/RII and 12.9% early RI/RII respectively. Two *in-vitro* sensitivity studies were conducted in Lahj (1988) and Hodieda and Taiz (1989). In the first study (in Lahj), 15 patients were enrolled out of which no resistant isolates were detected. In the second study (in Hodieda and Taiz), 7 resistant isolates were detected; 6 in Hodieda and 1 in Taiz.

1.8.5 The National Malaria Control Programme In Yemen

There is a very strong political commitment to roll back malaria and the National Malaria Control Programme has practically started in 2001 with the ultimate goal to reduce the incidence of malaria in the country by 50% by the year of 2010. Initially the programme focused on high-risk areas including the Tihama coastal belt, selected districts in foothills and mountainous areas and Socotra Island. The "*National*

Strategic Plan For Roll Back Malaria In The Republic Of Yemen" was prepared and issued in 2002 with strategic directions including development of human resource, early and correct diagnosis followed by prompt and effective treatment of malaria cases including severe cases of malaria, selective integrated vector control, prevention of malaria in pregnancy, efficient forecasting and controlling malaria outbreaks, strengthening the information system and surveillance, increasing the capability of the community to recognize, prevent and control malaria. This includes the initiation of "Home management" in selective remote areas, where there is a problem with accessibility to disease management, and the development of the capacity to plan and implement operational field research and to utilize results in programme intervention.

According to the National Malaria Control Program, (2005), four sentinel sites were selected and established between 2002 and 2004 with the intention of establishing two additional sites in 2006 (Brom, and Madarba) (**Figure 1.16**). Selection of these sentinel sites was based on some epidemiological criteria, such as, high endemicity, predominance of *P.falciparum* specie, different transmission seasons, different geographical locations, different climatic changes, bordering sites with Saudi Arabia and areas closest to refugee camps.

Eight antimalarial drug efficacy studies (including this study) have been conducted in the four established sentinel sites in the period between 2002 and 2004. Chloroquine, SP and AQ monotherapy efficacy studies were conducted in Al-Musaimeer where treatment failure was 61% for CQ, 5% for SP and 43% for AQ. In Al-Odein sentinel site, CQ, SP and AQ+AS efficacy studies were conducted where treatment failure was 39% for CQ, no treatment failures were detected in the SP test and 14% treatment failures were detected in the AQ+AS test. The efficacy of chloroquine monotherapy was also studied in Bajil in the Tihama region and the study resulted in 42% treatment failure. The efficacy of SP was also studied in 2004 in Harad bordering Saudi Arabia and no treatment failures were detected in this study.

However, challenges remain; (i) the capacity of the NMCP is still limited, (ii) the diagnosis of malaria is still based primarily on clinical signs, (iii) surveillance needs to be strengthened and, (iv) national treatment guidelines need updating in view of

resistance to CQ, and should be actively promoted to improve compliance by physicians.

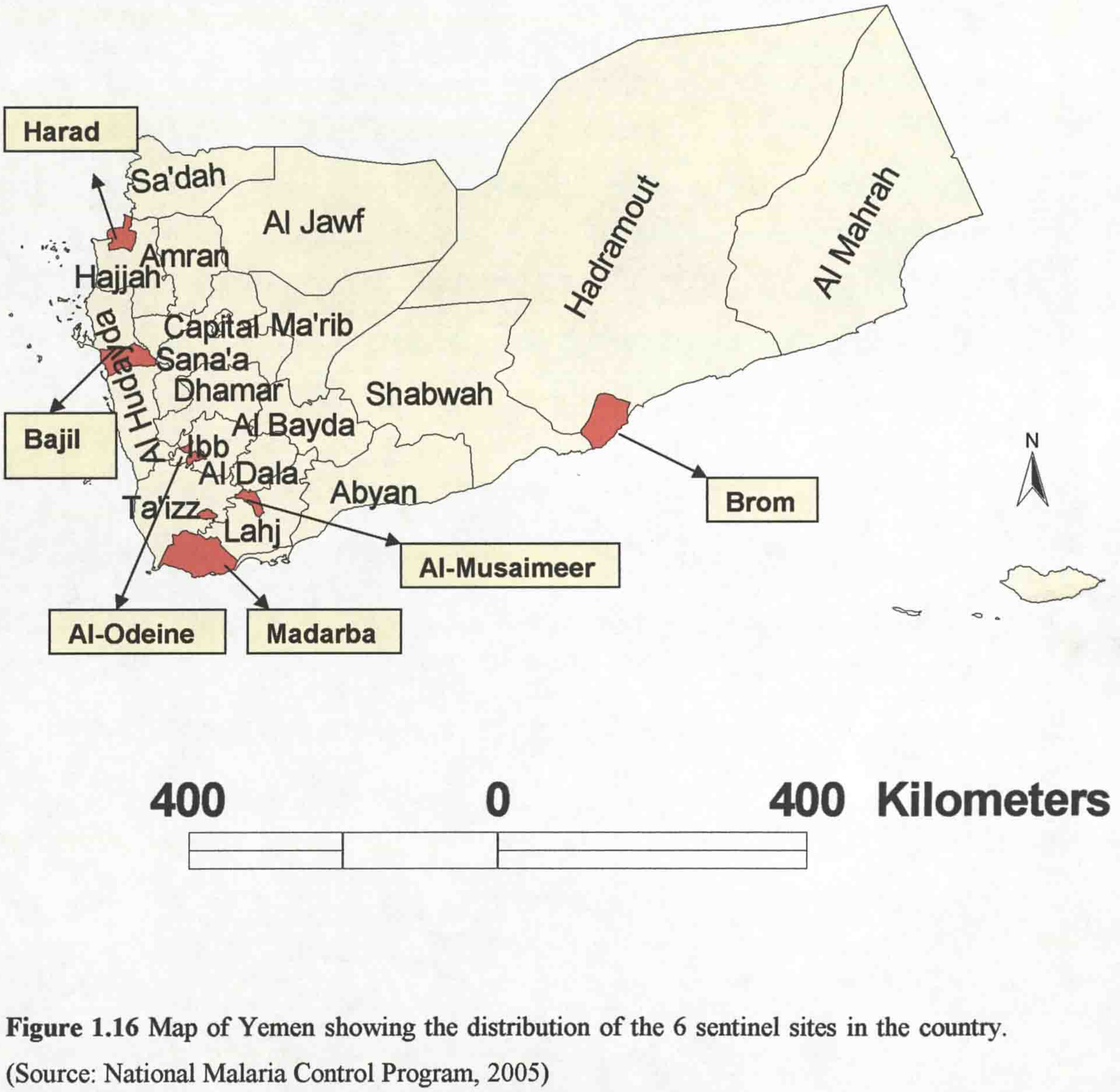


Figure 1.16 Map of Yemen showing the distribution of the 6 sentinel sites in the country.
(Source: National Malaria Control Program, 2005)

1.9 OBJECTIVES OF THE STUDY

This study was conducted with a general aim of establishing a practical system for continuous monitoring of therapeutic efficacy of antimalarial drugs in order to provide essential data for formulation and updating of antimalarial drug policy in Yemen.

Specific objectives of the study are:

- To determine, *in-vivo*, the prevalence of CQ resistance in *P. falciparum* acute uncomplicated malaria.
- To describe some easily identifiable predictors of CQ treatment failure.
- To formulate recommendations regarding methodology for monitoring therapeutic efficacy in sentinel sites in Yemen.
- To train some of the health workers on the methods for monitoring malaria drug resistance.
- To validate the use of *pfcrt*-K76T as a molecular marker for CQ resistance in Yemen.
- To use the *msp*-2 molecular marker to distinguish recrudescence from reinfection in treatment failure cases.
- To predict sulphadoxine/pyrimethamine resistance using *pfdhfr*-59 and *pfdhps*-540 mutations.
- To sequence the full length of the *pfcrt* gene in representative samples.
- To detect the possible presence of the newly discovered *pfcrt*-S163R mutation in Yemen.

CHAPTER 2

MATERIALS AND METHODS

2.1 IN-VIVO TEST

2.1.1 Study Site

The study was conducted between October 2002 and January 2003 in a rural hospital (Al-Musaimeer rural hospital, **Figure 2.1**) in Al-Musaimeer village (**Figure 2.2**), Al-Musaimeer district, Lahj governorate in the south of Yemen **(Figure 2.3)**.

Figure 2.1 Al-Musaimeer rural hospital

Figure 2.2 Al-Musaimeer village in Al-Musaimeer district

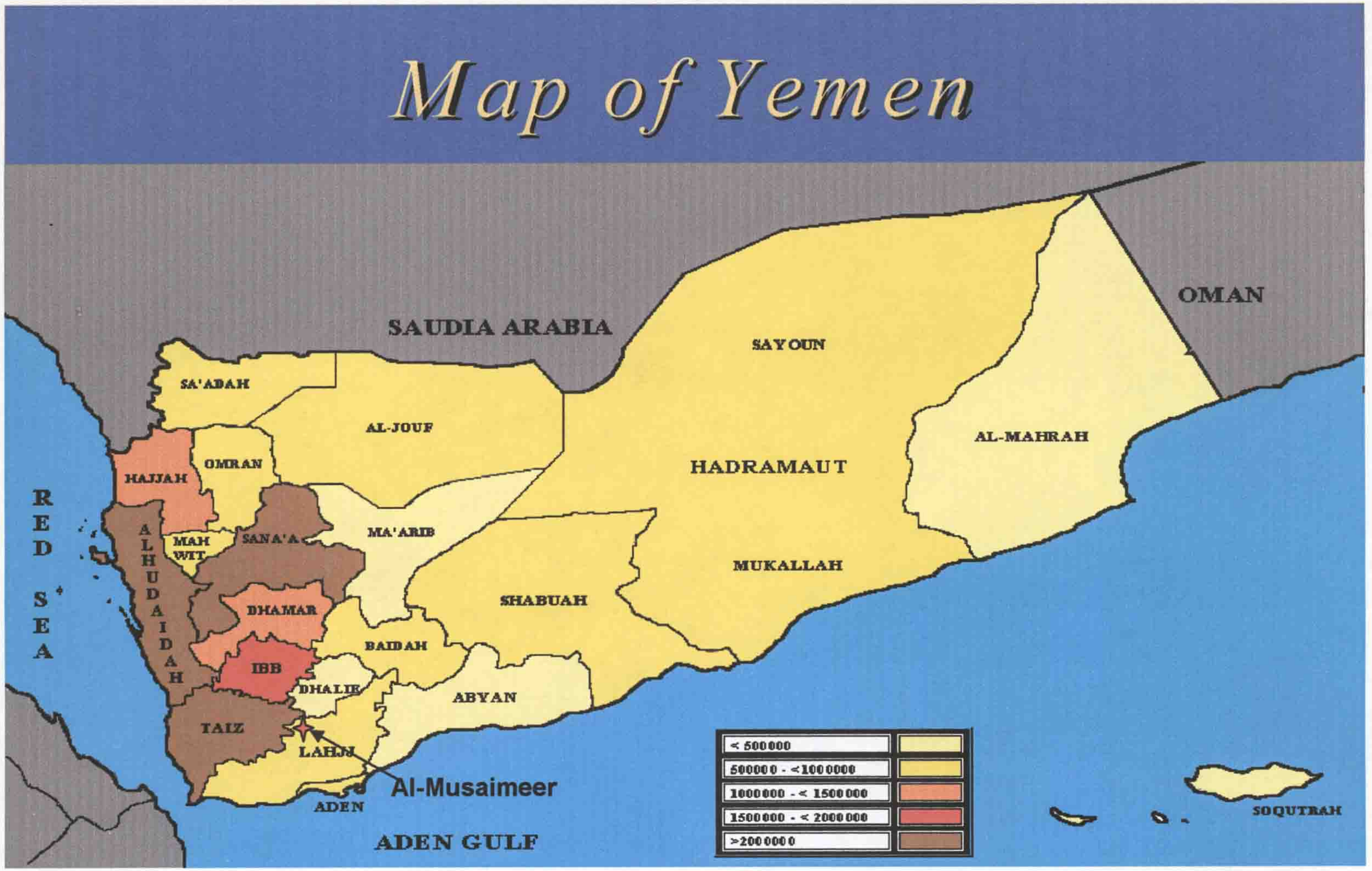


Figure 2.3 Map of Yemen. Lahj Govenorate (Yellow) is in the south of Yemen. The black arrow is pointing to Al-Musaimeer district (red star) (Adapted from: National Malaria Control Program, Yemen, 2005).

Malaria in the area is endemic and seasonal with the peak transmission season between October and March. The predominant malaria species in the area is *P. falciparum* and the predominant vector is *An. arabiensis*.

Note: The information about the area described below is obtained directly from people living there and through personal communication with Dr. Aref Naji (director of Al-Musaimeer Rural Hospital).

The area is rural and agricultural in nature where mango, lemon, banana, cereals and Qat (green leaves chewed by people in Yemen) are cultured. The surface area is about 900 Km^2, most of which are mountainous with intervening valleys that are used by the inhabitants for agriculture. The area is characterised by two main climatic seasons, the hot season, which may extend from May to August and during which the rain falls, and the cold season extending from September to April during which diarrhoeal cases, as well as, malaria cases are observed to be higher especially among children.

The population density in the area is about 30,000 and most of the inhabitants are working as farmers in their own fields. There is one rural hospital and 7 health centres in the area, however, they are not able to provide the required health care due to deficiency in supplies and equipment. Due to this deficiency in health care, in addition to the poverty of the people and the low level of education, some diseases including leishmaniasis, schistosomiasis, respiratory tract infections, tuberculosis, and diarrhoeal diseases in addition to malaria are prevalent in the area.

Proper water and electricity supplies are not available to all people in the area. There are three dams in the area where rain is collected in the hot season to be used for agriculture. This, together with the stagnant water of some open channels used for agriculture, provides a suitable breeding site for mosquitoes. There is also a valley in the area, which is considered as the major source of transmission (**Figure 2.4**). The area is one of the selected sentinel sites for monitoring anti-malarial drug resistance.

Figure 2. 4 Al-Musaimeer Valley, the major source of transmission

2.1.2 Study Subjects

All febrile patients coming to the hospital during the study period were screened for parasitaemia and were subjected to a pre-treatment examination. If the patient met all the inclusion criteria (see below), showed no danger signs or signs of severe malaria **(Appendix 2)** and informed consent **(Appendix 3)** had been obtained from the patient or the guardian, the patient qualified for enrolment in the test. Consent was obtained from patients or guardians after verbal explanation of the objectives and procedures of the study in a simple lay language.

2.1.2.1 Inclusion criteria

- Age >6 months
- Positive *P.falciparum* malaria parasite mono infection and parasite density of 1000-200,000 parasite/µl of blood (as long as, no danger signs encountered).
- Axillary temperature ≥37.5°C or a history of fever during the last 24 hours.
- Ability to take oral medication and no history of intolerance to chloroquine.
- Ability to come for follow-up visits and easy access to the health facility.
- Informed consent by the patient or their guardians for children.

2.1.2.2 Exclusion criteria

- Danger signs, severe malaria
- Severe malnutrition
- Pregnancy
- Concomitant other febrile illness that can interfere with the clear classification of the outcome.

***Note**: A history of previous anti-malarial drug use is not an exclusion criterion. As prior anti-malarial treatment is the rule rather than the exception, the restriction of the test to previously untreated patients would not yield a representative sample. However, the information on previous use was carefully collected and recorded for each patient.*

2.1.2.3 Exclusion criteria developing during follow-up:

- Occurrence of concomitant disease that would interfere with the clear classification of the outcome.
- Occurrence of infection, other than malaria, that requires the administration of medicaments with antimalarial activity, e.g. cotrimoxazole, tetracycline or doxycycline (patients given tetracycline eye ointments should not be excluded).
- Movement of the patient from the study site to a place outside the reach of active follow-up
- Failure to complete treatment due to withdrawal of consent
- Anti-malarial administered by a third party or self medication with antimalarial drugs or antibiotics with antimalarial activity as mentioned above.
- Detection, during follow-up of a mixed infection with another malaria species.
- Persistent vomiting of the treatment.

2.1.3 Sample Size And Sampling Technique:

Consecutive patients presenting to the health centre during the implementation of the study with symptoms suggestive of malaria and positive thick blood smear were enrolled to the study if they satisfied all the inclusion criteria. One hundred and twenty four cases were enrolled in the study. The sample size was calculated using Statcalc (*Epi info* Version 6) with an *expected prevalence* of chloroquine resistance (P) of 50%, a *precision level* of 10% and a *confidence level* of 95%. The sample size required was 96 patients. An *expected follow-up loss* of 10% was added to minimize the possible bias due to it.

2.1.4 Data Collection And Methods Of Measurements

Enrolled patients were given a consecutive number in the record book. Data were entered using special questionnaire forms designed by WHO. All necessary study variables were included in the form **(Appendix 4).**

2.1.4.1 Clinical assessment of cases

To guide the treatment doses and to assess the nutritional status of children, patients were weighed on a reliably calibrated scale **(Figure 2.3)**. Axillary temperature was recorded to one decimal point with a reliable, tested electronic thermometer **(Figure 2.4)**. Patients were also assessed for the presence of severity or danger signs.

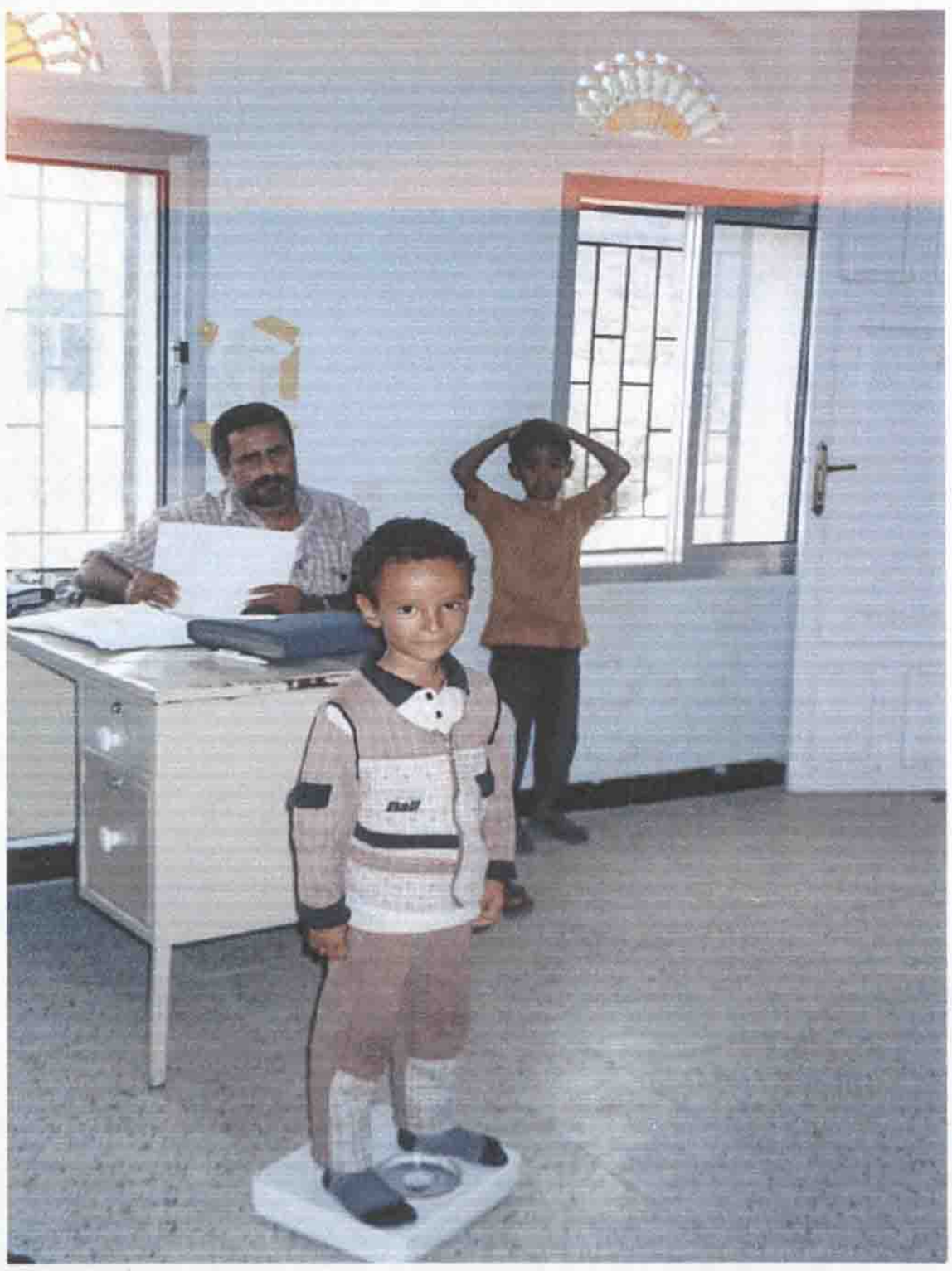


Figure 2.5 Weighing patients to guide treatment dose and nutritional status

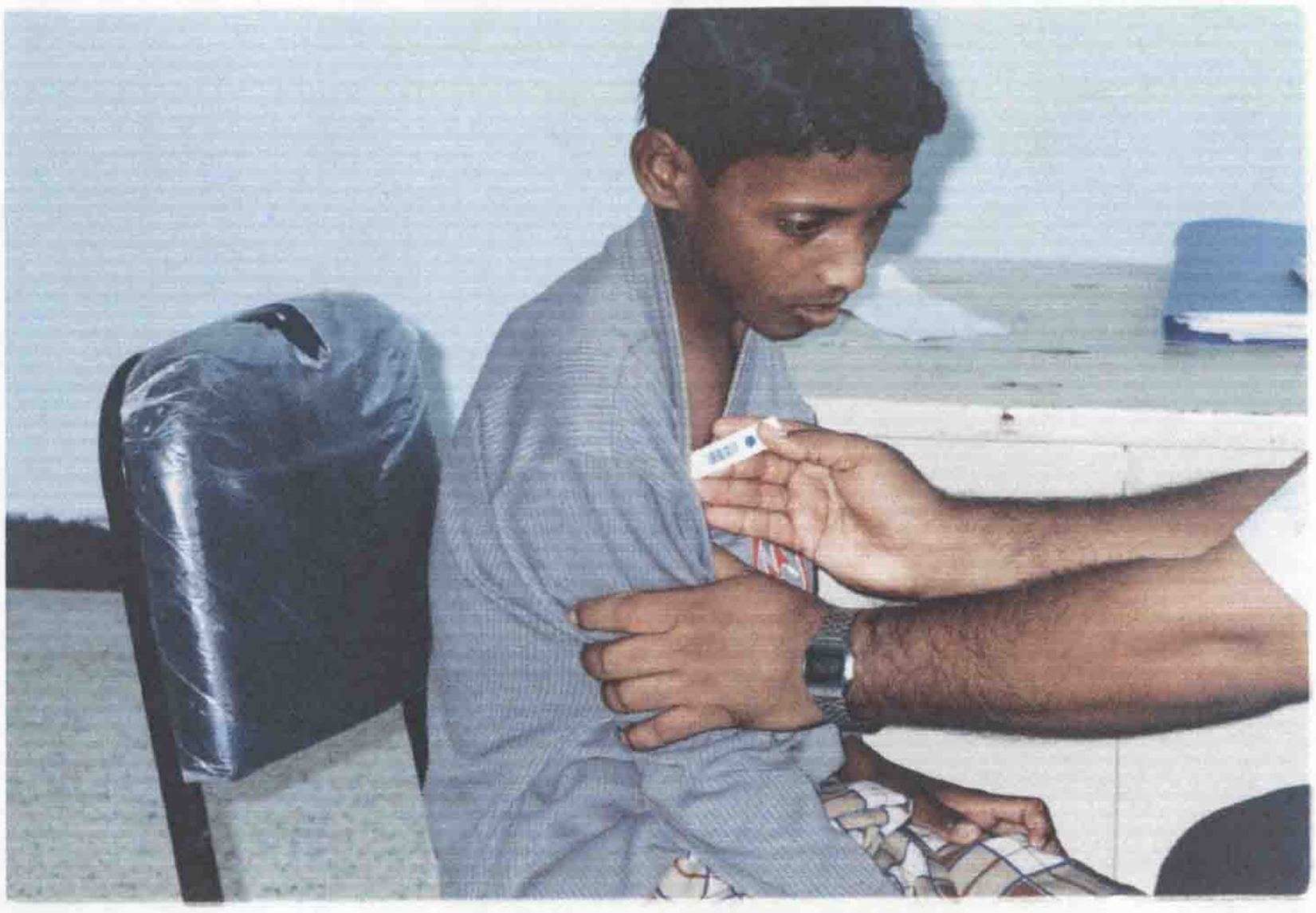


Figure 2.6 Clinical assessment of cases, taking axillary temperature with a reliable tested electronic thermometer

2.1.4.2 Microscopic blood examination

Preparation and staining of the blood slides followed the procedures outlined WHO, (1991). Two slides were always taken, one with a thick film (for rapid staining and screening for parasitaemia while the patient was in attendance), the other with a thick and a thin film on the same slide for subsequent standard staining to calculate parasite density.

Parasitaemia was examined by counting the number of asexual parasites and the number of white blood cells in a limited number of microscopic fields. Adequate parasitaemia for enrolment requires at least 1 parasite for every 6-8 white blood cells corresponding to 1000 asexual parasite/mm3. The parsitaemia per µl is calculated using the formula:

Parasites (per µl) = number of parasites x 8000 / number of leukocytes

A blood slide was announced negative when the examination of 100 thick film fields did not show the presence of asexual forms of *P. falciparum.* The presence of *P. falciparum* gametocytes was noted irrespective of asexual forms, but did not figure in the evaluation of the test.

Figure 2.7 Microscopic assessment of cases

2.1.5 Test Protocol And Follow-Up Procedure:

The standard 28-day *in-vivo* WHO draft protocol for assessment of therapeutic efficacy of antimalarial drugs for uncomplicated *falciparum* malaria, draft September 2002 (WHO, 2001c) was applied with some modifications (**Chapter 3, section 3.2**). WHO provided chloroquine (IDA-HOLLAND, Batch No.1636, expiry date October 2005), which was administered in a total dose, 25mg/kg body weight, under direct supervision, as a three-day course as follows: 10mg/kg in Day 0, 10mg/kg in Day 1 and 5mg/kg in Day 2. During the three days, patients, especially children, were observed for one hour after administration of the drug. If they vomited the drug within 30 minutes of its administration, the drug was re-administered with the same dose, if vomited again the patient was excluded from the study.

Patients were followed on an outpatient basis on Day 0, Day 1, Day 2, Day 3, Day 7, Day 14 and on any other day between the scheduled days in case of any worry (**Table 2.1**). The clinical condition, body temperature, parasitaemia were assessed in each visit.

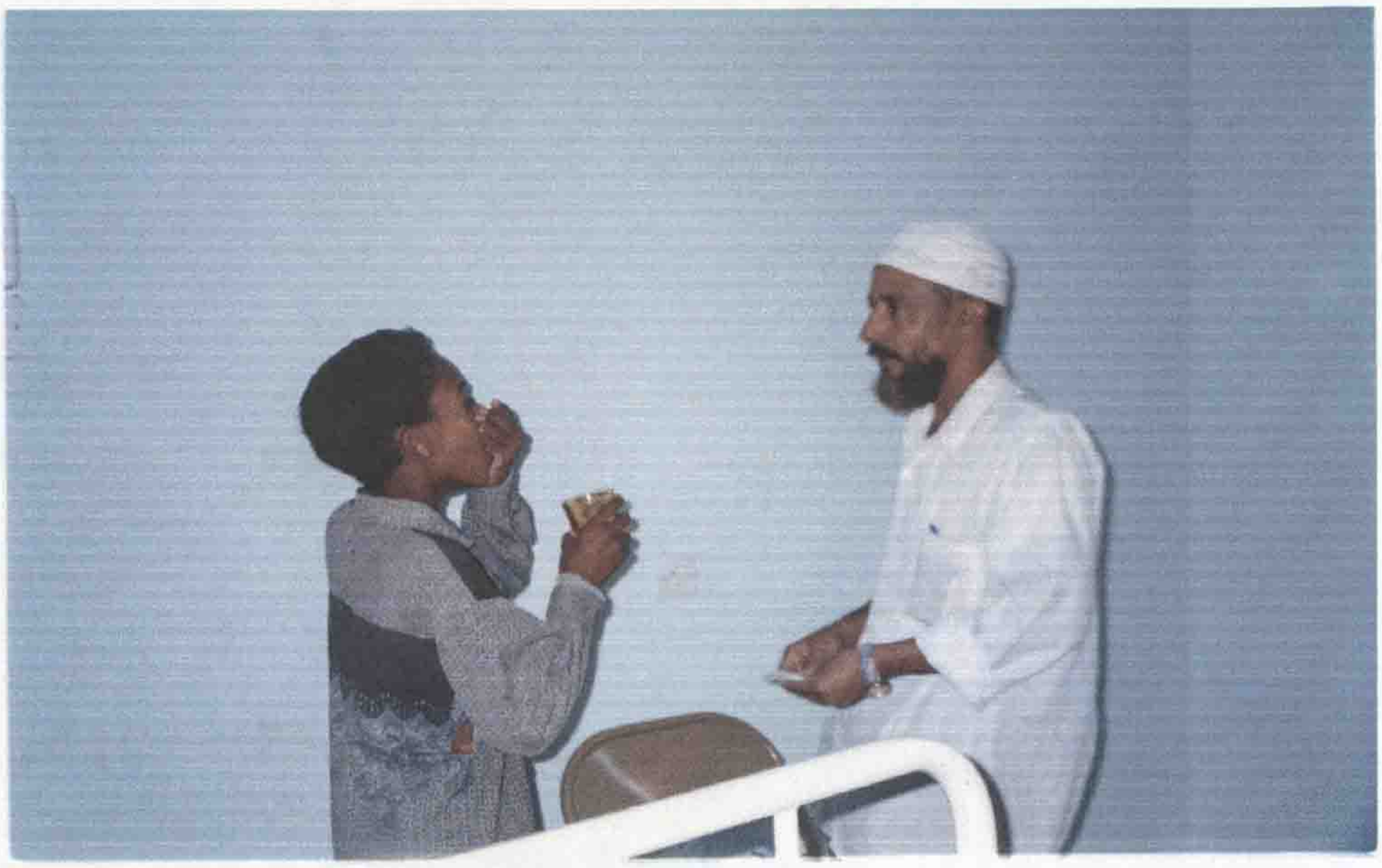


Figure 2.8 Directly supervised treatment

Day-0	Clinical assessment – referral in case of sever malaria/danger signs Measuring axillary temperature Parasitological assessment Informed consent – Enrolment Weighing – Treatment, first dose
Day-1	Clinical assessment – referral in case of sever malaria/danger signs Measuring axillary temperature Parasitological assessment, in case of sever malaria/danger signs second dose or alternative treatment in case of earlytreatment failure
Day-2	Clinical assessment – referral in case of sever malaria/danger signs Measuring axillary temperature Parasitological assessment, in case of sever malaria/danger signs Treatment, third dose or alternative treatment in case of early treatment failure
Day-3, Day-7, and Day-14	Clinical assessment – referral in case of sever malaria/danger signs Measuring axillary temperature Parasitological assessment, in case of sever malaria/danger signs Alternative treatment in case of treatment failure

Table2.1 Basic test schedule

2.1.6 Classification Of Therapeutic Response

The clinical and parasitological responses were c lassified as Early Treatment Failure (ETF), Late Clinical Failure (LCF), Late Parasitological Failure (LPF) and Adequate Clinical and Parasitological Response (ACPR) according to the criteria mentioned in the standard WHO protocol.

***Early Treatment Failure (ETF)*:** defined as

- Development of danger signs or severe malaria on Day 1, Day 2, or Day 3, in the presence of parasitaemia
- Parasitaemia on Day 3 with axillary temperature >37.5ºC
- Parasitaemia on Day 2 higher than Day 0 count
- Parasitaemia on Day 3 ≥25% of count on Day 0

Late Treatment Failure (LTF): divided to 2 sub groups

Late clinical (and parasitological) Failure (LCF): defined as

- Development of danger signs or severe malaria after Day 3 in the presence of parasitaemia
- Presence of parasitaemia with either a measured axillary temperature ≥37.5ºC or a history of fever in the last 24 hrs on any day between Day 4 to Day 14, without previously meeting any of the criteria of ETF

Late Parasitological Failure (LPF): defined as

- Presence of parasitaemia on either of the scheduled return days, 7 or 14, and axillary temperature < 37.5ºC without previously meeting any of the criteria of ETF or LCF

Adequate Clinical and Parasitological Response (ACPR): defined as

- Absence of parasitaemia on Day 14 irrespective of axillary temperature, without previously meeting any of the criteria of ETF or LCF or LPF.

Patients who were classified as a treatment failure were given the recommended dose of the second line treatment (sulfadoxine-pyrimethamine, Fansidar, *Roche*®). Two children developed signs of severe malaria (convulsions) during the follow-up period,

they were given the first dose of parenteral quinine and taken urgently to the appropriate health facility.

2.1 MOLECULAR MARKER ANALYSIS:

2.2.1 Blood Collection And Storage For DNA Extraction

Finger-prick blood was collected from each patient in filter papers on day 0 (before treatment), day 3, day 7 and day 14 or the day of classification in cases of early or late treatment failures (the day of classification represents the end point of the test for each case). Filter papers used were either ordinary Whatman No. 3 filter papers or FTA® Classic Cards (Whatman® BioSience) **(Figure 2.7)**. Each card or filter paper was labelled with the name of the patient, record number, date, and day of collection in relation to treatment (day 0 to day 14). Four spots of blood were taken for each sample. Blood was left to air dry at room temperature.

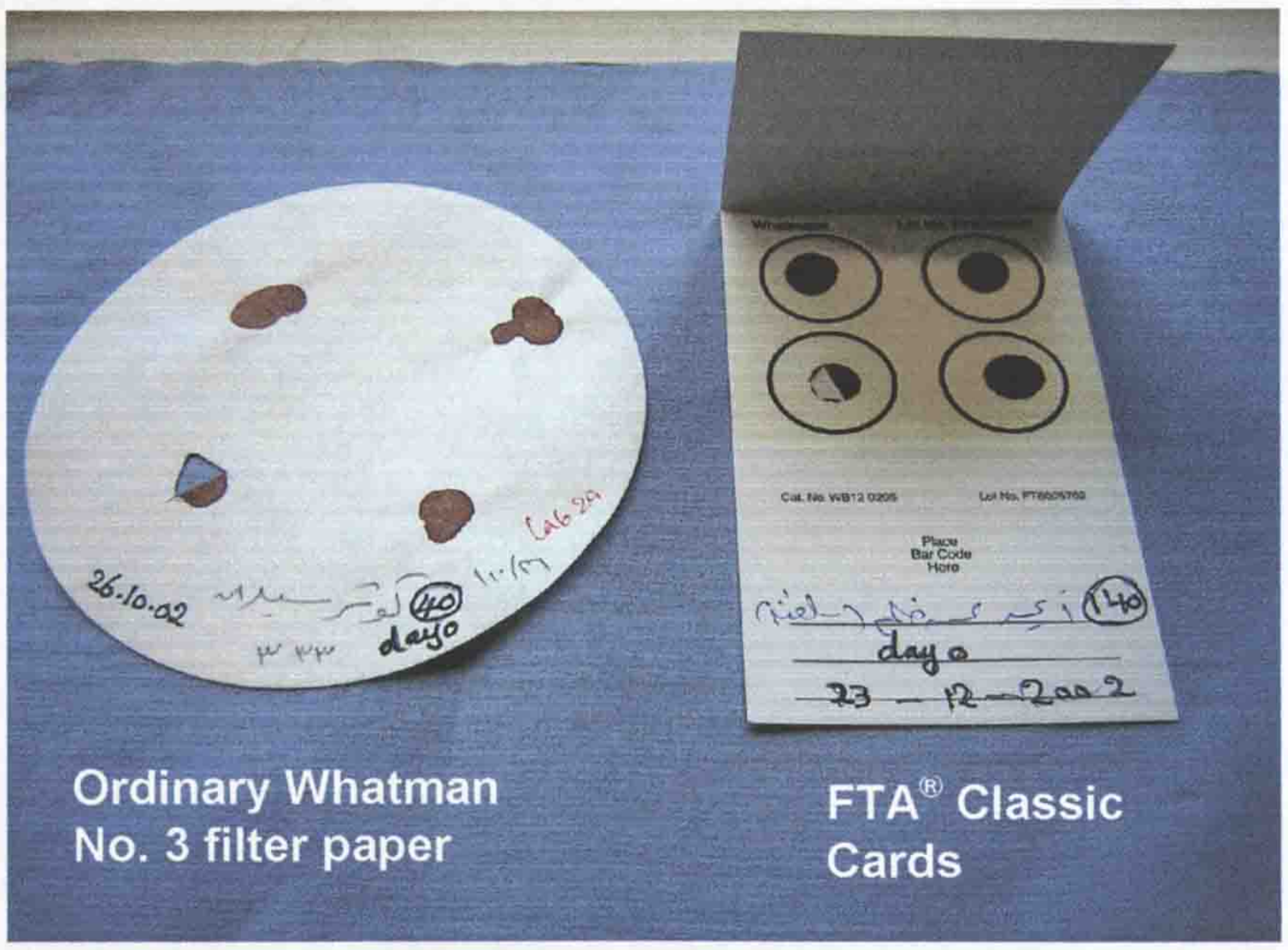


Figure 2.9 Blood samples collected on filter papers

Filter papers with dried blood spots were stored at room temperature in individual, closed plastic bags to prevent contamination between samples. Samples were stored with Silica Gel with humidity indicator (orangegel), MERCK KgaA, Germany for later on use for DNA extraction.

2.2.2 DNA Extraction

During DNA extraction, each sample was given a Laboratory number in addition to the original record number. Three alternative methods for DNA extraction were tested on both types of filter papers (ordinary filter papers and FTA® Cards) to choose the most to choose the most appropriate, convenient, practical, and cost-effective method. The methods were the methanol-fixation heat-extraction method (available at *http://medschool.umaryland.edu/CVD/plowe.html*), the FTA purification method and the QIAGEN kit extraction method (see **Section 2.2.2.1 to Section 2.2.2.3**). The amount of extracted DNA in 1µl was measured using a ND-1000 Spectrophotometer (Nanodrop Technologies).

2.2.2.1 Methanol-fixation/ heat-extraction method

1. A small piece ($4mm^2$) of blood-impregnated filter paper was cut using an individual carbon steel surgical blade (Swann-Morton®) for each sample to prevent contamination between samples.
2. The snippet was transferred to a 1.5 ml microcentrifuge tube and incubated with 50-100µl methanol for 15 minutes at room temperature.
3. After fixation, methanol was poured out while carefully retaining the snippet in the tube. The tube was left open on its side for fifteen minutes to allow the remaining methanol to evaporate.
4. To each tube containing a filter-paper snippet 50-70µl of distilled water was added. The tube was heated for 15 minutes at 95-100^{0}C, vigorously vortexing the tubes every two minutes or so during the incubation.
5. Filter paper snippets were removed from each tube using individual tips. Tubes were then centrifuged briefly in an MSE microcentaur.
6. The extracted DNA solutions were stored at -20^{0}C for later use. 5µl of extracted DNA solution was used in a 25µl primary PCR.

2.2.2.2 DNA extraction using FTA purification reagent

Buffers and reagents used were supplied by the manufacturer (Whatman Cat. No. WB120204) and used according to manufacturer instructions:

1. A sample disc (2mm diameter) from a dried blood spot was taken using a Harris 2.0 mm Micro-Punch™. The disc was transferred to a PCR tube. The tip of the

Harris Micro-Punch was cleaned between samples by taking a punch from blank filter paper.

2. 200µl of FTA Purification Reagent was added to PCR tube.
3. The mixture is incubated for 5 minutes at room temperature.
4. After incubation FTA Reagent was discarded using a pipette.
5. Steps 2-4 were repeated for a total of 3 washes with FTA Purification Reagent.
6. 200µl of TE Buffer (10 mM Tris-Hcl, 0.1 mM EDTA, pH 8.0) was added to the tube.
7. Mixture was incubated for 5 minutes at room temperature.
8. After incubation TE Buffer was discarded using a pipette.
9. Steps 6-8 were repeated for a total of 2 washes with TE Buffer.
10. The disc was allowed to dry at room temperature for about one hour before performing PCR.
11. The washed and dry disc was used as a template in the PCR reaction (the disc was included in the reaction). PCR Reagents were added directly to the disc in the PCR tube.

2.2.2.3 DNA extraction using QIAGEN kit

Buffers used are supplied by the manufacturer and used according to manufacturer instructions:

1. Using Harris 2.0 mm Micro-Punch™, 6 punched-out circles from a dried blood spot were placed into a 1.5 ml microcentrifuge tube and 180µl of Buffer ATL was added.
2. The tube was incubated at 85°C for 10 minutes and briefly centrifuged in M S E microcentaur to remove drops from inside the lid.
3. 20 µl Proteinase K stock solution is added, mixed by vortexing, and incubated at 56°C for one hour. The tube was briefly centrifuged to remove drops from inside the lid.
4. 200µl of Buffer AL was added to the sample, mixed thoroughly by vortexing, and incubated at 70°C for 10 minutes. The tube was briefly centrifuged to remove drops from inside the lid.
5. 200µl ethanol (96-100%) is added to the sample, and mixed thoroughly by vortexing. The tube was briefly centrifuged to remove drops from inside the lid.

6. The mixture from step 5 was applied carefully to the QIAamp spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed, and centrifuged at 6000 x *g* (8000 rpm) for 1 minute. The QIAamp spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
7. The QIAamp spin column was opened carefully and 500µl Buffer AW1 was added without wetting the rim. The cap was closed, and the coloumn was centrifuged at 6000 x *g* (8000 rpm) for 1 minute. The QIAamp spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
8. The QIAamp spin column was opened carefully and 500 µl Buffer AW2 was added without wetting the rim. The cap was closed, and the column was centrifuged at full speed (20,000 x *g*; 14,000 rpm) for 3 minutes.
9. The QIAamp spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. The column was centrifuged at 20,000 x *g* (14,000 rpm) for 1 minute to eliminate any chance of possible Buffer AW2 carryover.
10. The QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube, the collection tube with filtrate was discarded. The QIAamp spin column was opened carefully and 100-150µl Buffer AE (10 mM Tris.Hcl; 0.5 mM EDTA; pH 9.0) was added. The column was Incubated at room temperature for 1 minute, and then centrifuged at 6000 x *g* (8000 rpm) for 1 minute
11. The samples were stored at -20°C for later use. 5µl of elute was used in 50µl PCR.

The three methods of DNA extraction gave the same results when extracted DNA was used to amplify the *pfcrt* gene **(Chapter 4, Figure 4.1)**

2.2.3 Polymerase Chain Reaction (PCR)

The PCR method was used to detect mutations in a number of codons in *pfcrt, pfdhfr* and *pfdhps*. It was also used to genotype the *P. falciparum* parasite and to amplify DNA from *pfcrt* for sequencing of the gene. Amplification reactions were carried out using a Biometra® Personal Cycler™, Serial No.3507238.

2.2.4 Agarose Gel Electrophoresis

PCR or digestion products were analysed using 1-3% agarose or MicroSieve 3:1 agarose (*Flowgen*) gel stained with ethidium bromide (0.5mg/l in 1xTAE). Formulas for the preparation of 50x TAE and 1x TAE are found in **Appendix 5**. When digestion was carried out, undigested and digested PCR products were loaded into consecutive wells (to allow for comparison) after the addition of 1/5 volume of blue/orange 6X (Promega) loading dye. DNA ladders (50 bp-100 bp) were used as molecular weight markers. Electrophoresis was carried out at 70-100 V in gel lengths 5cm-8cm respectively, until the dye had electrophoresed ¾ of length of the gel. Separated products were visualised by UV transillumination.

2.2.5 Detection Of pfcrt Polymorphism At Codon 76

Two alternative methods developed by Djimde *et al.*, (2001a) were used to detect *pfcrt* polymorphism at codon 76. One method was based on a nested PCR followed by restriction enzyme digestion that detects either the wild-type codon (CQ-sensitive) or the mutant-type codon (CQ-resistant). The other method was a nested mutation-specific PCR that uses in the secondary reaction mutation- specific primers that detects either the wild-type codon (CQ-sensitive) or the mutant-type codon (CQ-resistant). Detailed methodology for the detection of *pfcrt*-76 polymorphism is available at *http://medschool.umaryland.edu/CVD/plowe.html*

For all reactions used to detect *pfcrt* polymorphism at codon 76, PCR amplification reactions were carried out in 25µl volumes consisting of 2x ReddyMix PCR Master Mix (*ABgene*®) containing 2.5 mM $MgCl_2$; 0.2mM each of dATP, dCTP, dGTP and dTTP; and 1.25U Thermoprime Plus DNA Polymerase. Primers were used at a final concentration of 1µM. Genomic DNA from the laboratory chloroquine-sensitive 3D7 strain (donated kindly by Dr. Yang Woo, Department of Molecular and biochemical Parasitology, LSTM) and from the chloroquine-resistant K1 strain (donated kindly by Professor Steve Ward's group, Department of Molecular and biochemical Parasitology, LSTM) were used as positive controls, while water was used as a negative control.

2.2.5.1 Detection of pfcrt polymorphism at codon 76 using a nested PCR and restriction enzyme digestion

For the first round reaction, primers *CRTP1* and *CRTP2* were to amplify a 537 base pair flanking region around the K76T mutation. Five µl of methanol extracted DNA was added as a template. Two µl of the first run product was used as a template for the second nested reaction using primers *CRTD1* and *CRTD2* to amplify a 145bp fragment surrounding the *Apo*I restriction enzyme site. PCR reaction conditions were used as described in **section 2.2.5**. Primer names, sequences and PCR cycling conditions are listed in **Table 2.2**

Primer name	**Primer sequence 5` to 3`**	**PCR cycling conditions** Temperature, time
CRTP1	CCGTTAATAATAAATACACGCAG	**Initial denaturation**: 94°C, 3 minutes **Denaturation**: 94°C, 30 seconds. **Annealing**: 56°C, 30 seconds. **Extension**: 60°C, 1 minute. **Number of cycles**: 45 **Final extension**: 60°C, 3 minutes.
CRTP2	CGGATGTTACAAACTATAGTTACC	
CRTD1	TGTGCTCATGTGTTTAAACTT	**Initial denaturation**: 95°C, 5 minutes. **Denaturation**: 92°C, 30 seconds. **Annealing**: 48°C, 30 seconds. **Extension**: 65°C, 30 seconds. **Number of cycles**: 35 **Final extension**: 65°C, 3 minutes.
CRTD2	CAAAACTATAGTTACCAATTTTG	

Table 2.2 Primer names, sequences and PCR parameters used to detect the *pfcrt* polymorphism at codon 76 (nested PCR followed by digestion method)

An aliquot (5µl) of the second round PCR was digested with one unit *Apo*I enzyme (*NEW ENGLAND, Biolabs*) at 50°C for 2 hours in a total reaction volume of 20µl according to the manufacturer's instructions. The enzyme cleaves off a fragment of 45 base pairs in the presence of CQ-sensitive allele (Lys). The laboratory sensitive isolate

3D7 was used to control for complete digestion. Water negative control was also used in all steps. Products were analysed by 2% agarose gel **(Section 2.2.4)**.

2.2.5.2 Detection of pfcrt polymorphism at codon 76 using a nested mutation-specific PCR

The first round reaction of the nested PCR amplification was performed using the same flanking primers *CRTP1* and *CRTP2* as described previously **(Section 2.2.5.1).** Primer sequences and cycling conditions are listed in **Table 2.2**. Second round reaction was performed using two mutation specific diagnostic PCRs, each using a common inner primer *CRTP3* coupled with an allele specific primer, either *CRTP4m*, which detects the resistant T76 genotype, or *CRTP4w*, which detects the sensitive K76 genotype. Thus each sample was subjected to two diagnostic PCRs, one to detect the resistant genotype and one to detect the sensitive genotype. PCR reaction conditions were used as described in **section 2.2.5.** The resulting amplicons were loaded into two consecutive wells on a 2% agarose gel containing ethidium bromide. A positive reaction for either genotype yields a 366 base pair band in the appropriate lane. Samples representing mixed infections containing both resistant and sensitive genotypes yield a band in both lanes, and negative samples yield no band. Sequences of the primers of the second round amplification and cycling conditions for the PCR reactions are shown in **Table 2.3**.

Primer name	**Primer sequence 5` to 3`**	**PCR cycling conditions** Temperature, time
CRTP3	TGACGAGCGTTATAGAG	**Initial denaturation**: 94°C, 3 minutes
CRTP4m	GTTCTTTTAGCAAAAATTG	**Denaturation**: 94°C, 30 seconds
CRTP4w	GTTCTTTTAGCAAAAATCT	**Annealing**: 47°C, 30 seconds.
		Extension: 64°C, 1 minutes
		Number of cycles: 45
		Final extension: 64°C, 3 minutes

Table 2.3 Primer names, sequences and PCR parameters used to detect of *pfcrt* polymorphism at codon 76 (nested mutation- specific PCR method)

2.2.6 Detection Of The Newly Discovered Mutation S163R

To detect the newly discovered mutation S163R at *pfcrt* the protocol developed by David Johnson (Johnson, 2003), which is based on a nested PCR followed by restriction enzyme digestion was performed as follows:

2.2.6.1 DNA amplification

The PCR reactions were carried out in 50µl volumes consisting of 1.1x ReddyMix™ PCR Master Mix (*ABgene*®) containing 2.5 mM $MgCl_2$; 0.2mM each of dATP, dCTP, dGTP and dTTP; 1.25U Thermoprime Plus DNA Polymerase; 75mM Tris-HCl (pH 8.8 at 25°C); and 0.01% (v/v) Tween® 20. Primers *PfCRT* 163-1 and *PfCRT* 163-2 were used at a concentration of 1µM each to amplify a 906 base pair fragment flanking the S163R mutation. Five µl of methanol extracted DNA was added as a template. Primers *PfCRT* 163-11 and *PfCRT* 163-12 were used to amplify a 290 base pair fragment surrounding the *Hin*fI restriction enzyme site. Five µl of the first run product was used as a template. DNA from the laboratory isolate K1AM that harbors the S163R mutation and DNA from 3D7 strain that lacks this mutation were used as positive controls, while water was used as a negative control. The sequences of each primer and the cycling conditions for the PCR reactions used to detect the PfCRT S163R polymorphism are shown in **Table 2.4**

Primer name	Primer sequence 5` to 3`	PCR cycling conditions Temperature, time
pfcrt 163-1	TTTTGTAACATCCGAAACTCACA	**Initial denaturation**: 95°C, 3 minutes. **Denaturation**: 95°C, 30 seconds **Annealing**: 54°C for 30 seconds **Extension**: 62°C, 90 seconds. **Number of cycles**: 40 **Final extension**: 62°C, 5 minutes
pfcrt 163-11	GGCCTTCATAGGTCTTACAAG	**Initial denaturation**: 95°C, 3 minutes **Denaturation**: 95°C, 30 seconds **Annealing**: 50°C, 30 seconds **Extension**: 62°C, 30 seconds **Number of cycles**: 45 **Final extension**: 62°C, 5 minutes
pfcrt 163-12	AACAATAATAACTGCTCCGAGAT	

Table 2.4 Primer names, sequences and PCR cycling conditions used to detect of *pfcrt* S163R

2.2.6.2 Restriction enzyme digestion

Five µl of the secondary amplicon was digested with one unit *Hin*f1 enzyme (NEW ENGLAND, Bio labs) at 37°C for 2 hours in a total 20µl reaction volume. The enzyme cleaves off a fragment of 61 base pairs in the presence of mutant allele (Arg). Products were analysed by 2% agarose gel electrophoresis **(Section 2.2.4)**

2.2.7 Detection Of pfdhfr Polymorphism At Codon 59

Two different PCR reactions followed by restriction enzyme digestion were used to detect PfDHFR-59 polymorphism, one developed by Plowe *et al.*, (1995) using *BsrG*I enzyme for restriction digestion. Details of this method are available at *http://medschool.umaryland.edu/cvd/2002_pcr_asra.html.* The other method was developed by (Duraisingh *et al.*, 1998) and uses *Xmn*I enzyme for restriction digestion. For all reactions used to detect *pfcrt* polymorphism at codon 59, PCR amplification reactions were carried out in a total volumes of 25µl consisting of 2x ReddyMix PCR Master Mix (*ABgene*®) containing 2.5 mM $MgCl_2$; 0.2mM each of dATP, dCTP, dGTP

and dTTP; and 1.25U Thermoprime Plus DNA Polymerase. Primers were used at a final concentration of 1μM. Genomic DNA from a laboratory sensitive 3D7 strain and resistant K1 strain were used as positive controls, while water was used as a negative control.

2.2.7.1 Detection of pfdhfr polymorphism at codon 59 using a nested PCR and restriction digestion with *BsrG*I enzyme

Five μl of methanol extracted DNA was used as a template to initiate the first round reaction, primers *FR519-A* and *FR519-B* were used to amplify a 147 base pair flanking region around the *C59R* mutation. For the second nested reaction 2-5 μl of the first round product were used with primers *FR51-D* and *FR59-D* to amplify a 100 base pair fragment. Five μl of the second round PCR was digested with one unit *BsrG* I enzyme (*NEW ENGLAND, Bio labs*) at 37°C for 2 hours in a total 20μl reaction volume according to the manufacturer instructions. The enzyme cleaves off a fragment of 35 base pairs in the presence of the wild allele (Cys) producing 2 cleaved products (35 and 65 bas pairs). Primer sequences and cycling conditions for the PCR reactions are listed in **Table 2.5**

Primer name	**Primer sequence 5` to 3`**	**PCR cycling conditions** Temperature, time
FR519-A	GCGCGCTAATAACTACACATTTA	**Initial denaturation**: 95°C, 5 minutes **Denaturation**: 92°C, 30 seconds. **Annealing**: 45°C, 30 seconds. **Extension**: 65°C, 45 seconds. **Number of cycles**: 45 **Final extension**: 72°C, 15 minutes
FR519-B	CCCGGGCTCTTATATTTCAATTT	
FR51-D	CTAGGAAATAAAGGAGTATTACCAT GGAAATGGA	**Initial denaturation**: 95°C, 5 minutes. **Denaturation**: 92°C, 30 seconds. **Annealing**: 45°C, 30 seconds. **Extension**: 65°C, 30 seconds. **Number of cycles**: 35 **Final extension**: 72°C, 15 minutes
FR59-D	ATTTTTCATATTTTGATTCATTCACAT ATGTTGTAACTGTAC	

Table 2.5 Primer names, sequences and PCR conditions used for the detection of *pfdhfr* polymorphism at codon 59 using restriction enzyme digestion with *BsrG*I enzyme

2.2.7.2 Detection of pfdhfr polymorphism at codon 59 using a nested pcr and restriction digestion with *xmn*I enzyme

The first round reaction was carried out using a pair of primers *M1 and M5* and 5 µl of methanol extracted DNA as a template. For the second nested reaction 2-5 µl of the first run product were used as a template and primers *F* and *M4* were used to amplify a 326 base pair fragment. Primer *F* was engineered by introducing mismatches in its 3′ end to create a restriction site which allows the detection of the polymorphic forms of codon-59 following restriction digestion with *Xmn*I. The PCR product also contains another *Xmn*I site, which serves as an internal control for digestion. Five µl of the second round PCR was digested with one unit *Xmn*I enzyme (*NEW ENGLAND, Bio labs*) at 37°C for 2 hours in a total 20µl reaction according to the manufacturer's instructions.

In the presence of the wild-type allele (Cys) the enzyme produces 2 cleaved products (189 bp and 137 bp). The enzyme produces two equally sized fragments of 163 base pairs in the presence of the mutant-type allele (Arg). Products were analysed by 3% agarose gel electrophoresis (**Section 2.2.4**). The sequences of each primer and the cycling conditions for the PCR reactions are shown in **Table 2.6**

Primer name	**Primer sequence 5` to 3`**	**PCR cycling conditions** Temperature, time
M1	TTTATGATGGAACAAGTCTGC	**Initial denaturation**: 94°C, 3 minutes **Denaturation**: 94°C, 1 minute. **Annealing**: 45°C, 1 minute. **Extension**: 72°C, 1 minute. **Number of cycles**: 45 **Final extension**: 72°C, 10 minutes.
M5	AGTATATACATCGCTAACAGA	
F	GAAATGTAATTCCCTAGATATGGAATATT	**Initial denaturation**: 94°C, 3 minutes.**Denaturation**: 94°C, 1 minute. **Annealing**: 45°C, 2 minutes for the first 5 cycles then 1 minute for the rest 40 cycles **Extension**: 72°C, 1 minute. **Number of cycles**: 45 **Final extension**: 72°C, 10 minutes.
M4	TTAATTTCCCAAGTAAAACTATTAGAG CTTC	

Table 2.6 Primer names, sequences and PCR conditions used for the detection of *pfdhfr* polymorphism at codon 59 using restriction enzyme digestion with *Xmn*I enzyme.

2.2.8 Detection Of pfdhps Polymorphism At Codon 540

A nested PCR reaction followed by restriction enzyme digestion was used to detect *pfdhps* plymorphism at codon 540. The method used was developed by Plowe *et al.*, (1995) and is available at *http://medschool.umaryland.edu/cvd/2002_pcr_asra.html*

2.2.8.1 DNA amplification

The same PCR reaction conditions used to detect mutation at codon 540 were applied for both the primary and secondary reactions. A pair of primers *PS500-A* and *PS500-B* was used in the first round reaction to amplify a 256bp fragment. For the second round reaction, primers PS500-D1 and PS500-D2 were used to amplify a 201bp fragment. Positive and negative controls were used in each reaction. Primer sequences and the cycling conditions are listed in **Table 2.7**

2.2.8.2 Restriction enzyme digestion

Five microliters of secondary reaction product were digested with 1 unit Fok1 (New England Biolabs) at 37°C for 2 hours in a total reaction volume of 20µl. The enzyme cleaves the mutant-type allele (Glu) and not the wild-type allele (Lys) resulting in two product sizes 56 bp and 145 bp. Products were analysed by 2% agarose gel electrophoresis **(section 2.2.4).**

Primer name	Primer sequence 5` to 3`	PCR cycling conditions
PS500-A	GGGCCCAAACAAATTCTATAGTG	**Initial denaturation**: 95°C for 5 minutes **Denaturation**: 92°C for 30 seconds **Annealing**: 45°C for 30 seconds **Extension**: 65°C for 45 seconds **Number of cycles**: 45 **Final extension**: 72°C for 15 minutes
PS500-B	GGCCGGTGGATACTCATCATATA	
PS500-D1	GCGCGCGTTCTAATGCATAAAAGAGG	**Initial denaturation**: 95°C for 5 minutes **Denaturation**: 92°C for 30 seconds **Annealing**: 45°C for 30 seconds **Extension**: 65°C for 30 seconds **Number of cycles**: 25 **Final extension**: 72°C for 15 minutes
PS500-D2	CCCGGGTAAGAGTTTAATAGATTGATCA*GC*TTTCTTC	

Table 2.7 Primer names, sequences and PCR conditions used for the detection of *pfdhfr* polymorphism at codon 540.

2.3 GENOTYPING P. FALCIPARUM PARASITE USING MSP-2

The distinction between recrudescence and reinfections in treatment failure cases, and the genetic structure of the parasite population in the area were determined by nested family-specific polymerase chain reaction (PCR) amplification of polymorphic regions of block 3 of P. *falciparum* antigen gene *msp-2* as described by Snounou *et al.,* (1999) and (Magesa *et al.*, 2002).

2.3.1 DNA Amplification

The primary and secondary PCR reaction conditions were carried out in 25ul volumes consisting of 2 x ReddyMix PCR Master Mix (*ABgene*®) containing 2.5 mM $MgCl_2$; 0.2mM each of dATP, dCTP, dGTP and dTTP; and 1.25U Thermoprime Plus DNA Polymerase. Primers were used at a final concentration of 1uM. Methanol extracted DNA from samples of patients on day 0 (before treatment), day 3 and on the day of classification were used separately as templates for the primary reaction. The primers that amplify the conserved-nest 1, *M2-OF* and *M2-OR,* were used in the first round amplification. The product of the first round reaction of each sample was subjected to

two separate second round reactions, one reaction using a pair of FC27 family-specific primers (*M2-FCF* and *M2-FCR*) that detect the FC27-type variants, and the other reaction using a pair of 3D7/IC family-specific primers (*M2-ICF* and *M2-ICR*) that detect the 3D7/IC-type variants of MSP2. DNA from 3D7 strain was used as positive control, while water was used as a negative control. Primer names, sequences and PCR conditions used to genotype P. *falciparum* parasites using the polymorphic genetic marker *msp*-2 were listed in **Table. 2.8**

PCR products were analysed by MicroSieve 3:1 agarose (*Flowgen*) gel electrophoresis as described in **(section 2.2.4).** Samples before and after treatment for each patient were loaded into adjacent wells for comparison in order to distinguish reinfection from recrudescence.

Primer name	**Primer sequence 5` to 3`**	**PCR cycling conditions**
M2-OF	ATGAAGGTAATTAAAACATTGTCTATTATA	**Initial denaturation**: 94°C for 11 min. **Denaturation**: 94°C for 1 min. **Annealing**: 58°C for 2 min. **Extension**: 72°C for 2 min. **Number of cycles**: 25 **Final extension**: 72°C for 5 min.
M2-OR	CTTTGTTACCATCGGTACATTCTT	
M2-FCF	ATACTAAGAGTGTAGGTGCA**R**ATGCTCAA	**Initial denaturation**: 94°C for 11 min. **Denaturation**: 94°C for 30 sec. **Annealing**: 61°C for 1 min. **Extension**: 72°C for 1 min. **Number of cycles**: 30 **Final extension**: 72°C for 5 min.
M2-FCR	TTTTATTTGGTGCATTGCCAGAACTTGAAC	
M2-ICF	AGAAGTATGGCAGAAAGTAA**K**CCT**Y**CTACT	
M2-ICR	ATTGTAATTCGGGGGATTCAGTTTGTTCG	

Table 2.8 Primer names, sequences and PCR conditions used to genotype P. *falciparum* parasites using the polymorphic genetic marker *msp*-2. **R=A+G, K=G+T, Y=C+T**

2.4 SEQUENCING THE *pfcrt* GENE

Direct DNA sequencing of the *pfcrt* gene from three representative field samples was carried out by Lark Technologies, Inc (Saffron Walden, UK). DNA was amplified using a number of primers that flank the 13 exons of the *pfcrt* gene (Johnson D.J., personal communication). The 13 exons were amplified using nested PCR in 3 different reactions; i) amplification of exons1 and 2, ii) amplification of exons 3 to 8, and iii) amplification of exons 9 to 13. Products of amplification were purified either directly from PCR or after gel extraction before sending to Lark Technologies for sequencing.

2.4.1 Amplification Of Exons 1 and 2

Both the primary and secondary PCR reactions were carried out in 50µl reaction volumes consisting of 2x ReddyMix PCR Master Mix from *ABgene* containing 2.5 mM $MgCl_2$; 0.2mM each of dATP, dCTP, dGTP and dTTP; and 1.25U Thermoprime Plus DNA Polymerase. Primers were added at a final concentration of 0.5µM. To initiate the primary reaction 5µl of methanol extracted DNA was added as a template and 5µl of the product of the primary reaction was used as a template for the secondary product. The resulting amplicon was purified and sent with to Lark Technologies, Inc (Saffron Walden, UK) for sequencing.

2.4.2 Amplification Of Exons 3-8 And Exons 9-13

Both the primary and secondary PCR reactions were carried out in 50µl reaction volumes consisting of 1.1x ReddyMix PCR Master Mix from *ABgene*® containing 1.5 mM $MgCl_2$; 0.2mM each of dATP, dCTP, dGTP and dTTP; and 1.25U Thermoprime Plus DNA Polymerase. Primers were added at a final concentration of 0.5µM. To initiate the primary reaction 5µl of methanol extracted DNA was added as a template and 5µl of the product of the primary reaction was used as a template for the secondary product. The resulting amplicon was purified and sent with to Lark Technologies™ for sequencing. The primer sequences used to amplify and to sequence the 13 exons of *pfct* and the PCR cycling conditions used to amplify different exons of *pfcrt* are listed in **Table 2.9** and **Table 2.10** respectively.

Reaction	Primer	Primer sequence 5` to 3`
Primary (Exon1-2)	**Forward**	CGACATTCCGATATATTATATTTTTAGAC
	Reverse	TATATGTGTAATGTTTTATATTGG
Secondary (Exon1-2)	**Forward**	CCGTTAATAATAAATACACGCAG
	Reverse	AATGTTTTATATTGGTAGGTGG
Sequencing reaction	**Exon1**	CCAATAGGTTGATTTATCTA
	Exon2	GATTCTCTTATAAATCCATC
Primary (Exon3-8)	**Forward**	CCACCTACCAATATAAAACATTAC
	Reverse	GTTAAAATATATATAAATGTCTC
Secondary (Exon3-8)	**Forward**	TATATATATATGTATGTATGTTG
	Reverse	AATGTCTCTTATAATTTTGAAATT
Sequencing reaction	**Exon3**	GTATGTATGTTGATTAATTTG
	Exon4	AATAAAAACAAAGTTTAAGTG
	Exon5-6	TATATATATGGGCACATTC
	Exon7-8	TAAATAAATGAATGTGCCC
Primary (Exon9-13)	**Forward**	CTTATAATAAAATTTCAAAATTATAAGAGAC
	Reverse	GAGATCTCTATACCTTCAACATTATTCC
Secondary (Exon9-13)	**Forward**	GAGACATTTATATATATTTTAAC
	Reverse	CCTTATAAAGTGTAATGCG
Sequencing reaction	**Exon9-10**	CATATATATCATATATTTCAC
	Exon11-12	TTATGATGGTACAACGTATC
	Exon13	ATTTTTATATTTCCATCTGTC

Table 2.9 Primer sequences used to amplify and to sequence the 13 exons of *pfcrt* gene. Primers were designed by D.J.Johnson and were synthesized by Sigma-Genosys (Pampisford, UK; http://www.sigma-genosys.co.uk)

Amplification of Exon 1-2 (For both primary and secondary reactions)	Amplification of Exon 3-8 and Exon 9-13 (For both primary and secondary reactions)
Initial denaturation: 94°C for 3 minutes **Denaturation**: 94°C for 30 seconds **Annealing**: 54°C for 30 seconds **Extension**: 62°C for 1 minute and 30 seconds **Number of cycles**: 35 **Final extension**: 62°C for 5 minutes	**Initial denaturation**: 94°C for 5minutes **Denaturation**: 94°C for 1 minute **Annealing**: 54°C for 1 minute **Extension**: 62°C for 3 minutes **Number of cycles**: 45 **Final extension**: 62°C for 5 minutes

Table 2.10 The PCR cycling conditions used to amplify different exons of *pfcrt*.

2.4.3 Purification Of DNA For Sequencing

The product of each amplification reaction was purified either directly from PCR using QIAquick PCR Purification Kit (*QIAGEN*) if the PCR product showed a single band on agarose gel electrophoresis or, in case of multiple bands, the desired band was cut from gel and the piece of gel containing the band was purified using QIAquick Gel Extraction Kit (*QIAGEN*).

2.4.3.1 Purification using QIAquick PCR purification kit (QIAGEN)

All Centrifuge steps were at 13,000 rpm (~17,900 x g). All buffers used were supplied by the manufacturer and were used according to manufacturer's instructions:

1. Five volumes of Buffer PB was added to one volume of PCR sample and mixed in a1.5 ml microcentrifuge tube.
2. A QIAquick spin column was placed in a provided 2 ml collection tube, the sample was applied to the column and centrifuged for 1 minute.
3. The flow-through discarded. The column was placed back into the same tube.
4. To the QIAquick column 0.75 ml of washing Buffer PE was added and the column centrifuged for 1 minute.
5. The flow-through was discarded. The column was placed back into the same tube, and centrifuged for an additional 1 minute.

6. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and 30 µl of elution Buffer EB (10 mM Tris.Hcl, pH 8.5) was added to the centre of the QIAquick membrane.
7. The column was left to stand for 1 minute, and then centrifuged for 1 minute.
8. DNA concentration was measured in 1µl using ND-1000 Spectrophotometer (Nanodrop Technologies) and samples sent for sequencing. Samples could be stored also at –20°C until required.

2.4.3.2 Purification using QIAquick gel extraction kit (QIAGEN)

All Centrifuge steps were at 13,000 rpm (~17,900 x g). All buffers used were supplied by the manufacturer and were used according to manufacturer's instructions:

1. The desired DNA fragment was excised from agarose gel with a clean sharp scalpel.
2. The gel slice was weighed in a 1.5 ml microcentrifuge tube. Three volumes of Buffer QG were added to 1 volume of gel.
3. The sample was incubated at 50°C for about 10-15 min. until the gel slice has completely dissolved. The mixture was mixed by vortexing every 2-3 min. during the incubation to help dissolve the gel.
4. After the gel slice had dissolved completely, the QIAquick spin column was placed in a provided 2 ml collection tube, the sample was applied to the column and centrifuged for 1 minute.
5. The flow-through was discarded. The column was placed back into the same tube.
6. To the QIAquick column 0.75 ml of washing Buffer PE was added and the column centrifuged for 1 minute.
7. The flow-through was discarded. The column was placed back into the same tube, and centrifuged for an additional 1 minute.
8. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube and 30 µl of elution Buffer EB (10 mM Tris.Hcl, pH 8.5) was added to the centre of the QIAquick membrane.
9. The Column was left to stand for 1 minute, and then centrifuged for 1 minute.
10. DNA concentration was measured in 1µl using ND-1000 Spectrophotometer (Nanodrop Technologies) and samples sent for sequencing. Samples were either sent directly for sequencing or stored at –20°C until required.

CHAPTER 3

Assessment Of The Therapeutic Efficacy Of Chloroquine For The Treatment Of Uncomplicated Falciparum Malaria In Yemen (In-Vivo Test)

3.1 INTRODUCTION

Early, correct diagnosis and prompt, proper treatment are essential for any malaria control program. However, the effectiveness of treatment is being limited by the development of drug-resistant strains of *P. falciparum* that are likely to have been introduced into Yemen during the last decade through population movement and the influx of refugees from some African countries (Somalia, Ethiopia, Eritria) due to political conflicts (Roll Back Malaria Program, Yemen, 2000). Chloroquine is still used as the first-line drug for acute uncomplicated *falciparum* malaria in Yemen,

The emergence of low-grade (RI level) chloroquine resistant *Plasmodium falciparum* in Republic of Yemen was reported in 1987. Since then, however, only a few studies have been conducted, which were non-systematic and used a test protocol developed only to evaluate the parasite response to treatment rather than evaluating the therapeutic outcome. Therefore the distribution and extent of chloroquine resistant parasites are not known and there is no system for monitoring antimalarial drug efficacy.

As the Roll Back Malaria centre in Yemen is newly established, recruitment and training of national staff in various areas of malaria control including assessment of the sensitivity of commonly used antimalarials is recommended (WHO, EMRO, 1999).

The work described in this chapter had the following specific objectives:

- To determine *in-vivo*, the prevalence of chloroquine resistance in *Plasmodium falciparum* acute uncomplicated malaria in a sentinel site in Yemen,
- To describe some easily identifiable predictors of chloroquine treatment failure.
- To formulate recommendations regarding methodology for monitoring therapeutic efficacy in sentinel sites in Yemen.
- To train some of the health workers in the Ministry of Public Health in clinical, epidemiological and laboratory methods regarding monitoring drug resistance in malaria in Yemen.

3.2 MATERIALS AND METHODS

To establish a practical system for continuous monitoring of therapeutic efficacy of antimalarial drugs and to provide and update essential data for antimalarial drug policy in Yemen, an in-vivo sensitivity test to chloroquine was applied in Al-Musaimeer rural hospital, Al-Musaimeer district, Lahj governorate from October/2002 to January/2003. The standard 28-day *in-vivo* WHO draft protocol for assessment of therapeutic efficacy of antimalarial drugs for uncomplicated *falciparum* malaria, draft September 2002 (WHO, 2001c) was used with slight modifications due to factors observed in the field during the implementation of the test. Modifications included:

- Parasite density of 1000-**200,000** asexual parasite/µl of blood (as long as no danger signs encountered).
- Follow-up period of **14**-days.

The reasons associated with those modifications are discussed in **section 3.4.**

All febrile patients coming to the hospital during the study period were screened for *P. falciparum* mono infection and were subjected to clinical assessment (**Chapter 2, section 2.1.4.1**), and parasitological assessment (**Chapter 2, section 2.1.4.2**), if they satisfied all the inclusion and exclusion criteria (**Chapter 2, sections 2.1.2.1 and 2.1.2.2 respectively**) and informed consent was obtained (**Appendix 3**), they were enrolled into the study. Patients were given chloroquine according to body weight and followed up to monitor the clinical and parasitological response (**Chapter 2, section 2.1.5**). Their response to treatment was classified according to the draft WHO protocol into: Early Treatment Failure (ETF), Late Clinical Failure (LCF), Late Parasitological Failure (LPF) and Adequate Clinical and Parasitological Response (ACPR) (**Chapter 2, section 2.1.6**).

Enrolled patients were given a consecutive number in the record book. Data were entered using special questionnaire forms designed by WHO (**Appendix 4**). Data were analysed using SPSS package (version 11.0). Odds ratios [OR] were obtained using Statcalc (*Epi info* Version 6).

To ensure good quality data, each patient had a study number which was the same in the record book, slides, patient form, and filter papers (to study molecular markers) to prevent mixing patients information. Slides of all patients were also re-examined separately by 2 microscopists in the central laboratory belonging to the Roll Back Malaria, Yemen. The re-examination was blinded to the results of the original examination, and focused on negativity, asexual *P.falciparum* counts, and *Plasmodium* species. Trivial discrepancies were found in counting parasite densities, 2 negative cases on day 14 were found to be positive (with low parasitaemia) when re-examined. These 2 cases were reclassified as late treatment failures.

This study has been approved by the Ministry of Public Health in the Republic of Yemen (**Appendix 1A**), the Faculty of Medicine, Aden University, Yemen (**Appendix 1B**), and the Liverpool School of Tropical Medicine, University of Liverpool, UK (**Appendix 1C**).

3.3 RESULTS

3.3.1 Enrolment And Patient Characteristics

Of 644 febrile patients referred for screening, 225 (34.9%) were positive for mono infection with falciparum malaria of which 124 (55.1%) were enrolled while 101 were excluded for different reasons (**Table 3.1**).

	No.	**%**
Screened	644	
Negative	419	65.1
Positive	225	34.9
• **Included**	**124**	**55.1**
• **Excluded**	**101**	**44.9**
Reasons for exclusion		
Reason	**No.**	**%**
Presence of other febrile illness	15	14.8
Parasite count <1000	22	21.8
Age <6 months	2	2
Pregnancy	1	1
Mixed infection (*P.falciparum+P.vivax*)	2	2
Severe malaria and danger signs	3	3
Acute renal failure	1	1
Severe anaemia	1	1
Far place of residence	36	35.6
Sensitivity to chloroquine	2	2
Refuse to participate	16	15.8
Total	101	100%

Table 3.1 number of patients screened for parasitaemia, number of patients included and excluded and the reasons for their exclusion.

Of the 124 cases enrolled to the study, 122 cases (98.4%) completed the follow-up and their treatment outcome was classified according to the standard WHO protocol, 2 cases were lost (1.6%) and non was withdrawn from the study. The two lost cases were excluded from all subsequent analysis.

Patients were almost exclusively children under 15 years of age (80.3%), however, children below 5 years of age were only 18% of the cases. Hyperparasitaemia (parasite density > 100,000/µl) was not common; it appeared in only 2 cases (1.6%). Fever (axillary temperature ≥ 37°C) at presentation was present in 35.2% of the cases.

3.3.2 In-Vivo Treatment Outcome

Of the122 cases that completed the follow-up, 48 (39.3%) had adequate clinical and parasitological response (ACPR), the remaining 74 (60.7%) were treatment failures. They were classified as Early Treatment Failures ETF 28 (23%), Late Clinical Failures LCF 16 (13.1%), and Late Parasitological Failures (LPF) 30 (24.6%, **Figure 3.1**).

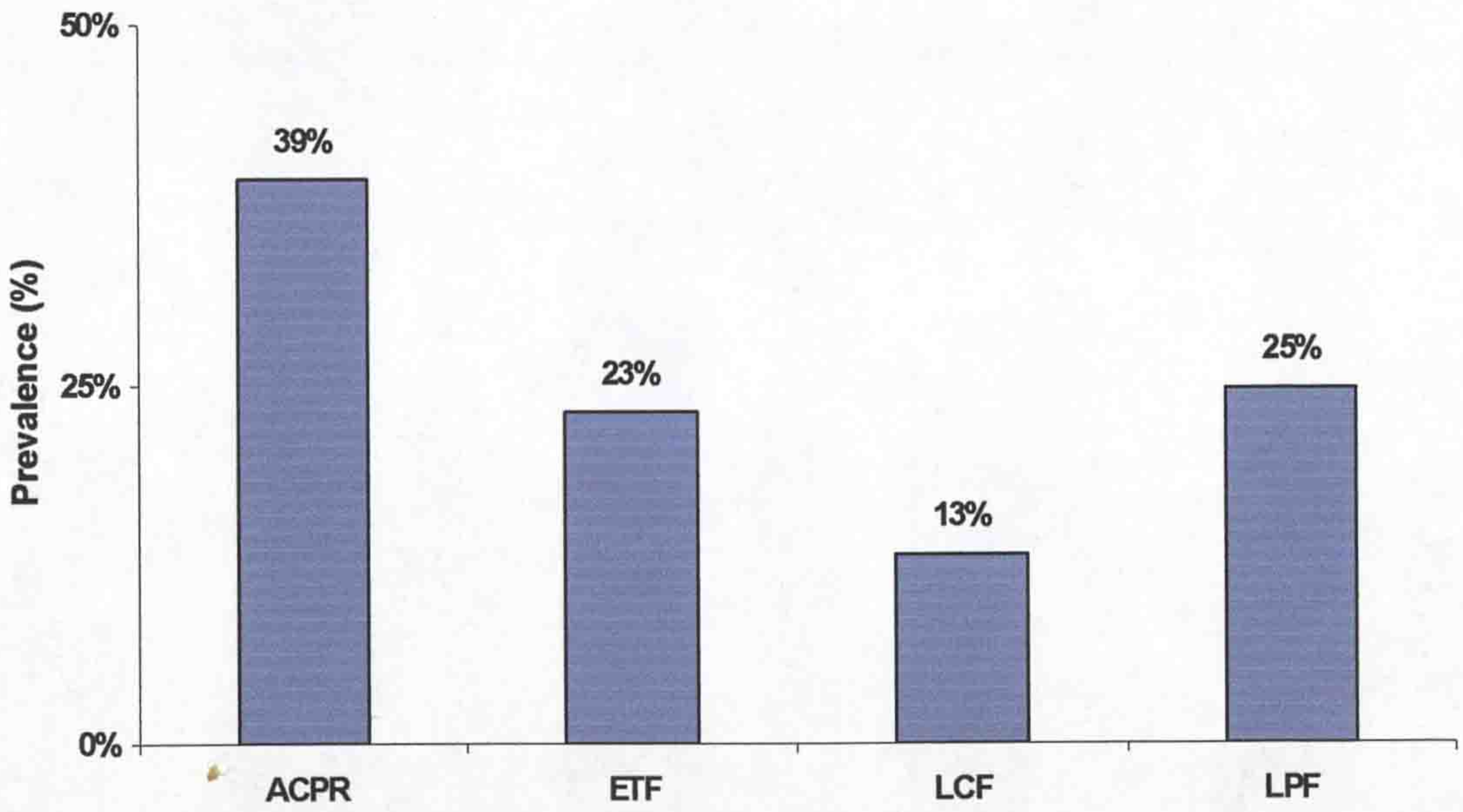


Figure 3.1 In-vivo chloroquine treatment outcome, Lahj governorate,Yemen, 2002-2003.

Among treatment failures, 28 (38%) were early treatment failures (ETF), 16 (22%) were late clinical failures (LCF), and 30 (40%) were late parasitological failures (LPF) (**Figure 3.2**).

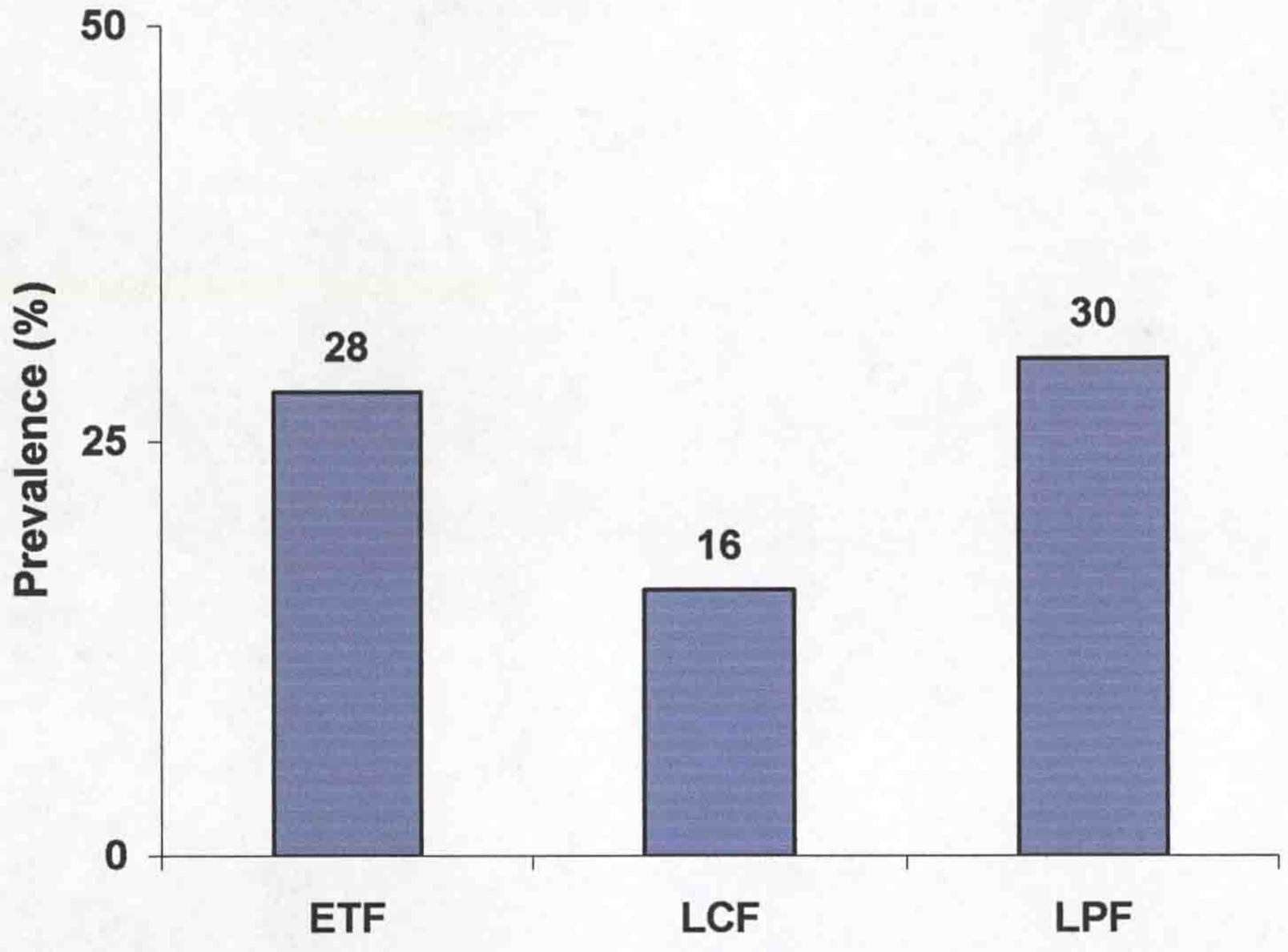


Figure 3.2 Treatment failure categories, showing the participation of each category in the total treatment failure

The base-line characteristics of cases that completed the follow-up and association with treatment outcomes are shown in **Table 3. 2**

	All subjects	ACPR	ETF	LCF	LPF	*P*-VALUE
Number (%) of cases	122 (100)	48 (39.3)	28 (23)	16 (13.1)	30 (24.6)	
Age (year)	9.5 (1-40)	13.5 (3-40)	6 (1-14)	6 (2.2-13)	9.75 (2.5-19)	.000
Sex (% male)	57.4	36.6	21.4	14.3	25.7	.930
Body weight (Kg)	21.52 (8-72)	31.5 (12-72)	16 (8-42)	16 (11-36)	21.50 (11-46)	.000
Axillary temp. (°C)	36.9 (35.2-40.9)	36.65 (35.7-40.9)	37.95 (35.3-40.4)	36.65 (35.9-39.5)	36.80 (35.2-39.3)	.013
Parasite count (parasites/µl)	7860 (1040-136000)	6699 (1040-67200)	10540 (1320-112000)	7300 (1360-13600)	8320 (1320-69040)	.228

Table 3.2 Effect of patient's characteristics on the in-vivo response to treatment

Data are medians (range), or sex (%male). Tests used: Kruskal-Wallis (for the 4 categories of treatment outcome), or Chi-square for sex

3.3.4 Predictors Of Chloroquine Treatment Failure

For the purpose of analysing some potential predictors of chloroquine treatment failure, treatment outcomes were grouped into two groups; Adequate Clinical and Parasitological Response (ACPR) comprising 39.3% of the total treatment outcome and Treatment Failure (TF) comprising 60.7% of patient's treatment outcome. The TF group includes ETF, LCF and LPF. In univariate analysis, younger age (<10 years), fever (axillary temperature ≥37.5°C) and parasite density of ≥25000 asexual parasites/µl blood at presentation were found to be significantly associated with increased risk of chloroquine treatment failure (**Table 3.3**).

Variable (no.)	Number of treatment failure	Odds ratio	95% CI	*p*-value
Age (122)				
≥10 years (60)	22	1.00	-	
<10 years (62)	52	8.98	3.54-23.33	0.000
Sex		0.93	0.42-2.06	0.839
Axillary temperature (122)				
<37.5°C (79)	42	1.00	-	
≥37.5°C (43)	32	2.56	1.06-6.30	0.022
Parasite count (122)				
<25000 parasites/µl blood (100)	56	1.00	-	
≥25000 parasites /µl blood (22)	18	3.54	1.02-13.39	0.025
Body weight		.891	.853-.931	0.000

Table 3.3 Univariate analysis of some potential predictors of chloroquine treatment failure:

To control for the possible confounding effect of the variables included in the univariate analysis, a multivariate backward selection logistic regression was

performed. Body weight was dropped from multivariate analysis since it has a high correlation with age; *Pearson correlation .890, p= .000* (Katz, 1999).

Multivariate analysis confirmed that age less than 10 years is an independent predictor of chloroquine treatment failure (OR=8.7, 95% CI=3.6-21.01; **Figure 3.3**), while the presence of fever and high parasite count lost their statistical significance.

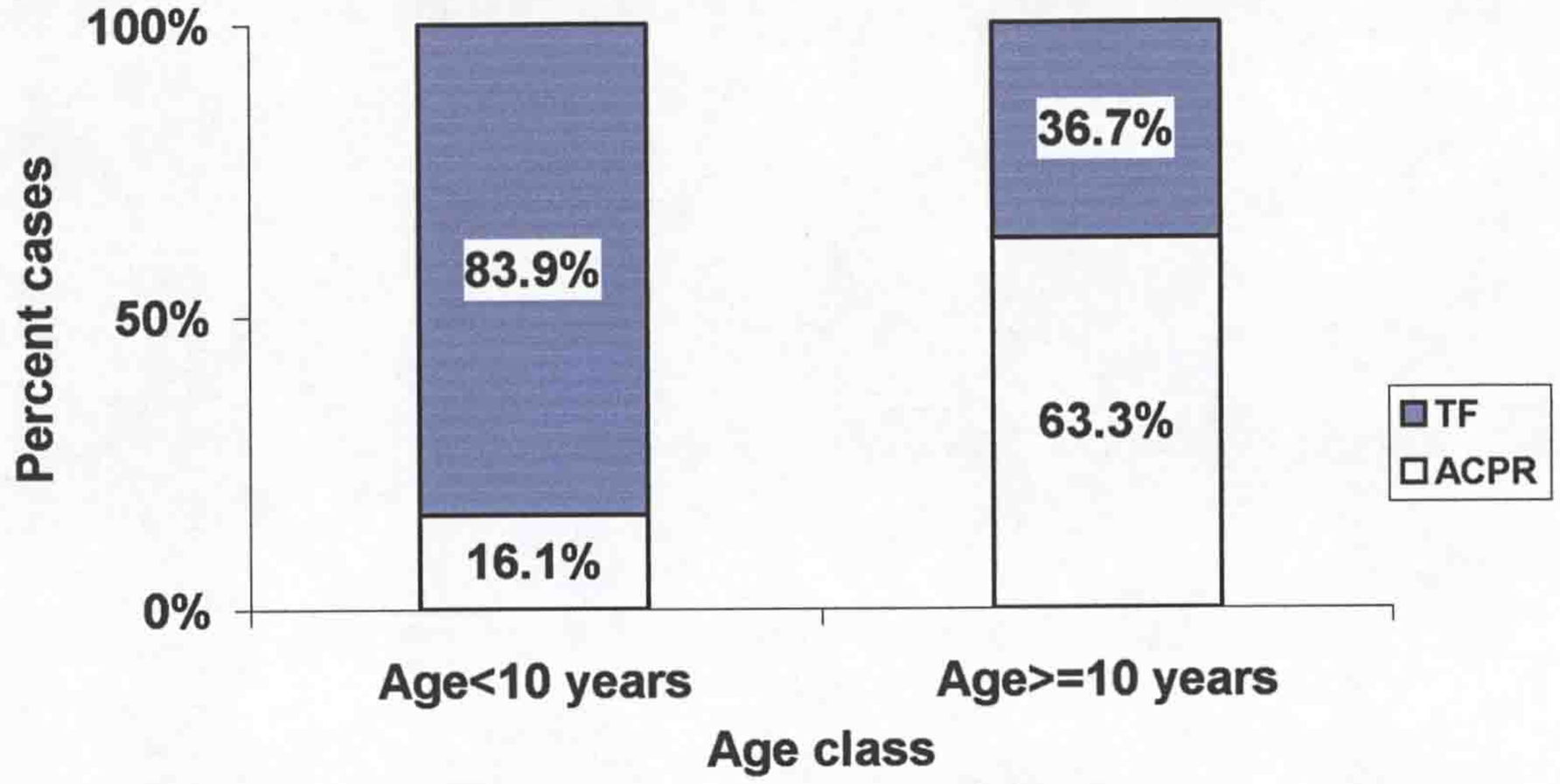


Figure 3.3 The effect of age on treatment outcome as found by multivariate analysis. TF=Treatment Failure (ETF+LCF+LPF), ACPR=Adequate Clinical and Parasitological Response

However, among the treatment failure group the presence of fever (axillary temperature ≥37.5°C) at presentation, in addition to younger age (<5 years) were found to be strong independent predictors of early treatment failure compared to late treatment failure (OR=5.7, 95% CI=1.96-16.58 and OR=3.42, 95% CI=1.04-11.21) for temperature and age respectively (**Figure3.4**).

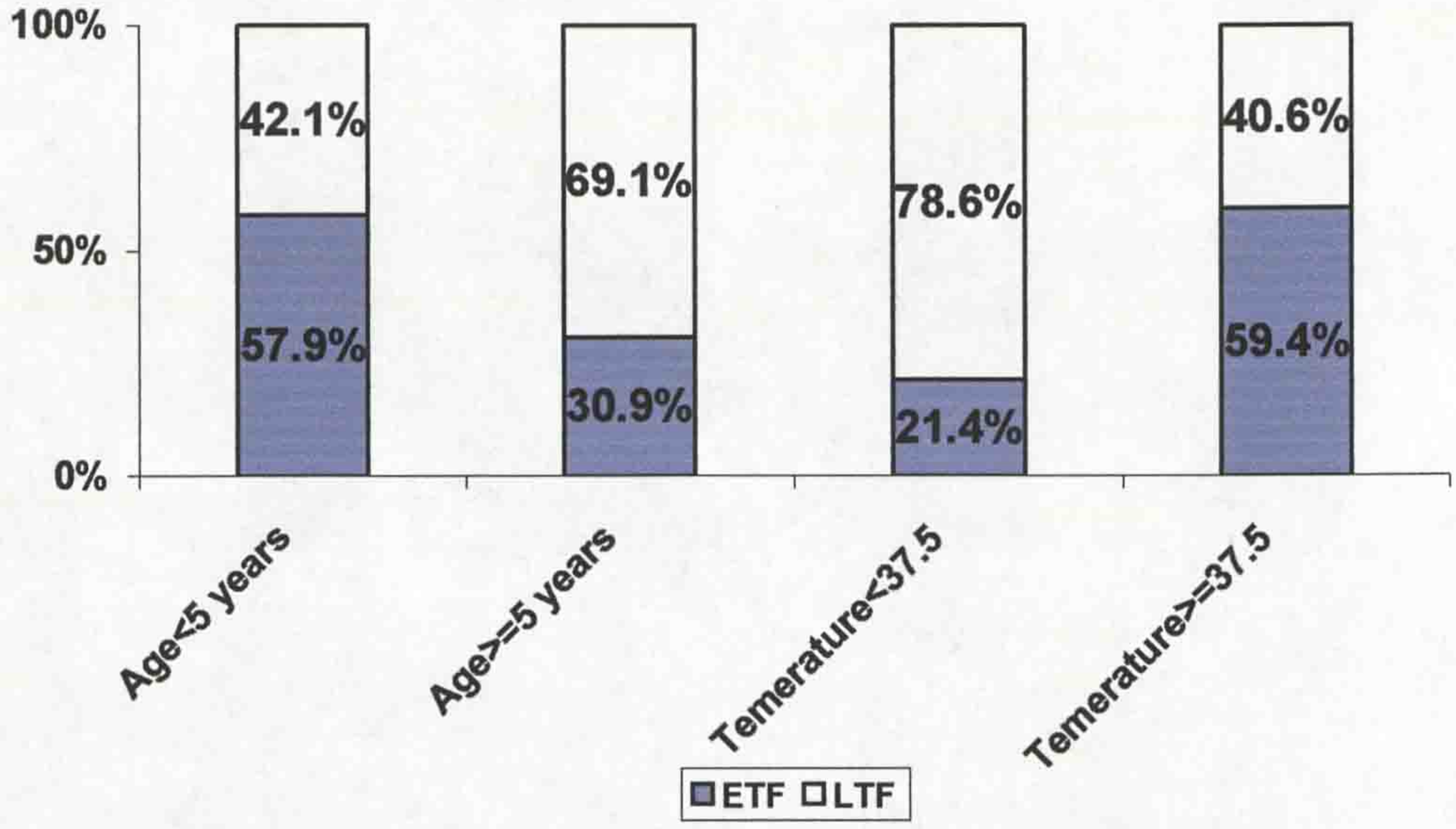


Figure 3. 4 Treatment failure stratified by age and body temperature. ETF=early treatment failure, LTF=late treatment failure including both late clinical failures (LCF) and late parasitological failures (LPF)

Among late treatment failures, children less than 10 years of age were significantly at higher risk of being late clinical failures, compared to late parasitological failures, than older children and adults (x^2 *p-value* $<.003$) (**Figure 5.3**)

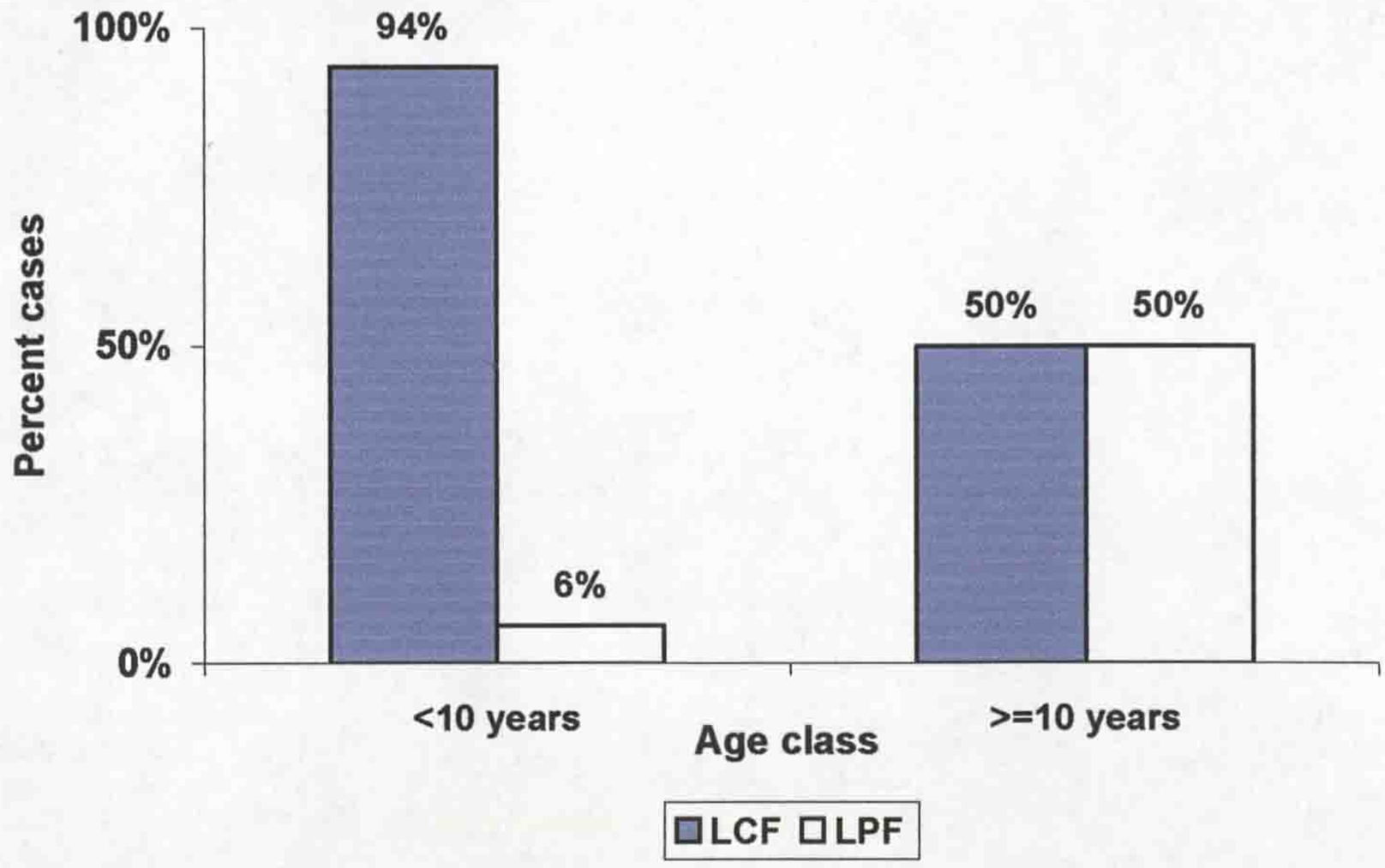


Figure 3.5 Association between age and late treatment failure categories. LCF=late clinical failure, LPF= late parasitological failure.

3.4 DISCUSSION

This study was conducted in collaboration with WHO/EMRO and the Ministry of Public Health represented by the National Malaria Control Program in Yemen with the main objective of establishing a practical system for monitoring therapeutic efficacy of antimalarials. The direct specific objective of the test was to determine the prevalence of CQ treatment failure and to compare it with the WHO thresholds for treatment failure (ETF plus LTF); grace (0 to 5%), alert (6 to 14%), action (15 to 24), and change (≥25%) in order to guide the antimalarial drug policy in the country. The intensity of malaria transmission in the area was not known and it was recommended by WHO/EMRO to use the standard 28-day *in-vivo* WHO draft protocol for assessment of therapeutic efficacy of antimalarial drugs for uncomplicated *falciparum* malaria, draft September 2002 (WHO, 2001c).

Possible bias that might be associated with this study has been taken in consideration during the preparation and implementation of the study. The most common source of bias in in-vivo tests is the follow-up loss, this has been avoided by 1) adding 10% expected follow-up loss to the originally calculated sample size (**Chapter 2, section 2.1.3**), 2) giving incentives to the patients (for transport) to come in the scheduled follow-up visits, and 3) rigorous follow-up of patients who did not show on the scheduled days. Selection bias, which is another important source of bias in this type of study, was not a great concern since there were no large differences between people living in the area with regard to education or socio-economic levels that might affect the treatment outcomes. Moreover, patients of all ages (>6 months) were included and the study was not restricted to only patients showing fever at presentation. The possibility of introducing selection bias through inclusion of febrile patients only is discussed below.

During the first two weeks of the test, it was noticed that treatment failure exceeded 25% of the estimated sample size required and more than 70% of those failures were early treatment failures and occurred in children less than five years. It was also noticed that recruited patients are almost exclusively less than 15 years of age and that most patients came to the hospital after taking antipyretic drugs like paracetamol

and more commonly oral or even parenteral diclofenac, which is an antipyretic and anti-inflammatory agent.

Accordingly, the area seemed to be a relatively high transmission area and the observations in the field led to the following suggestions:

- No need for 28 days of follow-up. A high level of in-vivo CQ treatment failure was expected and there was no concern of underestimating treatment failure rates to below the WHO recommended threshold (25%). Extending the follow-up to 28 days was not ethical and not cost-effective.
- The upper limit of parasite density at recruitment 100,000/µl of blood can be increased to 200,000 parasites/µl of blood, as long as no complications or danger signs encountered. This can help in reducing the possibility of underestimating the true prevalence of treatment failure due to the observed significant association between higher parasite densities and increased risk of treatment failure (Doherty *et al*., 1999), it also, avoids the exclusion of a substantial number of patients who would have been otherwise eligible, saving time for recruitment and consequently costs and logistics of the test.
- Inclusion of patients actively seeking treatment and having a history of fever during the last 24 hours but not febrile at the time of presentation, even though the area seemed to be a high transmission area, because it was noticed that it is a common practice in the area to take antipyretics and anti-inflammatory drugs to reduce fever and associated symptoms before going to the hospital. Restricting enrolment to only those with measured fever would result in the exclusion of many true cases of acute malaria thereby increasing the time, costs and logistics of the test.
- Inclusion of children >5 years and adults to get more comprehensive information by stratifying the results according to age.

Using the WHO outcome classification definitions (WHO, 2001c), (**Chapter 1, Table 1.4**), this study revealed a high proportion of chloroquine treatment failure, 60.7%, in Al-Musaimeer district in Lahj governorate in the south of Yemen where malaria is endemic and seasonal. Among treatment failures, Late Parasitological Failures (LPF) comprised 40% (30/74) followed by Early Treatment Failure 38% (28/74) while Late Clinical Failure (LCF) comprised only 22% (16/74) (**Figure 3.2**).

This high proportion of chloroquine treatment failure is reported for the first time in Yemen since it is higher than all the 14 previous chloroquine sensitivity studies performed between 1986 and 2002 in 5 different governorates in Yemen.

Comparing the result obtained from this study with results of other studies from within the country or from different countries is difficult due to variability in study methods, period of follow-up, type of subjects recruited, and definition of outcomes. For example the method used for measuring temperature to define fever as either axillary temperature of ≥37.5°C or rectal or tympanic temperature of ≥38°C is not specified in some test protocols. Excluding young children from some studies and restricting the follow-up to 7 days may underestimate the true rate of treatment failure. The use of different classification definitions of treatment outcomes also leads to discrepant results making comparability of different studies difficult.

The results of this study were not classified using the old WHO 1973 protocol, which depends on parasitological treatment outcomes only because this protocol has been distorted and adapted many times. At least 5 different definitions of RI-RII-RIII are available, which further complicates data comparability between studies (Ringwald, 2004). The new classification that has been used in this study (WHO, 2001c) is more appropriate for symptomatic patients and includes both clinical and parasitological criteria.

Out of the 14 in-vivo studies conducted in Yemen between 1986 and 2002, 11 were 7-day tests and 3 were 28-day tests. The three 28-day in-vivo test studies were conducted in Taiz governorate (1987), Hodeida governorate (1991) and Lahj governorate (1995). They reported the following results: 3% early RI/RII in Taiz, 17% early RI/RII in Hodeida and 12.9% early RI/RII in Lahj. However, all the previously conducted in-vivo studies were non-systematic and used a test protocol developed only to evaluate the parasites response to treatment rather than evaluating the therapeutic outcome (WHO, EMRO, 1999).

Recently, two other CQ in-vivo sensitivity studies were conducted in two other sentinel sites, both used the protocol for assessment of therapeutic efficacy of

antimalarial drugs for uncomplicated *falciparum* malaria, draft September 2002 (WHO, 2001c). Both studies were conducted between 2002 and 2003, and both had also a period of follow-up 14 days. One of the 2 studies was conducted in Bajel in Al-Hodieda governorate in the west of Yemen along the Red Sea (**Figure 1.16**) where malaria is considered to be meso to hyperendemic with the season of transmission mainly during the winter (from October to March). The other study was conducted in Al-Odein in Ibb governorate (**Figure 1.16**) in the foothills and middle altitude epidemiological region (**Chapter 1, section 1.8.1**) where malaria is mainly perennial or following rainy seasons that occur mainly in summer.

In the Bajel study, a hundred and six subjects were included of which 3 were lost and 4 were withdrawn from the test. Of the 99 who completed the follow-up 42 (42%) were treatment failures with LPF comprising 60% (25/42) of all treatment failures followed by ETF of 26% (11/42) and LCF of 14% (6/42) (National Malaria Control Program, 2005). In the Al-Odein study, 106 cases were also included and 101 completed the 14-day follow-up (5 cases were withdrawn). Of the 101 cases who completed the follow-up, 47 (47%) were treatment failures of which 64% (30/47) were LPF, 32% (15/47) were ETF and only 4% (2/47) (National Malaria Control Program, 2005). However, it is not known if history of fever during the last 24 hours was taken in consideration when classifying the treatment outcome, especially LCF. There is also no further information or analysis with regard to patient baseline characteristics in both studies.

Two in-vitro sensitivity studies were also conducted in Yemen, one in Lahj (1988) and the other in Hodieda and Taiz (1989). In the first study, 15 patients were enrolled out of which no resistant isolates were detected, whereas in the second study, 7 resistant isolates were detected; 6 in Hodieda and 1 in Taiz (National Malaria Control Program, Yemen, 2002).

In Somalia, two CQ in- vivo studies were conducted in Merca and Gabiley areas of high and low transmission of the disease respectively (Warsame *et al.*, 2002). They were both 14-day follow-up tests where all ages were included. According to WHO, 1996 protocol CQ treatment resulted in clinical failure of 33% and 51% of the patients in Merca and Gabiley respectively with corresponding parasitological

failures of 77% RII/RIII and 35% RII/RIII. Another 14- test was conducted in Tamale in northern Ghana (Ehrhardt *et al.*, 2002), where only children under five years were included. The WHO, (1996) classification definitions were also used together with the parasitological definitions (RI-RII-RIII). The study resulted in 29% treatment failure and 57% parasitological failure RI/RII/RIII. However, these results can't be compared with those of Somalia due to different inclusion criteria with regard to age and different measure for definition of parasitological failure (including RI in the definition of parasitological failure in the Ghana study but not in the Somalia study).

Talisuna *et al.*, (2004a) have reviewed and summarized the in vivo studies conducted in Africa, Asia and South America between 1996 and 2002 in an attempt to show general geographic differences in the prevalence of resistance to CQ and SP. The recent (1996 to 2002) prevalence and distribution of CQ in Africa, Asia, and South America are presented in **Tables 3.4, 3.5, and 3.6** respectively.

Region	Country	No. of data points identified	Period	Age range (yr)	Outcome classification	Period of follow-up (days)	Median %CQ-PF (range)[a]	Median %CQ-TTF (range)[a]
Central	Central African Republic	1	1998	0.5-5	WHO 1996	14		64
	Gabon	1	1996-1998	1-55	WHO 1973	14	19	
	Mozambique	1	1999	<5	WHO 1973, 1996	28	58	26
	Zambia	7	1998-2000	0.5-5	WHO, 1973	14, one study 7	55 (33-70)	42 (22-54)
	Zimbabwe	8	1996-1999	NS[b]	WHO 1973	7	27 (8-33)	
Southern	Botswana	3	1998	0.5-5	WHO 1996	14		25 (21-44)
Eastern and great lakes	Burundi	1	1998	0-14	WHO, 1973	7	41	
	Ethiopia	5	1998	0.5-5	WHO 1996	14		79 (51-93)
	Ethiopia combined with Eritrea	5	1989-1991	1-80	WHO 1973	7	88 (82-94)	
	Kenya	3 for clinical, 1 for parasitologic outcomes	1996-2001	0.5-5 for clinical, 2-12 for parasitologic outcomes	WHO 1996, WHO 1973	14, 28	71	64 (32-87)
	Kenya	1	1996-	All ages	Parasite	14	18	

Region	Country	No. of data points identified	Period	Age range (yr)	Outcome classification	Period of follow-up (days)	Median %CQ-PF (range)[a]	Median %CQ-TTF (range)[a]
			2001		clearance at day 14			
	Rwanda	6 clinical, 4 parasitologic	1999-2000	<5	WHO 1996	14	81 (73-88)	52 (19-60)
	Tanzania	9	1998-2002	0.5-5	WHO 1996, day 14 parasite failure	14	55 (11-98)	42 (17-71)
	Tanzania	2	1998-2002	1-14	WHO 1973, day 14 parasite failure	14, 28	55 (11-98)	42 (17-71)
	Uganda	17 clinical, 15 parasite outcome	1996-2000	0.5-5	WHO 1973, 1996	14	41 (10-96)	28 (9-89)
	Zanzibar	2	2000-2001	<5	WHO 1996	14		60-61
West	Burkina Faso	4 clinical, 2 parasite outcome	1998-2000	0.5-5	WHO 1973, 1996	14	42 (18-65)	18 (12-23)
	Cameroon	4	1994-2001	1-55	WHO, 1973	7, 14	31 (8-53)	
	Cote d'Ivoire	1	1996	>10	WHO 1973	28	33	
	The Gambia	1	1994-1996	0.5-10	Day 7 and 28 parasite failures	28	23, 50	
	Ghana	2	1999-2000	0.5-5	WHO 1973, 1996	14	58 (56-61)	41 (29-53)

Region	Country	No. of data points identified	Period	Age range (yr)	Outcome classification	Period of follow-up (days)	Median %CQ-PF (range)[a]	Median %CQ-TTF (range)[a]
	Guinea-Bissau	1	1998	0.5-5	WHO 1996	14		11
	Mali	5	1997	<5	WHO 1973, 1996	14	25 (20-30)	21 (18-24)
	Nigeria	1	1997	0.5-5	WHO 1973	14, 28	64	
	Nigeria	2	2001	1-12	WHO 1973	14, 28	31 (21-42)	
	Nigeria	2	1996	All	WHO 1973	14, 28	27 (13-54)	
	Senegal	4	1996-1998	1-55	WHO 1973	14	42 (24-59)	13 (10-16)
Northern	Somalia	2	2002	All	WHO, 1996	14	56 (35-77)	42 (33-51)
	Sudan	1	1994-1995	>5	WHO 1973	14	10	

Table 3.4 *P. falciparum* CQ treatment failure in vivo for selected countries in Africa, 1996 to 2002 (Source: Talisuna *et al.*, 2004a)

[a]CQ-PF, CQ parasitologic failure (either R1-R3 or cumulative parasite failure); CQ-TTF, clinical failure (either ETF + LTF or cumulative clinical failure).
[b]NS, not stated.

Region	Country	No. of data points identified	Period	Age range (yr)	Outcome classification	Period of follow-up (days)	Median %CQ-PF (range)[a]	Median %CQ-TTF (range)[a]
East Asia	China	1	1995-1996	6-60	WHO 1973	28	91	
	Laos	1	2001	>1	WHO 1973	28	46	
	Myanmar	2	1995	All	WHO 1973	28	82 (77-86)	
	Indonesia	5	1995-1999	≥5	WHO 1973	28	38 (25-82)	
	Malaysia	1	1996	5-51	WHO 1973	28	64	
Pacific	Philippines	1	1995	5-67	WHO 1973	28	38	
	Solomon Islands	1	1994-1996	5-14	WHO 1973	7	23	
Indian subcontinent	Bangladesh	1	1996-1997	12-60	WHO 1973	28	77	66
	India	1	2000	16-72	WHO 1973	7	82	
	Pakistan	1	1994	All	WHO 1973	28	46	
Middle East	Afghanistan	3	1999	>1	WHO 1973	28	69 (60-83)	
	Saudi Arabia	1	1997-1998	1-75	WHO 1973	14	12	
	Saudi Arabia	1	1997-1998	1-12	WHO 1973	28	18	

Table 3.5 *P. falciparum* CQ treatment failure in vivo for selected countries in Asia, 1996 to 2002 (Source: Talisuna *et al.*, 2004a).

Region	Country	No. of data points identified	Period	Age range (yr)	Outcome classification	Period of follow-up (days)	Median %CQ-PF (range)[a]	Median %CQ-TTF (range)[a]
South America/Caribbean	Colombia	3	1998	1-65	WHO	14, 28	50 (44-56)	44 (25-61)
	Guyana	1	1998	>5	Day 7, 14, and 28 cumulative parasite failure	28	33, 48, 56	
	Venezuela	1	1999	All	WHO, 14, 28	28		100
	Venezuela	2	1999	14-60	WHO, 14, 28	28	34 (20-47)	

Table 3.6 *P. falciparum* CQ treatment failure in vivo for selected countries in South America, 1996 to 2002 (Source: Talisuna *et al.*, 2004a)

[a]CQ-PF, CQ parasitologic failure (either R1-R3 or cumulative parasite failure); CQ-TTF, CQ clinical failure (either ETF + LTF or cumulative clinical failure).

Fever (axillary temperature ≥ 37°C) at presentation was present in only 35% of the cases, though most of them were children <15 years. This might be explained by the common practice of using antipyretics like paracetamol and diclofenac, it also could be explained by partial immunity acquired by the people in the area due to repeated exposure to the disease resulting in infection without fever. This is consistent with the finding that LCF comprised only 22% of all treatment failures compared to 40% LPF, which is also supported by the same finding of low prevalence of LCF in the other 2 studies in Bajel and Al-Odein.

This finding of high asymptomatic post-treatment infections (LPF) may reflect the transmission intensity in the area that leads to the development of partial immunity. It has been proposed that the development of partial immunity occurs in two stages: first, anti disease immunity develops protecting against symptomatic malaria followed by anti-parasite immunity responsible for reduction of parasite density (Rogier and Trape, 1993). While the risk of infection increases with transmission intensity, the risk of symptomatic disease per infection is inversely related to the intensity of transmission and age dependent acquisition of partial immunity (Rogier *et al.*, 1999). The finding of significantly higher risk of LCF in children below the age of 10 (**Figure 3.5**) is consistent with this explanation.

A practical implication of the finding that the prevalence of asymptomatic post-treatment infections (LPF) is high in this and other studies in areas of high transmission is that the use of WHO treatment outcome classification (WHO, 1996) (**Chapter 1, table 1.3**) clearly underestimates the true rate of treatment failure. For example if the WHO, 1996 classification criteria were used in this study, all the LPF group (25% of the overall treatment outcome) would be regarded as adequate response (ACR) due to the absence of fever hence the overall treatment failure would be 3 6% c ompared t o 6 1% a dequate r esponse (ACPR) w ith t he n ew c lassification, this a lso i llustrates t he d ifficulty o f c omparing studies w ith d ifferent c lassification criteria. While it can be argued that asymptomatic post treatment infections (LPF) are not clinically important in areas of high transmission because of common re-infection, the failure to clear infection completely may result in more frequent clinical episodes and chronic anaemia, contributing to morbidity and mortality (Zucker *et al.*, 1996).

Hyperparasitaemia (>100,000 asexual parasites/µl) was uncommon in the area. In general, only two patients had asexual parasite counts above 100,000/ul of blood at presentation, both were children less than five years, one responded with ETF at day 3 and the other responded with LCF at day 7. This also supports the conclusion that there is a high level of acquired immunity among the population in the area that prevents the acquisition of high parasite counts; this is "anti-parasite immunity".

Children below 5 years of age were only 18% of the enrolled cases. This finding has two possible explanations; 1) most children under 5 years were excluded from the study due to the presence of other concomitant febrile illness (15% of the excluded cases), 2) nature of father's job; since most fathers work either as farmers or most commonly in the capital of the governorate and so were not available to bring their children during the day time when mothers give the children antipyretics. The number of children less than five years in the area is not known.

It was found that the median age of persons seeking treatment for malaria and meeting the case definition of the study was 9.5, so restriction of the study to children under the age of five would provide no information on efficacy for much of the population suffering from the disease in this setting

Univariate analysis of some possible predictors of CQ treatment failure showed that age less than 10 years, fever at presentation (axillary temperature $\geq 37.5^{o}C$) and parasite count ≥25000 asexual parasites/µl of blood at presentation were significantly associated with treatment failure (p=0.000, 0.022, 0.025 respectively), however the lower limits of the 95% confidence intervals for both fever and parasite count were close to one (**Table 3.3**). The body weight was also significantly associated with treatment failure. As the body weight increased the risk of treatment failure was decreased (OR= .89 and 95% CI= .85-.93)

Multivariate analysis confirmed that young age is a strong, independent predictor of CQ treatment failure in this study (**Figure 3.3**). Children less than 10 years were eight times more likely to be treatment failure than older children and adults (OR=8.7). According to the 95% confidence intervals, the analysis showed that children <10 years were at least 3.6 times more likely to be treatment failure than

older children and adults (95% CI=3.6-21.01) However, the association between fever and the parasite count at presentation with treatment failure lost its statistical significance in multivariate analysis, which may be explained by the interaction between age with both fever and high parasite count due to the level of acquired immunity. The body weight was dropped from multivariate analysis due to its strong correlation with age.

Moreover, in multivariate analysis, among the treatment failures, febrile patients at presentation (axillary temperature ≥37.5°C) were at least 2 times more likely to be early treatment failure than older children and adults (OR=5.7 95% CI=1.96-16.58). In the same analysis, it was also found that those who were less than five years were significantly more likely to be early treatment failure than older children and adults though the lower limit of the 95% confidence intervals was close to one (OR=3.42 95% CI=1.04-11.21) (**Figure 3.4**).

The argument that the ETF might be overestimated in the WHO protocol though has its explanation and evidence (Plowe *et al.*, 2001; White, 2002), should be investigated carefully in children under five years. Having fever and parasitaemia in day 2 greater than day 0 in children under five years may lead to the development of severe malaria or danger signs with complications and sequelae if not managed appropriately. In this study 42% of patients having ETF on day 2 were children under five, they had more than double the parasite count of day 0.

The association between fever at presentation and treatment outcome has a practical implication in that temperature-based inclusion criteria could be a source of selection bias since it favours treatment failure outcome. This is consistent with the conclusion obtained by Dorsey *et al*, (2000). Analysis of the prevalence of treatment failure in only febrile patients at presentation in this study would result in 74.4% (32/43) treatment failure, which is an overestimation of the rate of treatment failure. Thus, recognition of this inclusion criterion is important when comparing the results of in-vivo studies.

The age association with treatment outcomes is also consistent with the findings in most studies in other countries e.g, Uganda (Dorsey *et al*, 2000), Ghana (Ehrhardt *et*

al., 2002), Indonesia (Sutanto *et al.*, 2004). This association between age and treatment outcome is usually observed in high transmission areas and reflects the state of high, acquired malaria immunity due to repeated exposure to the infection. In endemic areas with high levels of resistance, where the drug has no effect, host defence alone could control the infection (White, 2002). This is consistent also with studies in asymptomatic *P. falciparum* carriers, which showed that subjects in endemic countries do clear some of their malaria parasites without any antimalarial treatment in an age dependent manner (Franks *et al.*, 2001).

In less immune vulnerable young children the same drug-resistant infections are usually not cured, persisting for weeks or months, causing anaemia or expanding rapidly to fulminant disease. Thus, in high-transmission settings, a high adequate treatment response may merely mean that the host defence is adequate, not the drug treatment. This can mask high level drug resistance and potentially lethal treatment failures, when ineffective drugs are used unsupervised in the rural tropics (White, 2002).

This sentinel site (Al-Musaimeer sentinel site) was chosen to be a perminant sentinel site for monitoring the efficacy of antimalarial drugs. The research team participated in this study (phyisician, microscopist, nurse, clerk and a driver), had been trained on the use of standard WHO protocol for monitoring the efficacy of antimalarial drugs. Difficulties encountered during the implementation of this test including the process of inclusion of cases, proper case identification, case definition, definition of treatment outcomes, follow-up of cases, parasite counts, possible biases that might be introduced to the study. All those factors and their implications on the validity of data had been discussed with the research team. At the end of the study the research team was able to implement further in-vivo efficacy studies to monitor antimalarial drug resistance. The same team was responsible for two more efficacy studies of antimalarial drugs (for SP and AQ) in 2004 in Al-Musaimeer sentinel site.

CHAPTER 4

Studies On The Molecular Markers Of Chloroquine Resistance (*pfcrt*-K76T), Sulfadoxine-Pyrimethamine Resistance (*dhfr*-59 and *dhps*-540) And The Newly Discovered Mutation (*pfcrt*-S163R), In Al-Musaimeer District, Lahj Governorate, Yemen

4.1 INTRODUCTION

Despite the difficulties encountered in demonstrating the correlation between molecular markers and clinical treatment outcomes, molecular markers that can predict with reasonable reliability resistance to SP (Kublin *et al.*, 2002) and chloroquine (Djimde *et al.*, 2001a) are now available. K76T is established as a useful molecular marker for CQ-resistance that can be easily applied in field studies (Djimde *et al*, 2001b). However, this association between the presence of K76T and in-vivo CQ treatment failure was not perfect in some other studies suggesting the contribution of other factors like immunity and the presence of other unknown contributing mutations in *pfcrt* or other genes.

Prevalence rates of molecular markers for resistance have been found to be higher than the prevalence of in-vivo drug resistance in nearly all studies. Djimde *et al.*, (2001a) have addressed this problem in Mali by calculating ratios between rates of the chloroquine-resistant *pfcrt* genotype and therapeutic and parasitological outcomes. A genotype resistance index (GRI) and genotype failure index (GFI), derived by computing the ratio between the age-adjusted frequency of the *pfcrt* mutation at position 76 and the prevalence of parasitological and clinical CQ resistance, respectively, have been proposed for the surveillance of CQ resistance. After controlling for age, both GRIs and GFIs ranged from 1.6 to 2.8 at all study sites over the three-year period (Djimde *et al.*, 2001b), indicating that the prevalence of clinical or parasitological failure was two to three times lower than the prevalence of the gene mutation.

For SP resistance, there is growing evidence that the *dhfr* triple mutations with or without the *dhps* mutations can predict SP treatment failure. The triple (*dhfr* Asn-108, Ile-51, and Arg-59) mutant genotype was associated with SP treatment failure (Mutabingwa *et al.*, 2001; Nzila *et al.*, 2000). However, a study in Malawi observed that the quintuple mutant genotype (*dhfr* Asn-108, Ile-51, and Arg-59 plus *dhps* Gly-437 and Glu-540) was more strongly associated with SP treatment failure than was the *dhfr* triple mutant (Kublin *et al.*, 2002). Two mutations, *dhfr* Arg-59 and *dhps* Glu-540, were found to predict the presence of all five (quintple) *dhfr* and *dhps* mutations accurately (Kublin *et al.*, 2002; Kyabayinze *et al.*, 2003). In Uganda, the *dhfr* codon 59 M/W ratio, but not the prevalence of infections with the *dhfr* codon 59

mutation, was positively correlated with SP treatment failure in Ugandan sentinel sites (Talisuna *et al*., 2003).

Recently, Johnson *et al*., (2004) showed that stepwise selection for resistance to amantadine or halofantrine produced previously unknown *pfcrt* mutations (including S163R, **Figure 1.13**), which were associated with lose of VP-reversible CQ resistance. This was accompanied by a restoration of efficient CQ binding to haematin in these selected lines.

A non-culture-adapted Southeast Asian isolate (Pf164) was similar in its sequence to K1, However, it was found to contain an additional mutation (S163R). It was found to have a CQ IC_{50} value of 21 nM recorded with the WHO microtest, which classify it as CQS. Investigating the implications of this finding for surveillance protocols that use K76T as a marker for Chloroquine-resistant *P. falciparum* is now important, particularly in areas where halofantrine or mefloquine have been widely used (Johnson, 2003).

As a complementary tool, and not as a replacement for in vivo tests, molecular markers could be used for large-scale mapping and estimation of parasite resistance. In vivo tests could then be conducted in sites chosen on the basis of the molecular data to estimate more precisely the actual prevalence of resistance (Talisuna *et al*., 2004).

This chapter has the following objectives:

- To validate the use of PfCRT K76T as a molecular marker for CQ resistance in Yemen.
- To predict sulphadoxine/pyrimethamine resistance using dhfr-59 and dhps-540 mutations in order to guide malaria drug policy.
- To detect the possible presence of the newly discovered PfCRT mutation S163R in Yemen.

4.2 MATERIALS AND METHODS

4.2.1 Sample Collection And DNA Extraction

Finger-prick blood samples were collected from each patient participating in the in-vivo test (**Chapter 3**) on two types of filter papers filter papers (ordinary Whatman No. 3 filter papers and FTA® Cards, **Chapter 2 Figure 2.7**). Samples were collected before and after treatment. For method of collection and storage of blood samples please refer to **Chapter 2, section 2.2.1**.

To extract DNA from blood spots on ordinary Whatman No. 3 filter papers and FTA® Cards, three methods were tested on both types of filter papers. Extracted DNA using the three different methods was used to amplify the *pfcrt* gene to choose the most appropriate, convenient, practical, and cost-effective method. Full description of the three methods; the methanol fixation-heat extraction method, the FTA purification reagent and the QIAGEN kit extraction method is found in **chapter 2, sections 2.2.2.1 to section 2.2.2.3**

4.2.2 Detection Of Mutations At pfcrt, pfdhfr And pfdhps

The polymerase chain reaction (PCR) method followed by restriction enzyme digestion was used to detect mutations in a number of codons in *pfcrt* (K76T and S163R), *pfdhfr* (C59R) and *pfdhps* (K540E). Amplification reactions were carried out using a Biometra® Personal Cycler™. Digestion products were analysed using 2-3% agarose or MicroSieve 3:1 agarose (*Flowgen*) gel stained with ethidium bromide. Separated products were visualised by UV transillumination **(chapter 2, sections 2.2.4)**

4.2.2.1 Detection of mutations at *pfcrt* (K76T and S163R)

To detect Pfcrt polymorphism at codon 76, two alternative methods developed by Djimde et al., (2001a) were tested .One method is based on a nested PCR followed by restriction enzyme digestion *PCR/RFLP* **(chapter 2, sections 2.2.5.1)** and the other method is a nested mutation- specific PCR *MS-PCR* using mutation- specific primers that detects either the wild-type codon K76 or the mutant-type codon T76 **(chapter 2 , s ections 2 .2.5.2)**. The PCR reaction conditions used are described in **(Chapter 2, section 2.2.5).** Primer names, sequences and PCR cycling conditions used in the two methods are found in **(Chapter 2, Table 2.2 and Table 2.3).**

Detailed methodology for the detection of PFCRT 76 polymorphism is also available at *http://medschool.umaryland.edu/CVD/plowe.html*

For the detection of the newly discovered mutation S163R in *pfcrt* PCR was performed on pre-treatment samples of patients who despite the presence of pfcrt-K76T in their pre-treatment samples did respond adequately to CQ (classified as ACPR with the in-vivo test). The sample size required to determine the prevalence of pfcrt-S163R in 42 pre-treatment samples (those who carried the pfcrt-K76T but responded adequately to CQ) was calculated using statcalc, *epiinfo* version 6, with 50% expected prevalence of S163R, 10% worst acceptable error at 95% confidence level. Accordingly 30 randomly selected pre-treatment samples were studied using the PCR protocol developed by David Johnson (Johnson, 2003). The method is based on a nested PCR followed by restriction enzyme digestion *PCR/RFLP*. For full description of the method please refer to **Chapter 2, Sections 2.2.6.1 and 2.2.6.2**. Primer names, sequences and PCR cycling conditions used to detect pfcrt S163R are also found in **Chapter 2, Table 2.4**

2.4.2.2 Detection of *pfdhfr* polymorphism at codon 59

Two different nested PCR reactions followed by restriction enzyme digestion were used to detect *pfdhfr*-59 polymorphism, one developed by Plowe *et al*., (1995) using *BsrG*I enzyme for restriction digestion *PCR/RFLP* (**Chapter 2, section 2.2.7.1**). Details of this method are also available at *http://medschool.umaryland.edu/cvd/2002_pcr_asra.html.* The other method was developed by (Duraisingh *et al.*, 1998) and uses *Xmn*I enzyme for restriction digestion (**Chapter 2, section 2.2.7.2**). Description of PCR reaction conditions is found in (**Chapter 2, section 2.2.7).** Primer names, sequences and PCR cycling conditions used for the detection of *pfdhfr* polymorphism at codon 59 using restriction enzyme digestion with *BsrG*I enzyme and *Xmn*I enzyme are listed in **chapter 2, Table 2.5 and Table 2.6** respectively.

2.4.2.3 Detection of *pfdhps* polymorphism at codon 540

A nested PCR reaction followed by restriction enzyme digestion *PCR/RFLP* developed by (Plowe *et al.*, 1995) was used to detect *pfdhps* plymorphism at codon 540 (**Chapter 2. sections 2.2.8.1 and 2.2.8.2**). Primer names, sequences and PCR

cycling conditions used for the detection of *pfdhps* polymorphism at codon 540 are listed in **chapter 2, Table 2.7**. The method is also available at *http://medschool.umaryland.edu/cvd/2002_pcr_asra.html*

4.3 RESULTS

4.3.1 Results Of Testing DNA Extraction Methods

The three methods of DNA extraction (methanol fixation-heat extraction method, the FTA purification reagent and the QIAGEN kit extraction method) gave the same results when extracted DNA was used to amplify the *pfcrt* gene. The same results were also obtained when amplifying *pfcrt* gene from DNA extracted from both ordinary Whatman No. 3 filter papers and FTA® Cards. **Figure 4.1** shows the results of the second round reaction of the nested PCR amplification of *pfcrt* using the method developed by Djimde *et al.*, (2001a) and using the primer pair CRTP1 and CRTP2 for the first round reaction and the primer pair CRTD1 and CRTD2 for the second round reaction. The same PCR protocol and PCR cycling conditions are used to amplify all samples.

Figure 4.1_ Amplification of pfcrt gene using DNA extacted by three different methods. Genomic DNA of laboratory 3D7 strain extracted from both ordinary filter papers and FTA® Cards was used to test the effectiveness of the three methods of DNA extraction in amplifying the PfCRT gene. Lane M is DNA molecular ladder. Odd numbered lanes are DNA extracted from FTA® Cards while even numbered lanes are DNA extracted from ordinary filters. Lanes 1 and 2 represent amplification of DNA extracted using FTA extraction reagent, lanes 2 and 3 represent amplification of DNA extracted using QIAGEN kit and lanes 5 and 6 are amplifications of DNA extracted using the methanol method.

Based on these results and for reasons discussed in **section 4.4** all subsequent reactions were carried out using the methanol-fixation heat-extraction method.

4.3.2 Results Of Testing Two PCR Protocols For The Detection Of *pfcrt* Polymorphism At Codon 76

Two PCR protocols for the detection of pfcrt-76 polymorphism (Djimde et al., 2001a), one based on a nested PCR followed by restriction enzyme digestion (PCR/RFLP) and the other was a nested mutation specific PCR (MS-PCR) were compared and the results of comparison in six selected samples are shown in **Table 4.1**

Sample no.	Parasite clone		Technique			
			MS-PCR		PCR/RFLP	
			Lys	Thr	Lys	Thr
1		Dd2	–	+++	–	++
2		Dd2	+/– (e)	+++	–	++
3	3D7		– (e)	–	++	–
4	3D7	Dd2	+++	+++	++	++
5	3D7		+++	+/– (e)	++	–
4	3D7	Dd2	+++	+++	++	++

Table 4.1 Comparison of the two methods used to detect pfcrt polymorphism at codon 76. The *Plasmodium falciparum* clone 3D7 contains the *pfcrt* allele Lys76) and Dd2 contains the *pfcrt* allele Thr76. MS-PCR=mutation-specific polymerase chain reaction, PCR/RFLP= polymerase chain reaction followed by restriction enzyme digestion at polymorphic sites. +++=very strong band, ++=strong band, +/–=faint band, –=no band, (e)=erroneous result.

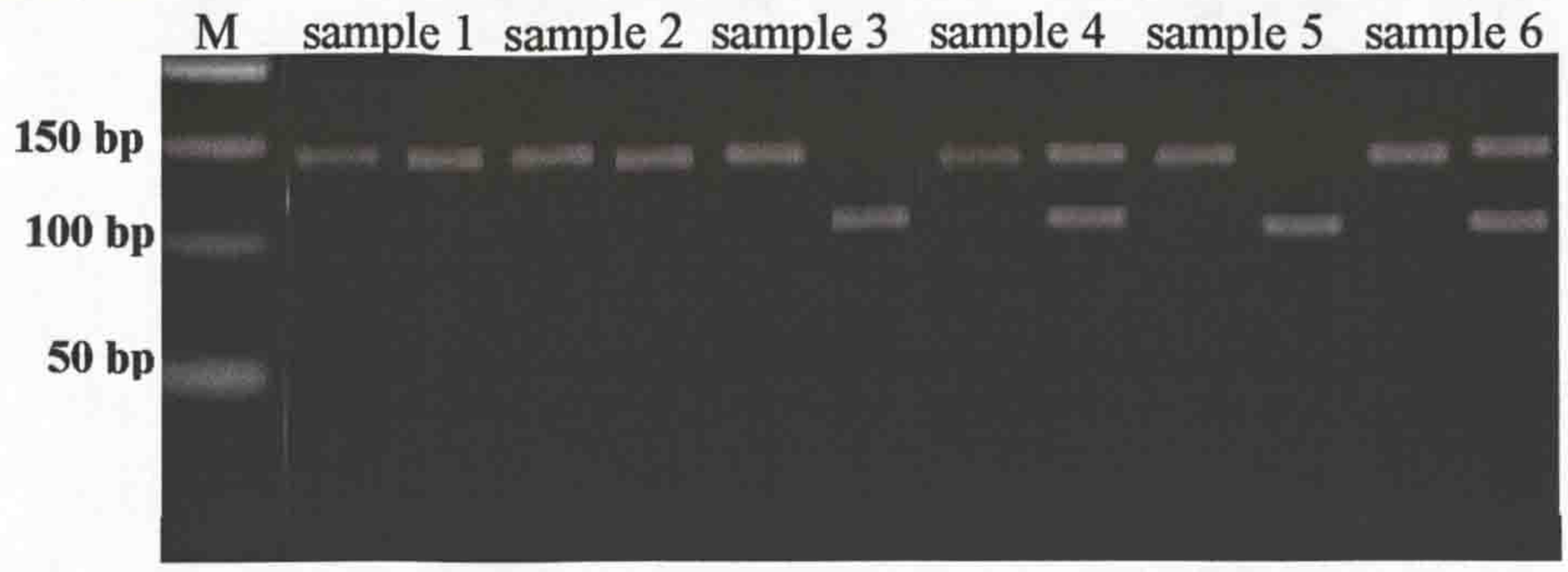


Figure 4.2 Detection of pfcrt-K76T using polymerase chain reaction followed by restriction enzyme digestion (PCR/RFLP). Samples run in 2% agarose gel before and after digestion in adjacent wells to allow for comparison. *Apo*1 enzyme should cleave the wild-allele Lys-76 but not the mutant-allele Thr-76. Sample 1 and 2 contained DNA from Dd2 with the mutant allele Thr-76, Sample 3 and 5 contained DNA from 3D7 with the wild-allele Lys-76, while samples 4 and 6 contained DNA from both Dd2 and 3D7 with both mutant and wild alleles.

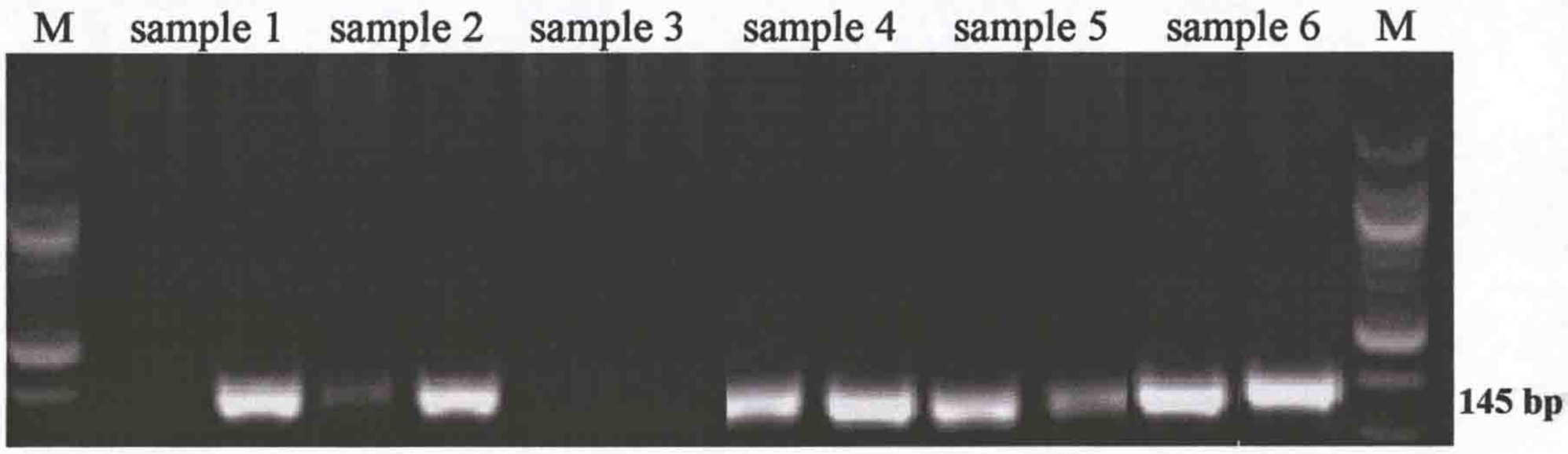


Figure 4.3 Detection of pfcrt-K76T using mutation specific-PCR (MS-PCR). Samples run in 2% agarose gel. Lanes M on both sides are 100 bp DNA molecular ladder. Each sample run in 2 wells, the first well detecting the wild allele Lys-76 while the next well detecting the mutant allele Thr-76. It was expected to see a band only in the first well of any sample containing DNA from only 3D7 and a band only in the second well of any sample containing DNA from only Dd2 while 2 bands were expected to be seen in the 2 wells (one in each well) of any sample containing DNA from both Dd2 and 3D7. Sample 1 and 2 contained DNA from Dd2 with the mutant allele Thr-76, Sample 3 and 5 contained DNA from 3D7 with the wild-allele Lys-76, while samples 4 and 6 contained DNA from both Dd2 and 3D7 with both mutant and wild alleles.

The results of comparing the 2 methods for detecting *pfcrt* polymorphism at codon 76 showed that the MS-PCR method had some problems and difficulties in interpretation of the results mainly due non-specific amplifications (sample 2 and 5) or false negative results (sample 3) resulting in erroneous result (see **Table 4.1 and Figure 4.3**). Subsequently it was decided to use the method based on nested PCR followed by restriction enzyme digestion (PCR/PFLP).

4.3.3 Analysis of pfcrt-76 Point Mutation

Samples of 112 patients of the 122 patients who completed the follow-up of the in-vivo test (**Chapter 3**) were tested for the detection of pfcrt-T76 polymorphism. PCR performed on samples obtained from patients before treatment with CQ (pre-treatment samples) and on samples obtained from patients after treatment with CQ on the day of classification (post- treatment samples).

4.3.3.1 Pre-treatment samples

DNA was successfully amplified from all the 112 pre-treatment samples with the following results; 109 (97.3%) carried the mutant T76 allele alone, 1 (0.9%) showed a mixed T76/K76 pfcrt genotype, and 2 (1.8%) contained only the wild K76 allele. Results did not show any pre-treatment negative sample (**Figure 4.4**)

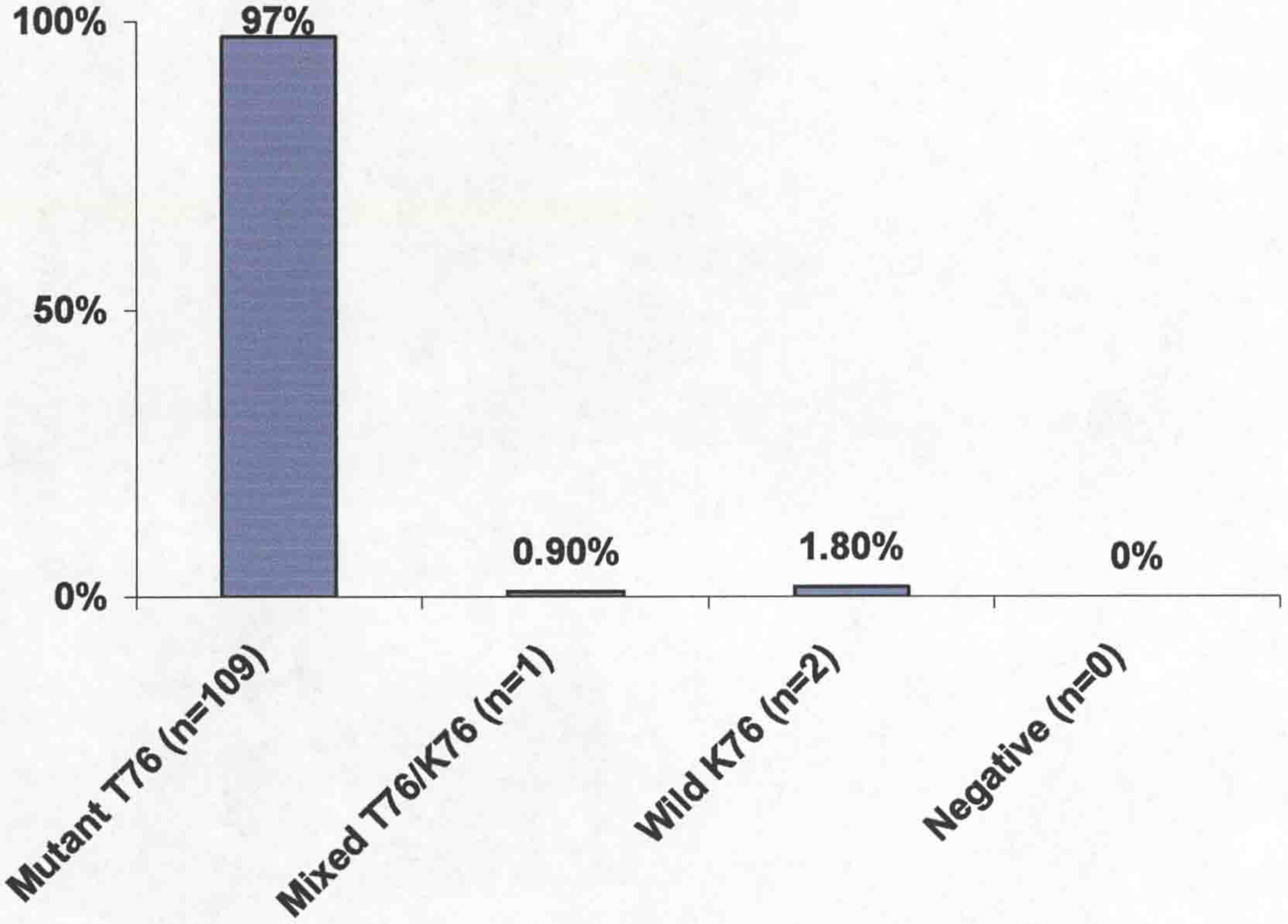


Figure 4.4 Distribution of pfcrt-76 polymorphism in pre-treatment samples.

4.3.3.2 Association between pfcrt-T76 point mutation in pre-treatment samples and treatment outcome

DNA of pre-treatment samples was available for 68 of the 74 cases that failed treatment with CQ. Analysis of pfcrt-76 polymorphisms in the pre-treatment samples of the 68 cases showed that 67 samples (98.5%) had only the mutant T76 allele. One sample (1.5%) had mixed T76/K76 genotype and none had the wild K76 allele alone. However, 42 of 44 cases (95.5%) who adequately responded to CQ and of whom DNA was available for analysis also appeared to have the mutant T76 allele alone in their pre-treatment samples (**Figure 4.5**). There was, therefore no association between the presence of either the mutant T76 or the wild pfcrt-K76

allele and the treatment outcome; *Fisher exact p-value=0.152* (mixed genotype was added to the mutant).

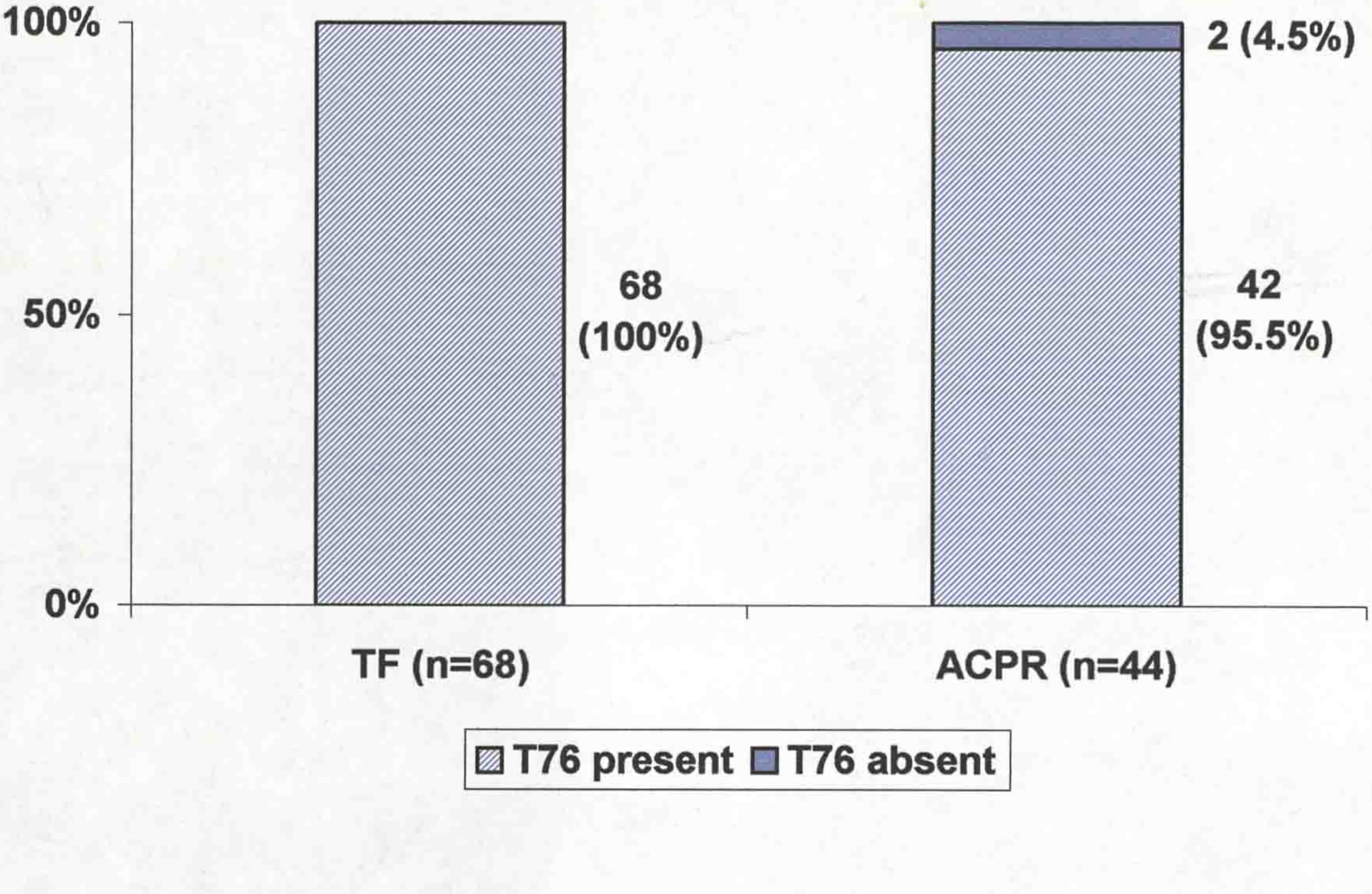


Figure 4. 5 Prevalence of pfcrt-T76 in pre-treatment samples of patients who failed CQ treatment (TF) and those who adequately respond to treatment (ACPR). TF=ETF+LPF+LCF

Parasites carrying the wild K76 allele before treatment were not able to survive CQ treatment, the only two pre-treatment samples that contained the wild K76 allele alone were belonging to patients who responded adequately to CQ. The prevalence of pre-treatment pfcrt-76 polymorphisms in different categories of treatment outcome is shown in **Table 4.2.**

Type of pfcrt-76 polymorphism	Treatment outcome				
	ACPR (%)	ETF (%)	LCF (%)	LPF (%)	All (%)
Pfcrt-T76 mutant-type	42 (95.5)	23 (100)	15 (100)	29 (96.7)	109 (97.3)
Pfcrt-K76 wild-type	2 (4.5)	0 (0)	0 (0)	0 (0)	2 (1.8)
Pfcrt-T76/K76 mixed-type	0 (0)	0 (0)	0 (0)	1 (3.3)	1 (0.9)
Total (%)	44 (100)	23 (100)	15 (100)	30 (100)	112 (100)

Table 4.2 Prevalence of *pfcrt*-76 polymorphisms of pre-treatment samples in different categories of CQ treatment outcomes.

The analysis showed that the pfcrt-T76 mutation was also prevalent (95.5%) in pre-treatment samples of patients who responded adequately to CQ treatment and classified in-vivo as ACPR (**Figure 4.5**)

4.3.3.3 Post-treatment samples

The amplification of the112 post-treatment samples resulted in 66 (58.9%) samples with the mutant (T76) allele, 4 (3.6%) with mixed pfcrt genotype, and 1 (0.9%) with the wild K76 allele alone while 41 (36.6%) post-treatment samples did not amplify (gave negative results, **Figure 4.6**). Excluding the negative results, the prevalence of T76 in post treatment samples (either alone or with the K76) was 98.6% (70/71), while only one sample (0.4%) was found to have the K-76 wild-type alone.

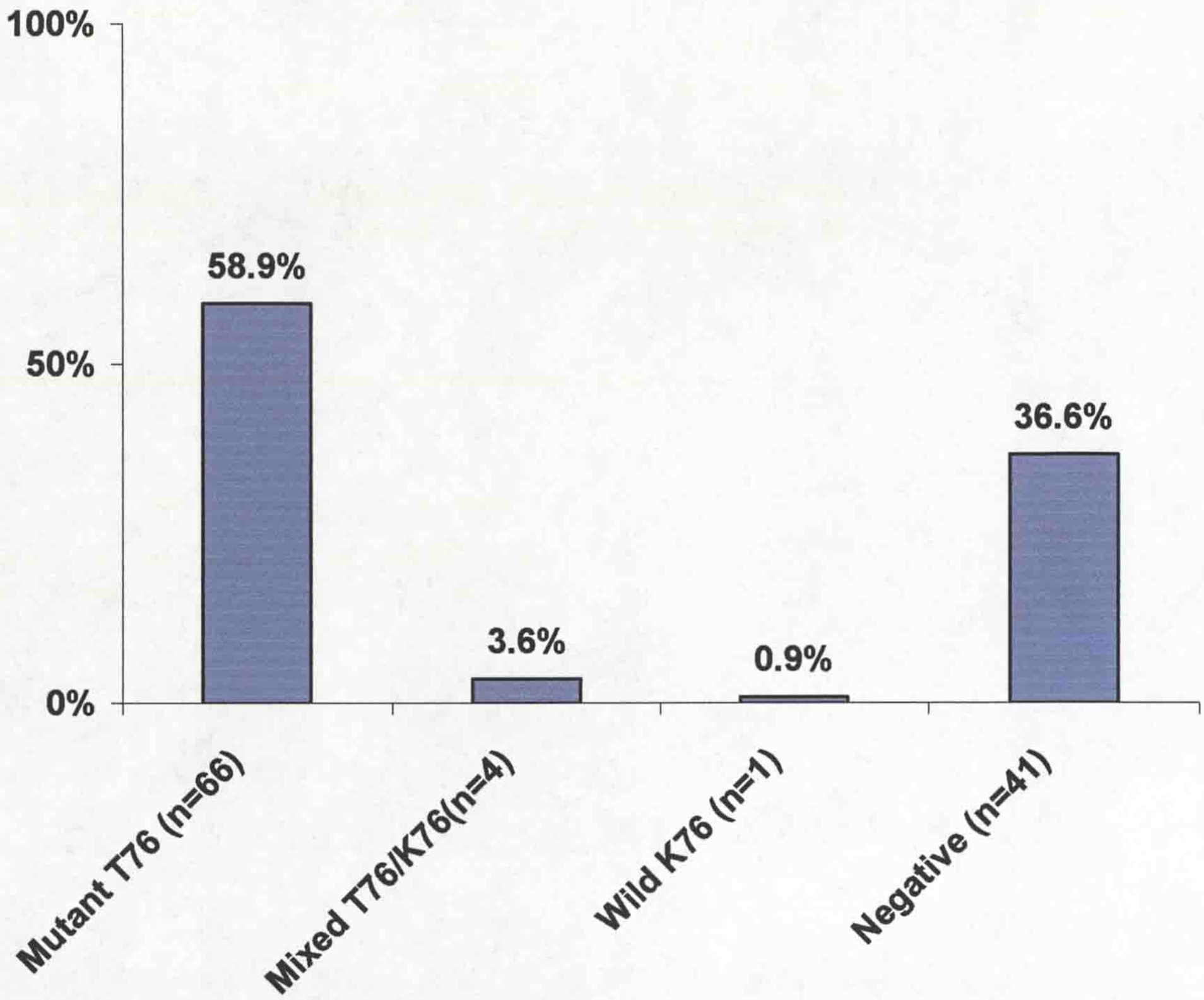


Figure 4.6 Prevalence of pfcrt-76 polymorphisms in post-treatment samples. Negative= no amplification.

The *pfcrt*-T76 was found in all post-treatment samples of patients who failed CQ treatment. Analysis of the post-treatment samples of patients who were classified as ACPR (responded adequately to CQ) showed that 13 samples of a total of 44 (29.5%) still had positive PCR results in day 14 after treatment (day of classification). The remaining 31 (70.5%) samples gave negative PCR results.

4.3.3.4 Effect of age on the association between pfcrt-76 mutation and outcome

The base-line prevalence of *pfcrt*-T76 (including the mixed T76/K76 genotype) 110/112 (98.2%) was higher than that of clinical chloroquine treatment failure 68/112 (60.7%) obtained by the in-vivo test. To determine whether partial immunity

developing with prolonged exposure to malaria contributed to the ability to clear infections caused by parasites carrying *pfcrt* T76, the proportion of infections caused by parasites carrying *pfcrt* T76 that cleared in children younger than 10 years of age were compared with the proportion of infections caused by parasites carrying *pfcrt* T76 that cleared in older children and adults.

In the younger group (<10 years), only 13% (7/55) of pre-treatment infections by parasites carrying the T76 mutation were successfully cleared after taking chloroquine, whereas in the older group 65% (35/54) of pre-treatment infections by parasites carrying T76 mutation were cleared after taking the drug, odds ratio= 0.04, 95% CI= 0.01-0.12, x^2 *p-value=0.000* (**Figure 4.7**).

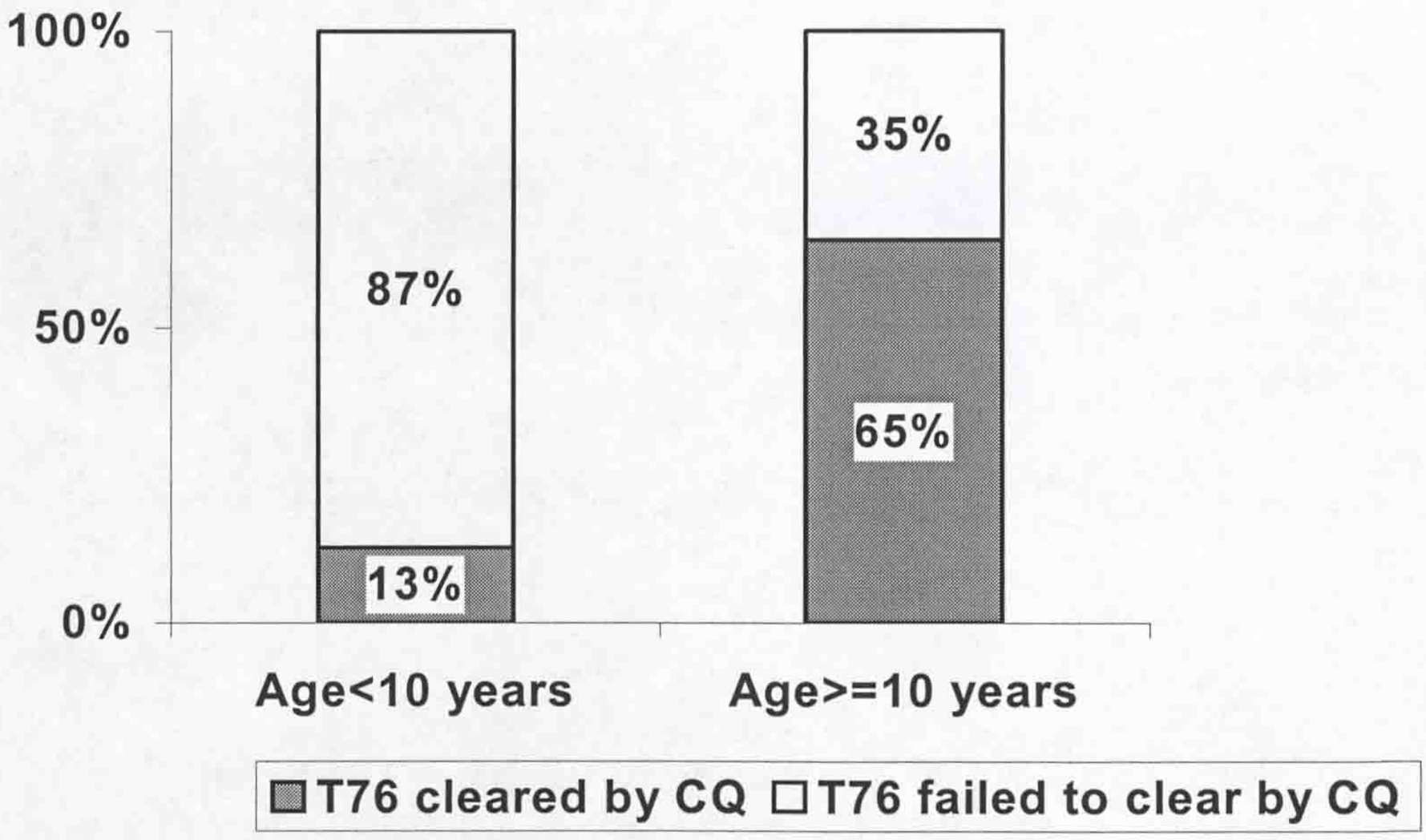


Figure 4.7 Effect of age on the in-vivo clearance of parasites carrying *pfcrt*-T76 mutation.

4.3.3.5 Validation of the use of pfcrt -T76, as a molecular marker of in-vivo chloroquine treatment failure

To validate the use of *pfcrt*-T76, as a molecular marker of chloroquine treatment failure and its use as a tool for surveillance in Yemen, the sensitivity of the test was calculated by categorising the treatment outcome into 2 categories; treatment failure (including ETF+LCF+LPF) and treatment success represented by ACPR. The sensitivity was found to be 100%, but the test was poorly specific (specificity =4.5%, 95% CI=0.0-10.7), The positive predictive value PPV was also low (PPV= 61.8%, 95% CI= 52.7-70.9). This result was expected due to the finding of high base-line (pre-treatment) prevalence of *pfcrt*-T76 compared to clinical chloroquine treatment failure (**section 4.3.3.4**).

Therefore, the genotype failure index GFI defined as the ratio of the prevalence of the resistant genotype (T76%) to the prevalence of chloroquine therapeutic failure both early and late (ETF%+LCF%+LPF%) was considered as a more reliable predictor of in-vivo treatment failure in this area. GFI was calculated and was found to be 1.6 (97.3/60.7) for all ages. The mixed genotype K76/T76 (1.8%) was excluded from this analysis. Controlled for age, the GFI was found to be 1.2 (98.4/83.9) in children less than 10 years, whereas in older children and adults it increased to 2.8 (98.3/36.7).

4.3.4 Screening for the presence of pfcrt-S163R:

The presence of the newly discovered mutation pfcrt-S163R was studied in 30 randomly selected pre-treatment samples of 42 samples of patients who despite the presence of pfcrt-K76T in their pre-treatment samples did respond adequately to CQ (classified as ACPR with the in-vivo test). None of the 30 samples was found to carry the pfcrt-S163R (**Figure 4.8**)

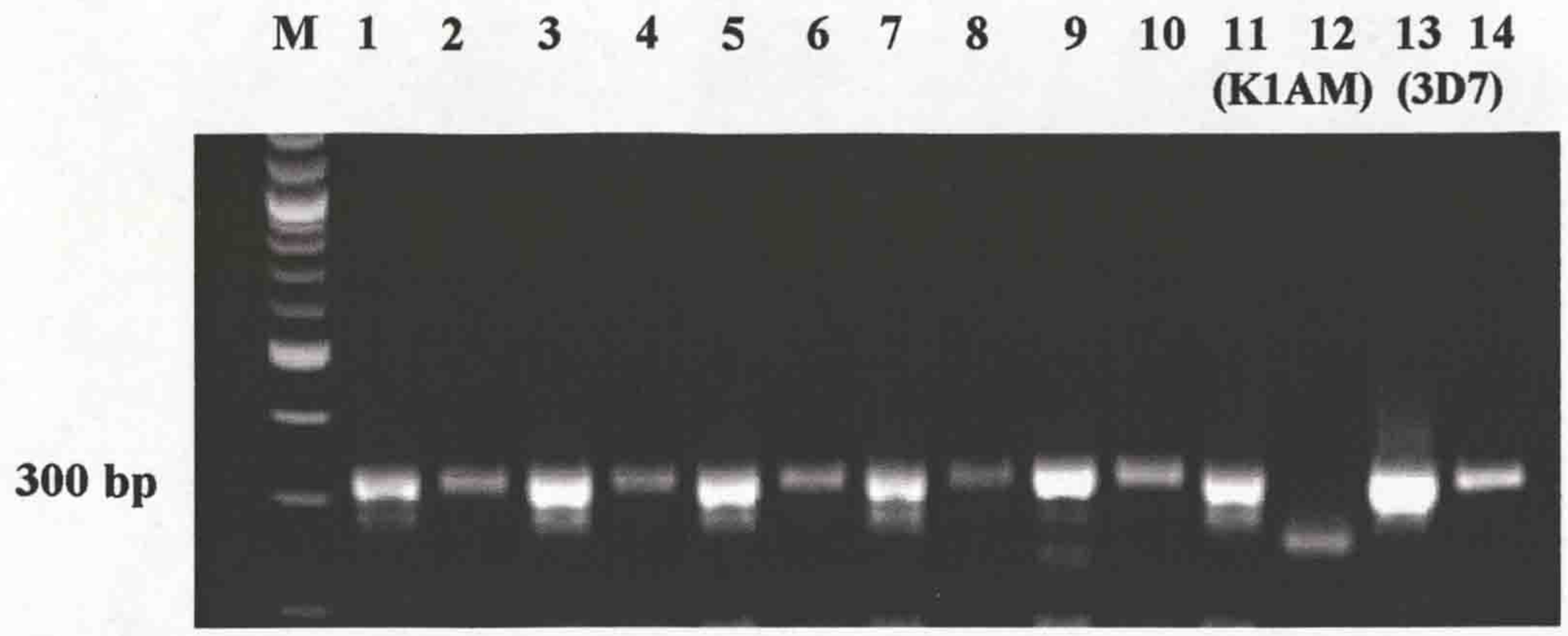


Figure 4.8 Detection of pfcrt-S163R in pre-treatment samples. Lane M is a 100 base pair DNA molecular ladder. Each sample run before and after digestion in adjacent wells to allow for comparison. Odd numbered lanes are for samples before digestion while even numbered lanes are for those after digestion. Lanes 1-10 represent field samples. Lanes 11 and 12 represent *K1AM* laboratory isolate that harbours the S163R mutation, while lanes 13 and 14 represent *3D7* isolate that lacks the S163R mutation. The only digested sample is *K1AM* (lane 12) that harbours the S163R mutation. No digestion occurred in any of the field samples nor in *3D7* that lacks the S163R mutation.

4.3.5 Detection of dhfr-C59R

Using the method developed by Plowe *et al.*, (1995) amplification of DNA to detect dhfr-C59R was performed in 119 pre treatment samples (samples obtained from patients before treatment with CQ). Amplification was successful in 80.7% (96/119) of the samples (**Figure 4.9**). Attempts to repeat and optimize the PCR in the 23 samples that failed amplification were not successful.

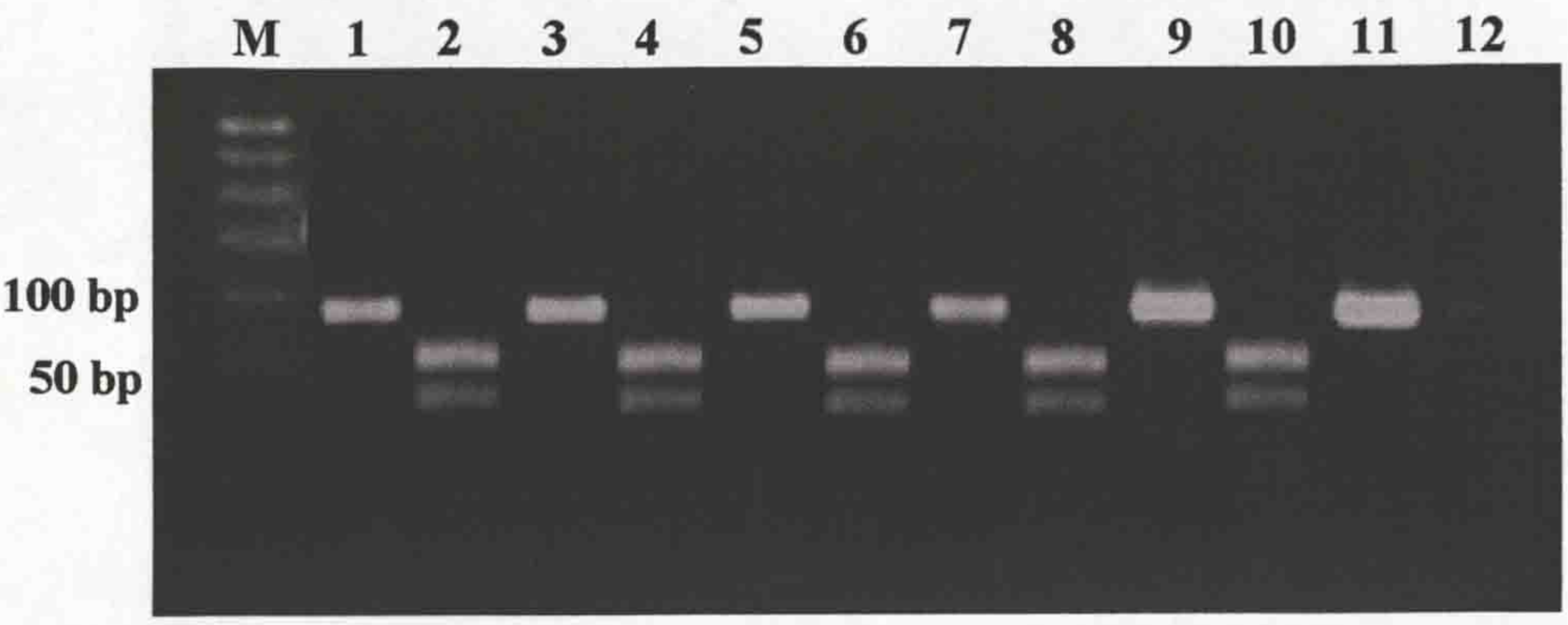


Figure 4.9 Detection of dhfr-C59R in pre-treatment samples using Plowe *et al.*, (1995) PCR protocol. Samples run in 3% MicroSieve-agarose gel. Each sample run before and after digestion in adjacent wells to allow for comparison. Lane M is a 50 bp DNA molecular ladder. Odd numbered lanes represent samples before digestion while even numbered lanes are those after digestion. Lanes 1-8 represent field samples. Lanes 9 and 10 are the results of amplification and digestion of the laboratory isolate 3D7, which has the wild-type dhfr-C95. Lanes 11 and 12 represent K1 that contains the mutant-type dhfr-C95R. BsrGI enzyme produced 2 cleaved products (65 bp and 35 bp) in the presence of the wild-type dhfr-C95 in 3D7 and in all the field samples in this figure. K1 was not digested by the enzyme (lane 12).

In order to increase the sensitivity of the detection of dhfr polymorphism at codon 59, amplification was repeated in the failed 23 samples using another PCR protocol developed by Duraisingh *et al.*, (1998). This resulted in successful amplification of only 13% (3/23) of the samples (**Figure 4.10**).

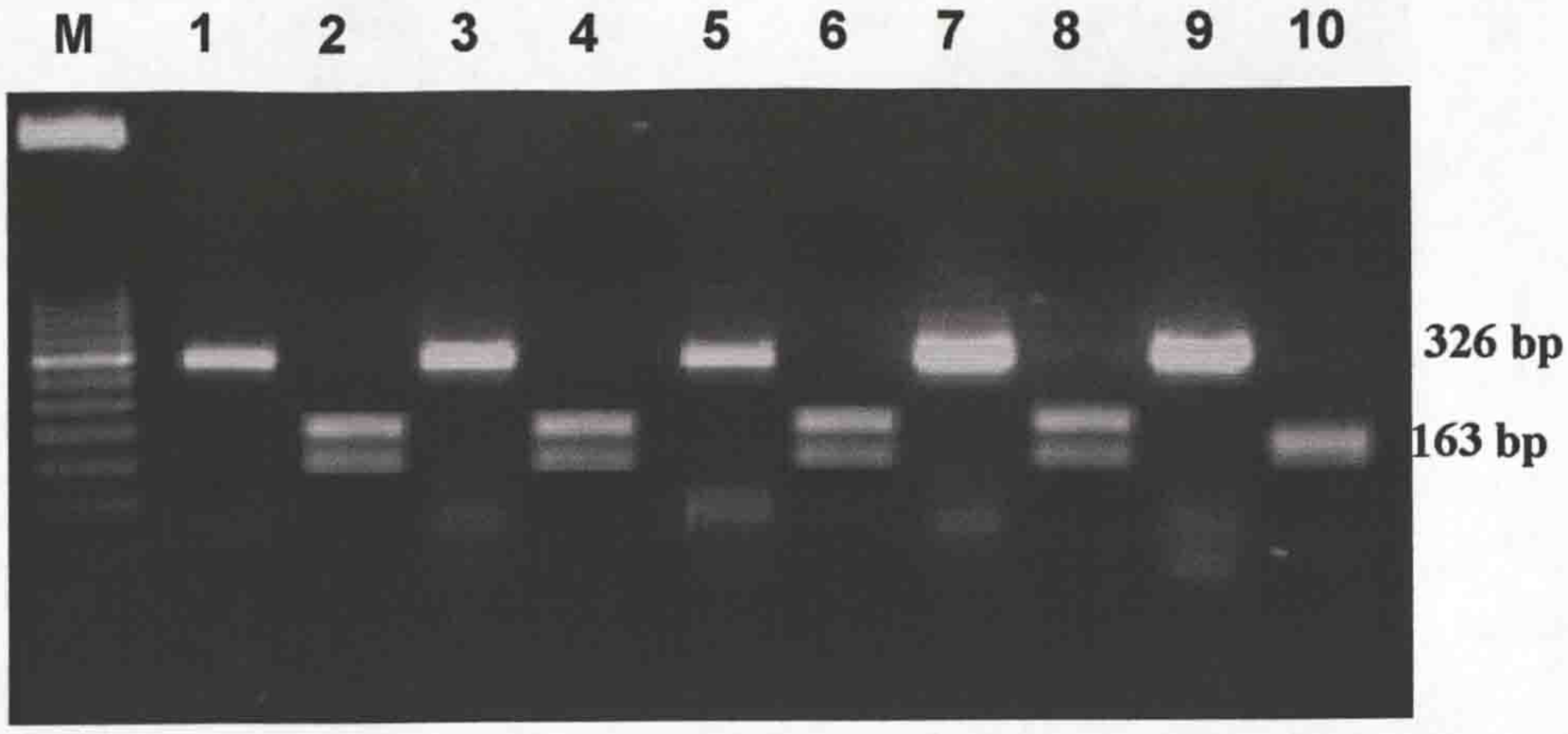


Figure 4. 10 Detection of dhfr-C59R in pre-treatment samples using Duraisingh *et al.*, (1998) PCR protocol. Samples run in 3% MicroSieve-agarose gel. Lane M is a 50 bp DNA molecular ladder with the approximate fragment sizes in bp. Each sample run before and after digestion in adjacent wells to allow for comparison. Odd numbered lanes represent samples before digestion while even numbered lanes are those after digestion. Lanes 1-6 represent field samples. Lanes 7 and 8 are the results of amplification and digestion of the laboratory isolate 3D7 that contain the wild-type dhfr-C95. Lanes 9 and 10 represent amplification and digestion of the laboratory isolate (K1) that has the mutant-type dhfr-C95R. *Xmn*I enzyme produced 2 cleaved products (189 bp and 137 bp) in the presence of the wild-type dhfr-C95 in 3D7 and in all the field samples in this figure. The enzyme produced two equally sized fragments of 163 bp in the presence of the mutant-type dhfr-C59R in K1 (lane 10).

Using the two PCR protocols for the detection of dhfr-C59R, amplification was successful in 83% (99/119) of the samples. Of the 99 successfully amplified samples 4 (4%) samples contained the dhfr-R59 mutant-type, one sample (1%) had the mixed-type C/R59 and 94 samples (95%) had the wild-type dhfr-C59 (**Figure 4.11**). The mutant to wild genotype prevalence ratio (M/W) was calculated and found to be 0.04 (4/95). The mixed type C /R59 (1%) was excluded in this calculation.

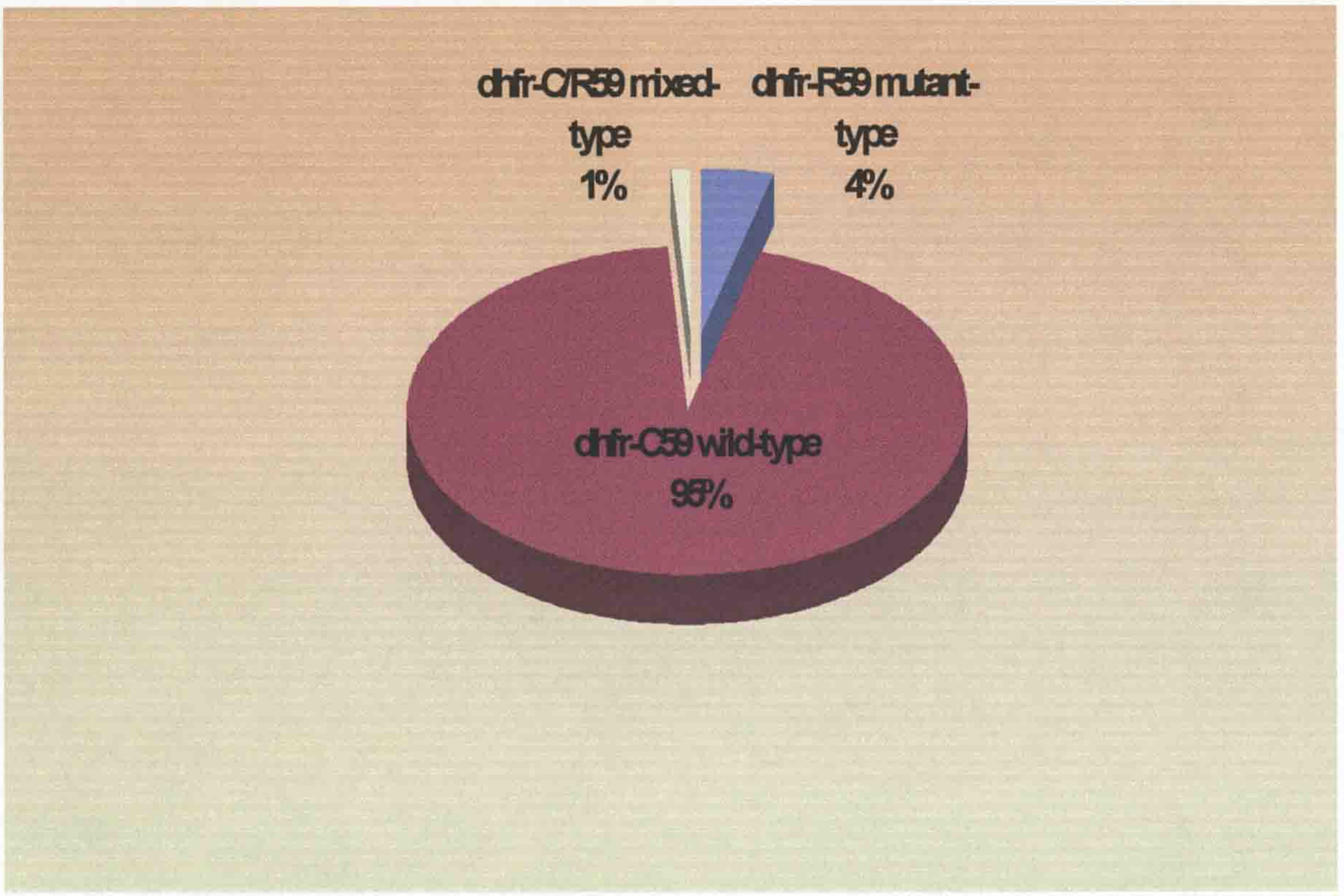


Figure 4.11 Prevalence of dhfr-59 polymorphism in pre-treatment samples.

4.3.6 Detection of dhps-K540E

Using the method developed by Plowe *et al.*, (1995) amplification of DNA to detect the dhps-540 polymorphism was successful in all the 119 pre-treatment samples tested. All samples (100%) had the wild-type dhps-K540 (**Figure 4.12**)

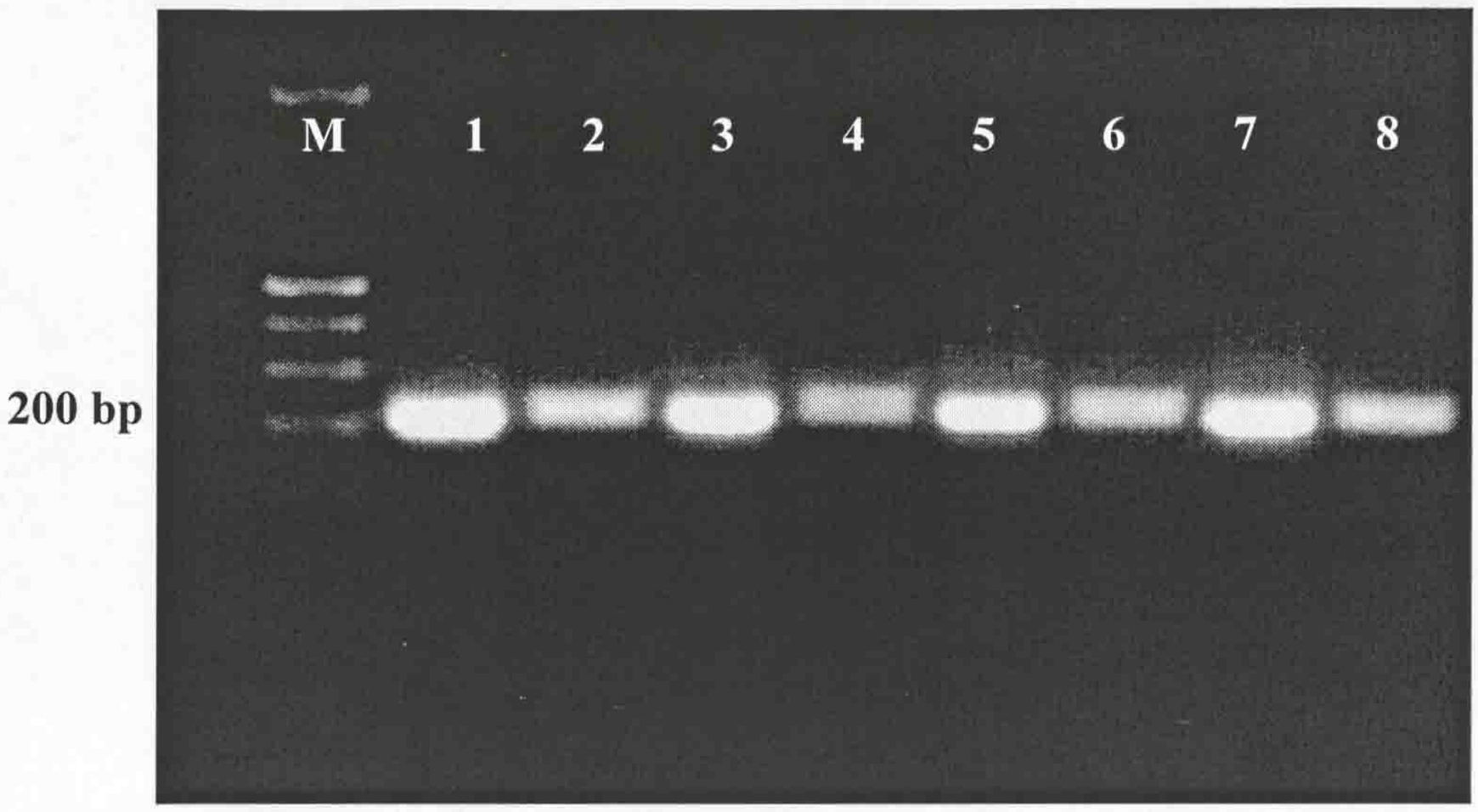


Figure 4.12 Detection of dhps-540 in pre-treatment samples. Field samples run in 3% MicroSieve-agarose gel. The figure shows 4 field samples, each sample run before and after digestion in adjacent wells to allow for comparison. Lane M is a 50 bp DNA molecular ladder, odd numbered lanes represent samples before digestion while even numbered lanes are those after digestion. *Fok*I enzyme did not cut any of the field samples in this figure indicating that they contain only the wild-type dhps-K540.

4.4 DISCUSSION

The molecular work started with testing three different methods for the extraction of DNA; namely the methanol fixation-heat extraction method, the FTA purification reagent and the QIAGEN kit extraction method from ordinary Whatman No. 3 filter papers and Whatman FTA® Card. The three methods of extraction gave the same results when extracted DNA (from both ordinary Whatman No. 3 filter papers and FTA® Cards) was used to amplify the *pfcrt* gene (**Figure 4.1**). The same PCR protocol to amplify *pfcrt* gene (Djimde *et al*., 2001a) was applied on DNA extracted by the 3 methods.

It was decided to use the *Methanol-Fixation Heat Extraction* method for the following reasons:

- It was effective in amplifying DNA extracted from both ordinary and FTA® Cards.
- Compared to QIAGEN kit extraction method, it was less expensive, simple, quick, and convenient method that didn't need too much manipulation.
- Compared to FTA Reagent extraction method, it yielded 50-70 µl of DNA in solution that is sufficient for 10-14 reactions. In FTA Reagent extraction method, the result of each extraction is a single disc of filter paper that is used as a template in the PCR reaction (**chapter 2, section 2.2.2.2**). To repeat the reaction or to use the same DNA in another reaction a new disc extraction was needed
- It is an economic, cost-effective method. Methanol is available everywhere and this also makes the method suitable for use in field studies in resource poor countries like Yemen.

To detect the PfCRT-K76T polymorphism two methods developed by Djimde *et al*., (2001a) comprised a nested PCR followed by restriction enzyme digestion (PCR/RFLP) and a mutation-specific PCR (MS-PCR) were tested. After several trials to compare the effectiveness of the two methods in detecting PfCRT-K76T polymorphism in laboratory isolates and field samples (**Table 4.1**, **Figure 4.2 and Figure 4.3**), the following conclusions were made:

- The *Restriction Enzyme Digestion*, although labour intensive, is robust in detecting PfCRT-K76T polymorphism.

- With *Restriction Enzyme Digestion* method, the discrimination step (restriction digestion) is separated from the PCR. This means that optimisation of PCR step is not as crucial as with the mutation-specific PCR.
- The Mutation-Specific (MS-PCR) method is sensitive to the amount of DNA used (Chaparro *et al.*, 2001). Some negative results were obtained with genomic DNA from laboratory isolates (**sample 3, Figure 4.3**), which might be caused by the high concentration of the template.
- Some difficulties in interpreting the results obtained by Mutation-Specific method were encountered when analysing some field isolates particularly the presence of non-specific amplifications represented by the appearance of some faint bands (**Figure 4.3, sample 2 and 5)** that resulted in erroneous typing of samples. This could be explained by the effect of the complex nature of those samples on the PCR amplification or the presence of low parasitaemia and mixed allele infection in field isolates (Chaparro *et al*. 2001; Ranford-Cartwright *et al.*, 2002).

Consequently it was decided to use the PCR followed by restriction enzyme digestion (PCR/RFLP). However, potential problems with the PCR/RFLP method noticed during testing were the difficulty in visualizing the digest fragments on agarose gels and the possibility of incomplete digestion although this was rare. Visualization problems were improved by the use of MicroSieve 3:1 agarose (*Flowgen*) gel and the problem of incomplete digestion could be overcome by increasing digestion time (overnight) or increasing the amount of the enzyme used.

This is the first study that analysed the prevalence of (PfCRT K76T) and that validated its use as a molecular marker for in-vivo chloroquine resistance in Yemen. The prevalence of *pfcrt* T76 (either alone or in mixed infections also carrying wild-type K76) was found to be extremely high (98.1%, 110/112) in pre-treatment samples. The pure wild-type K76 was detected in only 1.8% (2/122) of pre treatment sample. Talisuna *et al.*, (2002) found that the prevalence of infections carrying the K76 wild genotype was more closely related to CQ resistance than that of the T76 mutated genotype. They suggested that the disappearance of infections with the wild genotype may be one of the last stages of the long process resulting in CQ resistance, and that drug pressure must be an important factor in this process. According to

(Kyosiimire-Lugemwa *et al.*, 2002), increasing drug pressure would probably select the T76 mutation and would consequently decrease the prevalence of the wild type (K76). This was also supported by the finding that the prevalence of T76 alone, as well as, the ratio T76/K76 were significantly increased with increasing CQ use, mainly because of the disappearance of the K76 (Talisuna *et al.,* 2002). This also supports the idea that increasing drug pressure selects mutations involved in CQ resistance. The widespread use of CQ in this population might have highly selected for CQ resistant mutants in the parasite population. High parasite transmission rates in combination with a high proportion of hosts treated with drugs are key factors both for the survival of a newly arisen drug resistance mutant (Mackinnon, 1997) and its rapid spread once the frequency has reached a threshold level (Mackinnon and Hastings, 1998).

In this study the prevalence of T76 (either alone or in mixed infections also carrying wild-type K76) was higher than the prevalence of *in-vivo* CQ resistance (98.1% and 60.7% respectively). It was found that the T76 was present in all pre treatment as well as post treatment samples of patients who failed CQ treatment, and the only two pre-treatment samples that contained the wild K76 allele alone were belonging to patients who adequately responded to CQ. This indicates an absolute selection of the T76 mutation by the drug.

However, the T76 mutation was also highly prevalent (95.5%) in pre treatment samples of patients who adequately responded to CQ (**Figure 4.5**). Therefore no association was found between the presence of either the mutant or the wild *pfcrt*-76 allele and the treatment outcome; *Fisher exact p-value=0.152*. Similar finding of high prevalence of infection with theT76 genotype in pre treatment isolates, which was not predictive of in-vivo failure at the individual level was observed in Sudan (Babiker *et al.*, 2001) , Uganda (Dorsey *et al.*, 2001)et al., 2001), and in Loas (Pillai *et al.*, 2001). Those studies, like the present study, were conducted on patients seeking medical care, a selected group more likely to have taken antimalarial drugs as compared to the general population (Foster, 1995; McCombie, 1996). To avoid such a selection bias, Talisuna *et al.*, (2002) conducted a study in Uganda by randomly sampling individuals from the general population, however, they were also unable to link the overall T76 prevalence to that of CQ resistance in-vivo, mainly

because the mutant genotype (T76) was present in most of the malaria infections they identified (Talisuna *et al.,* 2002). These results are also consistent with those of previous field studies in which in vitro drug resistance were more common than in-vivo resistance (Baird *et al.*, 1991; Bickii *et al.*, 1998; Basco *et al.*, 1998).

Such results led to the conclusion that the presence of T76 may be necessary but not sufficient to predict in-vivo treatment outcome in all patients. In-vivo resistance may be influenced by a variety of factors, in addition to the K76T in *pfcrt*, including individual variations in drug absorption, pharmacokinetics, the underlying innate and acquired immune response, and the presence of additional mutations or compensatory changes in expression of other genes that may influence the level of resistance and ultimately the treatment outcome of patients infected with parasites that already display the K76T mutation.

Johnson *et al.*, (2004) described a mutation at PFCRT (*pfcrt*-S163R) selected in CQ-resistant lines that harbours the *pfcrt*-K76T. This mutation was selected by drugs like amantadine or halofantrine and is proposed to replace a positive charge in the transporter barrel, thereby compensating for the loss of positive charge associated with the K76T mutation and restoring the sensitivity to CQ of the parasite harbouring the K76T mutation (**Chapter 1, section 1.7.4.6.3**). Halofantrine is one of the available and prescribed drugs in Yemen (Abdo-Rabbo, 2003), therefore, the presence of this mutation (*pfcrt*-S163R) was studied in pre-treatment samples of patients who responded adequately to CQ in-vivo despite their infection with parasites harbouring the *pfcrt*-K76T. However, the mutation (*pfcrt*-S163R) was not found in any of the 30 samples studied which means that this mutation is not likely to explain the high prevalence of T76 compared to the prevalence of in-vivo CQ treatment failure in this study.

The role of age as the best surrogate for protective immunity in areas endemic for *P. falciparum* malaria was also assessed in this study. The ability to clear chloroquine-resistant parasites (parasite carrying the mutant T76) after CQ treatment was strongly associated with age. Only 13% of children<10 years of age compared to 65% of children above 10 years and adults were able to clear resistant parasites after chloroquine treatment (odds ratio [OR] = 0.04, 95% CI= 0.01-0.12, **Figure 4.7**). This

finding is consistent with those in earlier reports, which showed that even in areas of widespread chloroquine resistance, older children respond better to treatment than younger ones (Djimde *et al.*, 2001a; Fontanet and Walker, 1993; Dorsey *et al.*, 2000; Ekvall *et al.*, 1998; Nosten *et al.*, 1991; Dorsey *et al.*, 2001; Djimde *et al.*, 2003)

Although the other known *pfcrt* mutations and the *pfmdr1* mutations were not investigated in this study, report of Djimde *et al.*, (2001a) found no significant difference in the prevalence of *pfcrt* 74**I**, 75**E**, 220**S**, 271**I**, 326**S**, 356**T**, and 371**I** mutations in *P. falciparum* infections that cleared after CQ treatment and those that did not clear after CQ treatment. Reports also showed that the addition of *pfmdr1* mutations to *pfcrt*-T76 does not improve the predictive value of *pfcrt*-T76 alone, suggesting that mutations in *pfmdr1* are not likely to explain these differences in the ability to clear *pfcrt*-T76 parasites (Djimde *et al.*, 2001a; Jelinek *et al.*, 2002; Babiker *et al.*, 2001).

This result suggests that acquired immunity due to repeated exposure to *P. faciparum* infection is a dominant factor in clearing malaria parasites. It also supports the idea of the use of clearance of drug resistant parasites as a model for protective immunity in *P. falciparum* malaria (Djimde *et al.*, 2003). Moreover, Cravo *et al.*, (2001) showed that drug resistant parasites might behave as sensitive ones in the presence of partial immunity. They found that, in rodents, partial immunity in the presence of drugs (CQ and MQ) can reduce the growth of the drug resistant clone more significantly than in the absence of the drugs. This has an interesting implication in malaria vaccine development. If CQ can some times benefit individuals with preexisting immunity, then it might be possible to improve chemotherapeutic responses by erythrocytic-stage vaccines against *P. falciparum.*

Detection of T76 in *P.falciparum* isolates in pre-treatment samples provided 100% sensitivity for in-vivo resistance (all those who failed CQ treatment had infection with parasites carrying T76 in their pre-treatment samples). However, it was not highly predictive of treatment outcome (positive predictive value PPV = 61.8%). This is because the prevalence of molecular marker for resistance T76 was found to be higher than the in-vivo drug resistance due to the presence of T76 in pre-treatment samples of patients adequately responding to CQ. This finding has been noticed in

nearly all studies (Plowe, 2003). This makes the application of this marker as a tool for surveillance challenging. However, this problem was addressed by studies in Mali (Djimde *et al*., 2001b) where ratios between the prevalence of the resistance genotype and the prevalence of the therapeutic failure (genotype-failure index GFI) were calculated.

The GFI in this study was found to be 1.6 for all ages. Controlled for age, the GFI was found to be 1.2 in children <10 years and increased to 2.8 in older children and adults. This calculated GFI could be used in future surveillance activities to predict in-vivo drug resistance. For example, if the prevalence of pfcrt-T76 mutation is monitored in the area, and if this prevalence is found to be 50% at any one time, it means that the predicted in-vivo CQ resistance would be 42% in children <10 years and 18% in older children and adults. The increase in the GFI with age was consistent with Mali studies that showed that the GFIs were significantly associated with younger age in univariate and logistic regression analysis (Djimde *et al*., 2001b). The increase in the GFIs with age reflects an acquired immunity and a higher proportion of older persons who cleared parasites with the CQ-resistant genotype when treated with CQ, it also reflects the intensity of transmission in the area. After controlling for age the GFIs in Mali ranged from 1.6-2.8 at all study sites over three-year period (**Table 4.3**, Djimde *et al*., 2001b).

Site and Year	**Age ≤5 years**	**All ages**
	GFI	**GFI**
Mopti 1997	2.1	2.6
Bandiagara 1997	1.7	1.6
Bandiagara 1997	2.2	2.5
Kolle 1998	2.7	2.8
Kolle 1998	3.1	2.8
Mean	2.4	2.5

Table 4.3 Age adjusted Genotype-failure index (GFI) at three sites in Mali from 1997 to 1999 (Djimde *et al*., 2001b)

The finding of 29.5% positive PCR results in day 14 after treatment in post-treatment samples of patients who responded adequately to CQ (ACPR) could be explained by the high sensitivity of the PCR reaction compared to microscopy (Oster *et al.*, 2005), it can detect parasitaemia below the level of microscopic detection. Those cases might represent treatment failure cases if otherwise the follow-up period was extended beyond 14 days. Another possible explanation of this positive PCR results is the presence of gametocytes in day 14 after CQ treatment, which gives a positive PCR results. The presence of gametocytes in day 14 after CQ treatment was documented in Gambia (Sutherland *et al.*, 2002; von Seidlein *et al.*, 2001), in Nigeria (Sowunmi and Fateye, 2003) and in eastern Sudan (Ali *et al.*, 2006). This has its public health implications since it can favour the transmission and spread of CQ-resistant parasites. This is supported by the finding that gametocyte prevalence was higher among resistant compared to sensitive *P.falciparum* infections (Ali *et al.*, 2006). It was also found that the resistant genotype T76 was significantly over-represented among gametocytes emerging after treatment with CQ suggesting that under CQ selection this genotype is more likely to be transmitted than others, and that this is the mechanism by which the prevalence of this mutant allele may increase from generation to generation (Sutherland *et al.*, 2002).

As the prevalence of both in-vivo CQ treatment failure and the molecular marker for CQ resistance were unacceptably high in this study the prevalence of the molecular markers of the second-line drug SP were also studied to predict parasite resistance against this drug in order to guide anti-malarial treatment policy in Yemen. Studies showed that the quintuple mutant genotype (*dhfr* Asn-108, Ile-51, and Arg-59 plus *dhps* Gly-437 and Glu-540) was strongly associated with SP treatment failure (Kublin *et al.*, 2002). However, *dhfr* Asn-108 and Ile-51 were found to be too common to be useful predictors and that their inclusion in analysis did not add to the predictive value of *dhfr* Arg-59 (Kyabayinze *et al.*, 2003). Two mutations, *dhfr* Arg-59 and *dhps* Glu-540, were found to predict the presence of all five (quintple) *dhfr* and *dhps* mutations accurately (Kublin *et al.*, 2002; Kyabayinze *et al.*, 2003).

The prevalence of these two mutations (*dhfr* Arg-59 and *dhps* Glu-540) was studied in samples collected from patients before CQ therapy (pre-treatment samples). For the detection of *dhfr* Arg-59, the PCR protocol developed by Plowe *et al.*, (1995),

Chapter 2, section 2.2.7.1) was used and amplification obtained in 80.7% (96/119) of the studied samples. The remaining 23 samples were negative using this protocol although the same samples produced amplicons using the PCR protocol used to detect pfcrt-T76. It was decided to use another PCR protocol in order to increase the sensitivity of the detection of *dhfr* Arg-59, Oster *et al.*, (2005) also suggested that epidemiological studies in endemic areas require the use of more than one diagnostic technique due to the widely differing negative predictive values (NPVs) of various PCR protocols.

The Duraisingh *et al.*, (1998), **Chapter 2, section 2.2.7.2**) PCR protocol was used to detect *dhfr* Arg-59 in the 23 samples and amplification succeeded in 3 of them. Using the 2 protocols amplification was successful in 83% (99/119) of the samples, it is worth noting that amplification was also successful in all these 119 samples using the PCR protocol used to detect pfcrt-T76 (Djimde *et al.*, (2001a), **Chapter 2, sections 2.2.5.1**) indicating higher sensitivity of this protocol in diagnosis, this is also consistent with the finding of higher sensitivity of the pfcrt- PCR protocol than some other PCR protocols (Oster *et al.*, 2005).

The prevalence of *dhfr*-R59 (either alone or with *dhfr*- C59) was found to be 5% (5/99, **Figure 4.11**), however, *dhps*-E540 was not detected in any of the 119 samples studied indicating that this mutation is very rare in Yemen. The results suggest that the prevalence of *dhfr* Asn-108 and *dhfr* Ile-51 mutations is higher than 5% because previous studies showed that the prevalence of *dhfr*- C59 has been found to be lower than that of the other two *dhfr* mutations (*dhfr* Asn-108 and Ile-51) (Sirawaraporn *et al.*, 1990; Peterson *et al.*, 1990; Plowe *et al.*, 1997). This might be explained by the idea of stepwise accumulation of *dhfr* mutations that follows the order Asn-108 → Ile-51 → Arg-59. This idea was further supported by the finding that the prevalence of infections with only 2 *dhfr* mutations (*dhfr* Asn-108 and Ile-51) was significantly and inversely correlated to the prevalence of infections with 3 *dhfr* mutations (*dhfr* Asn-108, Ile-51 and Arg-59). The absence of *dhps*-E540 is similar to the finding of (Khalil *et al.*, 2002) in Khartoom in Sudan and similar to that of the Middle East were parasites are generally of wild-type *dhps* (Wang *et al.*, 1997).

The stepwise process in selection of SP resistance might also explain the absence of *dhps*-E540 in the studied samples. It was proposed that selection of *dhfr* mutations gradually decreases the sensitivity of the parasite to the pyrimethamine component of SP and once the triple *dhfr* mutations are well established in the population, selection of mutations in *dhps* occurs and sensitivity to the sulphadoxine component diminishes, resulting in a decrease in the efficacy of the drug (SP) so that when three or more mutations in both *dhfr* and *dhps* are common in the population, clinical failure of the drug results (Nzila *et al.*, 2000b). This is supported by findings from South Africa, where five years after establishment of *dhfr* triple mutants, an increase in the risk of SP treatment failure from 20% to 70% coincided with the emergence of the *dhps* double mutants (Roper *et al.*, 2003). The report by (Dorsey *et al.*, 2004) of the presence of *dhps* Glu-540 in 23% of samples in the absence of the *dhfr*- Arg-59 in Uganda contradicted the previous suggestions by (Sibley *et al.*, 2001) that key *dhps* mutations are rare in the absence of dhfr triple mutant. However, the same report (Dorsey *et al.*, 2004) concluded that the *dhfr* Arg-59 mutation was associated with treatment failure only in the presence of *dhps* Glu-540 and that *dhps* Glu-540 is a strong predictor of SP treatment failure independent of *dhfr* Arg-59 indicating that the overall prevalence of *dhps* Glu-540 was generally lower than *dhfr* Arg-59.

A study conducted in 2 areas of different endemicities in Kenya concluded that in areas where the level of immunity is low like in epidemic areas, mutations in *dhfr* and *dhps* were found to be important and the occurrence of ≥1 mutation in *dhps* predicted SP treatment failure, whereas in holoendemic areas, potentially resistant parasites, especially those with mutations in *dhps* only, were cleared by a combination of drug response and acquired immunity (Omar *et al.*, 2001). Thus, in areas of a high level of immunity, triple mutant *dhfr* with or without mutant *dhps* could be the main genetic determinant of SP treatment failure (Nzila *et al.*, 2000a; Mutabingwa *et al.*, 2001b; Khalil *et al.*, 2002).

Talisuna *et al.*, (2003) suggested that *dhfr* codon 59 mutant to wild genotype ratio M/W estimated through cross-sectional surveys that involve a random sample from the general population is a simple and robust molecular marker that could be used for early detection of low SP treatment failure. They concluded that an M/W genotype ratio for the *dhfr* codon 59 of < 1 would suggest SP treatment failure of <10% while

an M/W ratio of 1-1.5 would indicate a higher SP treatment failure of 10%-15% (Talisuna *et al*., 2003). In the present study, although not a population-based study, the M/W genotype ratio for the *dhfr* codon 59 was calculated to provide base-line information and help in monitoring the emerging SP resistance in Yemen. The ratio was found to be 0.05 (5/95) and according to Talisuna *et al.,* (2003), this may predict <10% SP treatment failure, however, it should be noted again that the present study was not population-based.

The result of the study of the SP molecular markers indicated generally that at the time of the collection of the samples used for this study (2002) the prevalence of the triple mutation indicated by *dhfr* Arg-59 was relatively low, 5%, whereas the *dhps* Glu-540 was very rare suggesting that the selection process had not reached *dhps*. However, four-years have passed since the start of the study, and in this area where the people are aware that CQ is no longer effective in treating malaria and where SP is readily available even over the counter in pharmacies further selection of *dhfr* and *dhps* mutations might have rapidly taken place. In a study conducted in Uganda, it was noticed that the prevalence of pure mutant *dhfr* Arg-59 and *dhps* Glu-540 increased over a four-year period during which samples were collected where the average prevalence of *dhfr* Arg-59 increased from 40% to 60% between 1999-2003 and that of *dhps* Glu-540 increased from 40% to 70% during the same period (Dorsey *et al*., 2004). This will compromise the efficacy of SP and the possibility of its use as a partner for artimisinin-based combination as a future long-term, first-line treatment in Yemen.

CHAPTER 5

Distinguishing *P. falciparum* Recrudescence From Re-infection By PCR Genotyping Of MSP2 In Al-Musaimeer District, Lahj Governorate, Yemen

5.1 INTRODUCTION

In-vivo studies using microscopy are the gold standards for the assessment of drug efficacy against *P. falciparum* malaria and upon which decisions regarding drug policy largely rely. However, in areas of high endemicity, reappearing parasites might be derived from new inoculations (re-infections) and could be classified falsely as treatment failures (recrudescence) due to the inability of the in-vivo tests (microscopy) to differentiate parasites resulting from the initial infection from those due to new infections. Molecular genotyping is used to help distinguish recrudescence (that results from exacerbations of persistent, undetectable parasitaemias in the absence of an exo-erythrocytic cycle) from reinfection (Snounou and Beck 1998).

PCR techniques using polymorphic markers such as the merozoite surface proteins (MSP1 and MSP2), glutamate-rich protein (GLURP), or microsatellite markers (Leclerc *et al.*, 2002) and based on the assumption that genetically different parasites at recruitment and at follow-up indicate a new infection (Ranford-Cartwright *et al.*, 1997) are employed. Using these markers new infections could be differentiated from true cases of treatment failures. The diversity of parasite populations and multiplicity of infection could also be obtained as a secondary outcome measurement. Among those markers *msp*-2 was found to be the most highly polymorphic gene and its analysis effectively distinguished recrudescence from reinfection (Snounou *et al.*, 1999; Cattamanchi *et al.*, 2003; Happi *et al.*, 2004). PCR analysis showed that reinfection might occur between day 7 and day 14 after chemotherapy with CQ and SP (Irion *et al.*, 1998; Magesa *et al*, 2001).

The multiplicity of infection in any one infected host may be related to the endemicity of malaria (Konate *et al.*, 1999) and the degree of immunity against *P. falciparum*, and the risk of clinical malaria (Al-Yaman *et al.*, 1997; Beck *et al.*, 1997) reflecting the intensity of transmission of the parasite. The multiplicity of infection may also have important implications for the epidemiology of drug-resistant *P. falciparum* malaria and the outcome of treatment in patients.

However, PCR techniques have some limitations (Snounou and Beck 1998) such as the sensitivity and resolution power of the PCR. Some other limitations are related

to the biological characteristics of the parasite's life cycle, such as sequestration and asynchrony, presence of gametocytes that can give false positive results overestimating the rate of recrudescence, and daily differences in the diversity of an infection. Host immunity and the pharmacokinetics of the drug are also important factors to be taken in consideration.

There is also no agreed standard on whether mixed infections (isolates where both new and recrudescent genotypes are present) should be classified as new, recrudescent, or indeterminate genotypes. However, if sufficient number of consecutive samples are analysed, and the efficiency and resolution power of the PCR are improved, meaningful results can be obtained with reasonable confidence.

The objectives of this chapter are:

- To use the *msp-2* molecular marker to distinguish recrudescence from reinfection in treatment failure cases.
- To describe the diversity and complexity of *P. falciparum* infection in Al-Musaimeer district, Lahj, Yemen.

5.2 MATERIALS AND METHODS

5.2.1 Genotyping of *P. falciparum* using PCR for msp-2

To distinguish recrudescence from reinfections in treatment failure cases and to determine the genetic structure of the parasite population in the area, samples of patients who failed CQ treatment between day 7 and day 14 after therapy (LCF+LPF) were considered. DNA from samples obtained before treatment (on day 0) and on day 3 after treatment and on the day when the case was classified as either LCF or LPF (day7 to day 14) was extracted from filter papers using the methanol-fixation heat-extraction method (**Chapter 2, section 2.2.2.1**). DNA was then amplified using a nested family-specific PCR for the amplification of polymorphic regions of block 3 of *P. falciparum* antigen gene *msp-2* as described by Snounou *et al.,* (1999) and Magesa *et al*., (2002) (**Chapter 2, section 2.3.1**). The primer names, sequences and PCR conditions used are listed in **Table 2.8** (**Chapter 2**). PCR products were analysed by MicroSieve 3:1 agarose (*Flowgen*) gel electrophoresis as described in (**Chapter 2, section 2.2.4**). Samples before and after treatment for each patient were loaded into adjacent wells for comparison in order to distinguish reinfection from recrudescence.

5.2.1.1 Sample definitions

A sample obtained before treatment (day 0) is referred to as a Primary sample (sample P) whereas any sample obtained at the day of classification (day 7 to day 14 after CQ therapy) is referred as a Recrudescence sample (sample R). Samples obtained on day 3 were added to improve the efficiency and resolution power of the PCR and they are considered as baseline or primary samples (sample P).

5.2.1.2 Outcome classification

In the presence of identical genotype patterns (bands having the same molecular size) in both sample P and sample R, the infection was classified as a recrudescence (**Figure 5.1a**), the presence of genotype patterns in the post-treatment samples that are identical to a subset of genotype patterns in the pre-treatment samples was classified also as a recrudescence (**Figure 5.1b**). In contrast, if the genotype patterns (bands) in sample P and sample R were different and the sample on day 3 did not show the band appearing in sample R, the infection was classified as reinfection (**Figure 5.1c**). Some mixed patterns were seen where sample R had the same

genotype patterns (bands) as those in sample P plus other new bands that were not detected in sample P nor in day 3 sample, these mixed patterns were classified as indeterminate (**Figure 5.1d**). Estimating DNA fragment sizes from an agarose gel is not particularly accurate and has an estimated error of ± 10-15 base pairs. However, comparison of pre-treatment and post-treatment fragment pairs for each patient was made by running the primary and recrudescent PCR products in adjacent lanes on the same gel to allow for simple visual comparisons to be made making the accurate estimate of the absolute fragment size less important with regard to distinguishing recrudescence from reinfection.

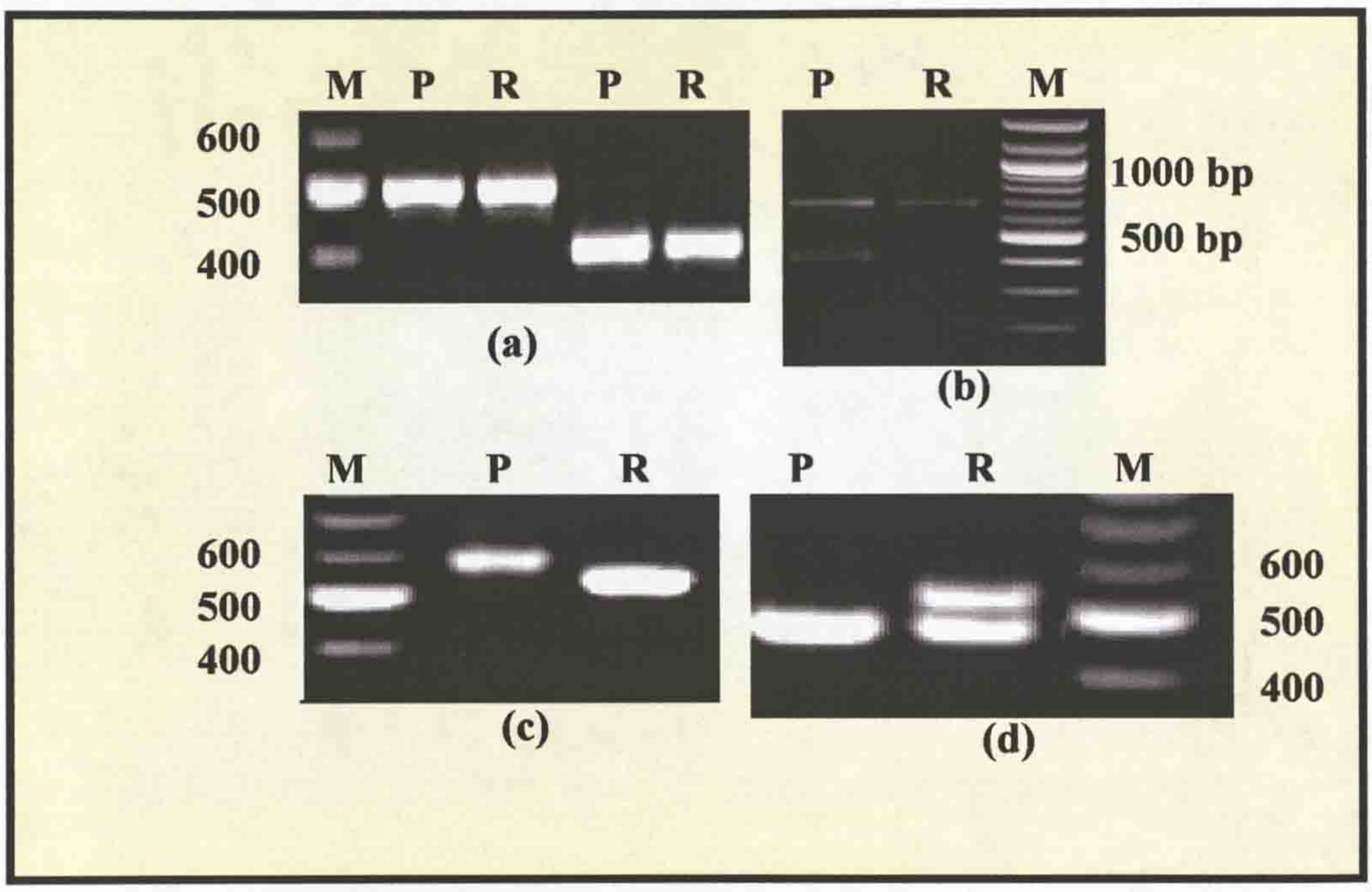


Figure 5.1 Distinguishing recrudescence from reinfection by *msp-2* family-specific PCR. Samples run on 1.3% MicroSieve 3:1 agarose (*Flowgen*) gel in pairs before and after treatment. P=primary (pre-treatment) sample, R=recrudescent (post-treatment) sample, M=100 bp DNA molecular ladder. **(a)** Two cases of recrudescence where the patterns (bands) detected in recrudescent samples (R) were exactly similar to those in primary samples (P). **(b)** A case of recrudescence where only a subset of bands present in primary sample was detected in the recrudescent sample. **(c)** A case of reinfection where the band detected in sample R was different in size from that present in sample P. **(d)** An indeterminate case where in sample R a band similar to that in sample P was detected together with a novel band not appearing in sample P.

5.3 RESULTS

5.3.1 Performance Of Polymerase Chain Reaction

Polymerase chain reaction to genotype *P. falciparum* using *msp-2* molecular marker was performed on a total of 65 paired samples (pre-treatment and post-treatment) samples. The amplification was successful in 48 (74%) of pre-treatment samples and in 41 (63%) of post-treatment samples.

5.3.2 Parasite Genotyping

5.3.2.1 Distinguishing recrudescence from reinfection

Twenty-four paired samples (pre-treatment and post-treatment) of patients who failed CQ treatment between day 7 and day 14 (LCF and LPF) were successfully amplified and thus included in the genotyping analysis to distinguish recrudescence from reinfection. Seventy-one percent (17/24) of the samples had exactly the same alleles in both the pre-treatment sample (primary sample) and the post-treatment sample (recrudescent sample) and were classified as recrudescence. Twenty-one percent (5/24) of the samples had recrudescent alleles plus new alleles in the recrudescent sample and were classified as indeterminate and 8% (2/24) of the samples had only new alleles in the recrudescent sample and were classified as reinfection. The prevalence of recrudescence, reinfection or indeterminate infections in the 2 classes of late treatment failure (late clinical failure and late parasitological failure) is shown in **Table 5.1**.

Treatment response	No. examined	Recrudescence	Indeterminate (recrudescent alleles+new alleles)	Reinfection
LCF (%)	7	5 (71.4)	1(14.3)	1 (14.3)
LPF (%)	17	12 (70.6)	4 (23.5)	1 (5.9)
Total	24	17 (71)	5 (21)	2 (8)

Table 5.1 Prevalence of recrudescence, reinfection and indeterminate infections in the 2 classes of late treatment failure.

5.3.2.2 Parasite diversity

Genotyping of a total of 89 individual infections (48 pre-treatment infections and 41 post-treatment infections) produced up to 21 fragments of MSP2 families IC/3D7 and FC27 with molecular size variations. The IC/3D7 family produced up to 11 bands with molecular sizes ranging from 400 bp to1000 bp (**Figure 5.2a**), and the FC27 family produced up to 10 bands ranging in size from 300 bp to1100 bp (**Figure 5.2a**).

Figure 5.2 Diversity of msp-2 allelic families. **(a)** represents amplification of IC/3D7 family in 11 different samples with size variation from 450 bp to 750 bp. Some samples contain multiple clone infection (sample 3,5,6,8,10 and 11). **(b)** represents amplification of FC27 family in 16 different samples with size variation from 400 bp to 1100 bp. Some samples contain multiple clone infection (sample 3,6,7 and 10). Samples run on 1.3% MicroSieve 3:1 agarose (*Flowgen*) gel. Lane M represents a 100 bp DNA molecular ladder.

The distribution of the alleles of the two families was almost similar in pre-treatment samples (FC27 family comprising 48% and IC/3D7 family comprising 52%). In post-treatment samples, the IC/3D7 family alleles were slightly more prevalent (58%). The genotypic structure of alleles in pre-treatment samples was conserved in post-treatment samples for both families, the most abundant FC27 family allele in pre-treatment samples (350 bp) was also the most abundant in post-treatment samples. Similarly the most abundant IC/3D7 family allele in pre-treatment samples

(600 bp) was also the most abundant in post-treatment samples. The presence of genetically novel alleles in post-treatment samples that differ from pre-treatment alleles was not observed. However, the high molecular weight FC27 alleles (1000 bp and 1100 bp) detected in some pre-treatment samples (**Figure 5.2(b), samples 3,6,7,9 and 10**) were not detected in post-treatment samples. The frequencies of different alleles of the two families in pre-treatment and post treatment samples are shown in **Table 5.2, Figure 5.3** and **Table 5.3, Figure 5.4** respectively.

Approximate molecular Weight in base pair	Frequency of each allele variant (indicated by its molecular weight) of the 2 MSP2 families observed in pre-treatment samples		
	FC27 Family (%)	**3D7/IC Family (%)**	**Total**
300	4 (9)	0	4 (4.4)
350	11 (26)	0	11 (12.2)
400	5 (12)	9 (19)	14 (15.6)
450	4 (9)	3 (6.4)	7 (7.8)
500	6 (14)	3 (6.4)	9 (10)
550	0	3 (6.4)	3 (3.3)
600	4 (9)	14 (29.8)	18 (20)
650	0	3 (6.4)	3 (3.3)
700	0	3 (6.4)	3 (3.3)
750	0	3 (6.4)	3 (3.3)
800	0	3 (6.4)	3 (3.3)
900	3 (7)	3 (6.4)	6 (6.7)
1000	2 (5)	0	2 (2.2)
1100	4 (9)	0	4 (4.4)
Total	43 (100)	47 (100)	90 (100)

Table 5.2 Frequency of individual allelic variants for the 2 families of MSP2 observed in samples collected before CQ treatment. Shaded cells indicate the most frequent allele appeared in the samples.

Approximate molecular Weight in base pair	Frequency of each allele variant (indicated by its molecular weight) of the 2 MSP2 families observed in post-treatment samples		
	FC27 Family (%)	**3D7/IC Family (%)**	**Total**
300	6 (21.4)	0	6 (9)
350	10 (35.7)	0	10 (15)
400	6 (21.4)	3 (8)	9 (14)
450	2 (7.1)	4 (10)	6 (9)
500	1 (3.6)	5 (13)	6 (9)
550	0	5 (13)	5 (8)
600	1 (3.6)	11 (29)	12 (18)
650	0	3 (8)	3 (4.5)
700	0	3 (8)	3 (4.5)
750	0	1 (3)	1 (1.5)
800	1 (3.6)	2 (5)	3 (4.5)
900	1 (3.6)	0	1 (1.5)
1000	0	1 (3)	1 (1.5)
Total	28 (100)	38 (100)	66 (100)

Table 5.3 Frequency of individual allelic variants for the 2 families of MSP2 observed in samples collected after CQ treatment. Shaded cells indicate the most frequent allele appeared in the samples.

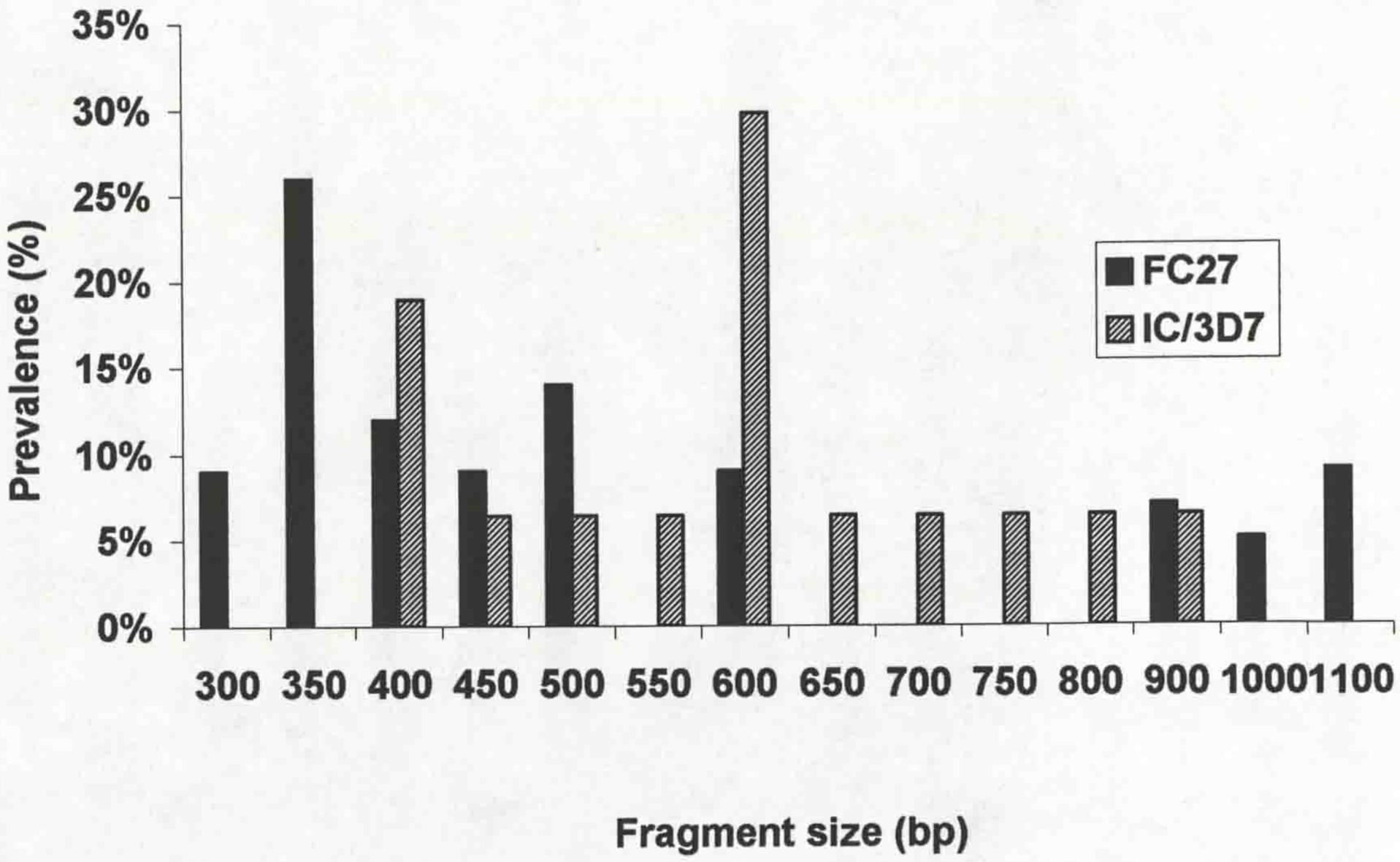


Figure 5.3 Prevalence of individual allelic variants for the 2 families of MSP2 observed in samples collected on day 0 before CQ treatment.

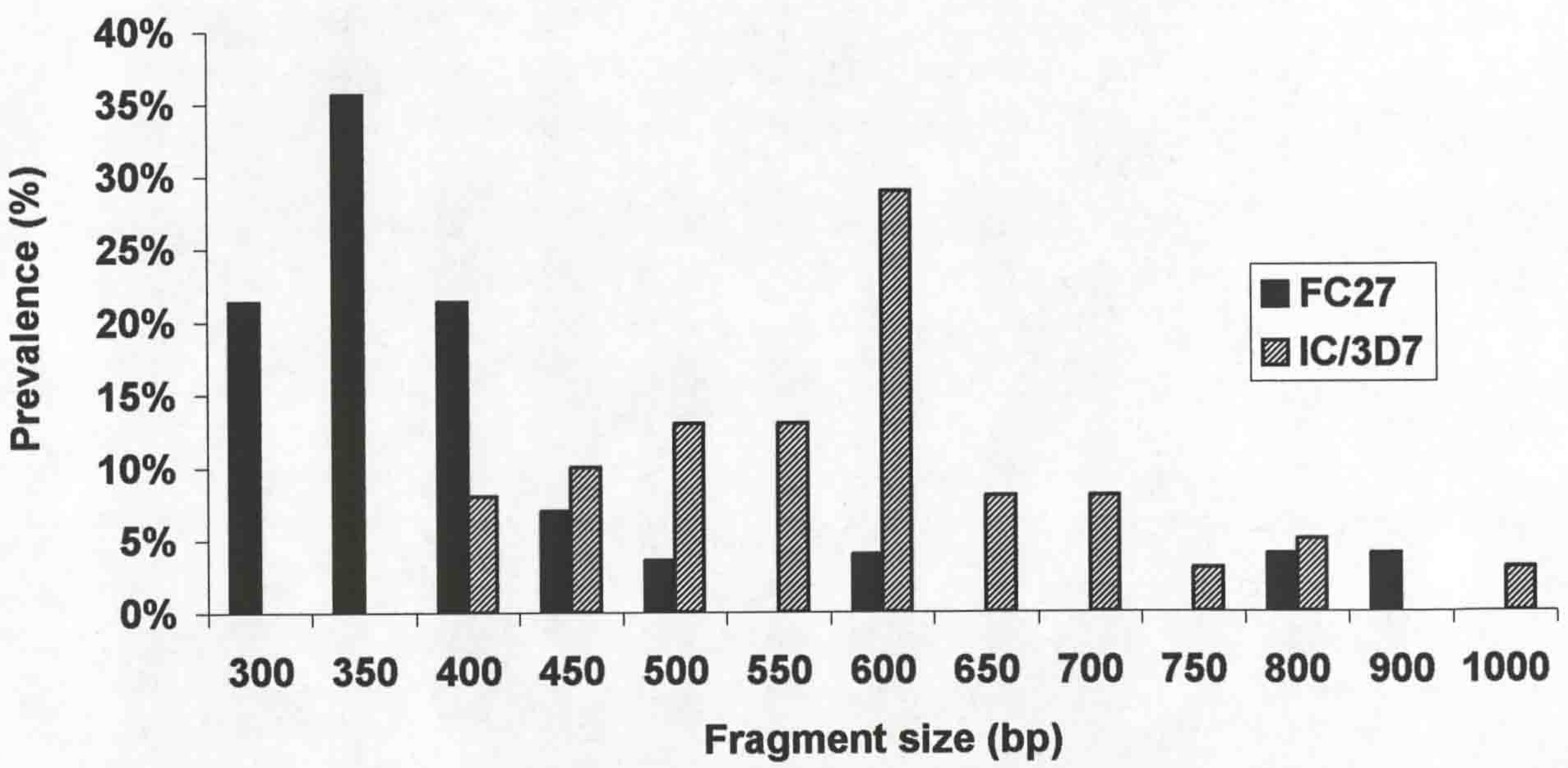


Figure 5.4 Prevalence of individual allelic variants for the 2 families of msp-2 observed in samples collected on day 14 after CQ treatment.

Analysis of the distribution of msp-2 allelic families in pre-treatment samples showed that the IC/3D7 alleles were the most prevalent comprising 46% (22/48), while the mixed type alleles (IC/3D7 plus FC27) were the least prevalent comprising 25% (12/48) **(Figure 5.5)**

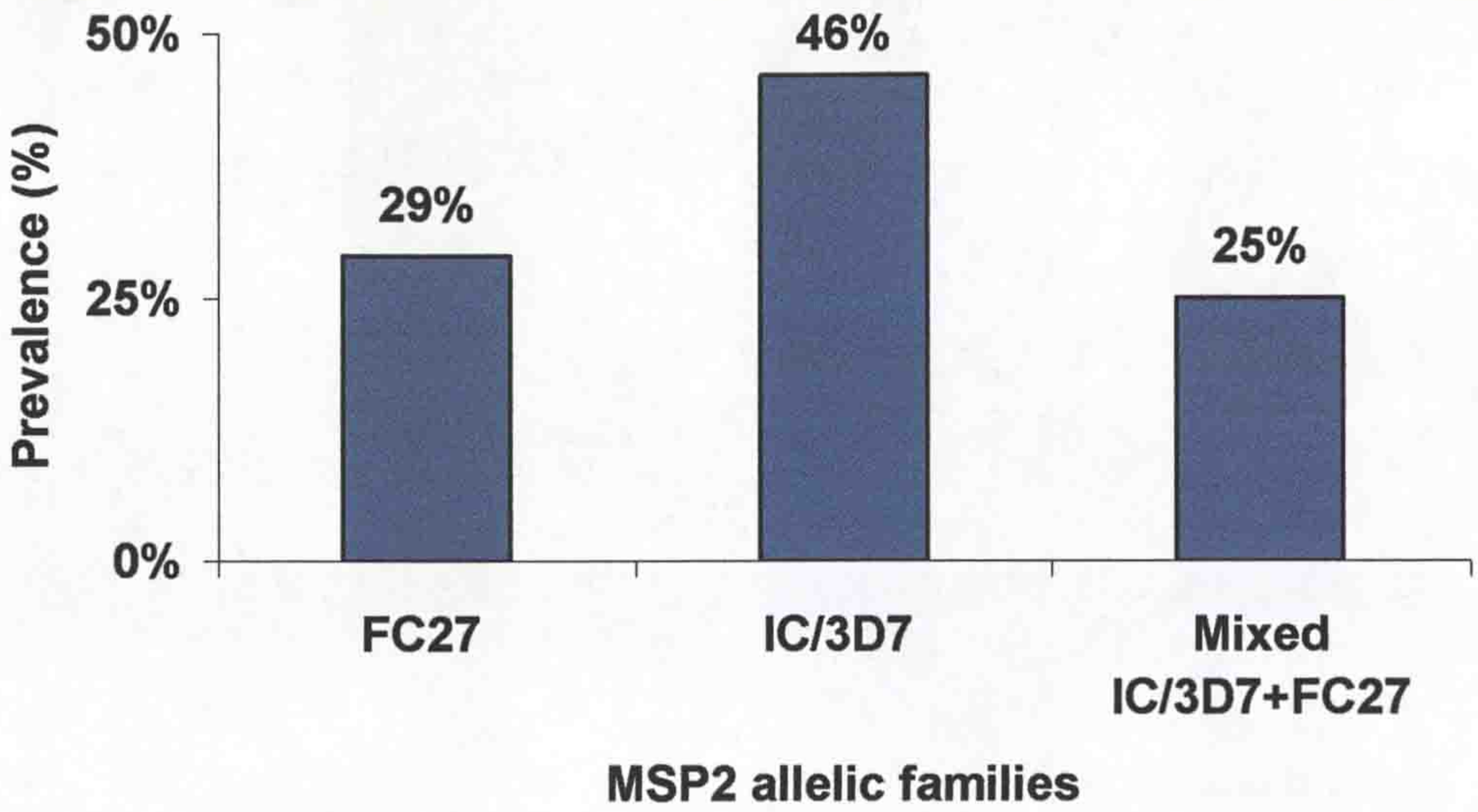


Figure 5.5 Prevalence of FC27 and IC/3D7 alone or mixed in pre-treatment and post-treatment samples.

The association between the presence of either FC27 or IC/3D7 allele in pre-treatment samples and some factors like age, sex, fever, parasite density and treatment failure outcome was studied using Chi-square or Fisher's Exact tests. Despite the failure of the test to detect any significant effect, most probably due to the small sample sizes of the comparison groups, it was noticed that the IC/3D7 allele was most common in children ≥5 years (82%), in non-febrile patients (64%), in infections with parasite density <25,000 parasite/µl (76%), in late treatment failures (89%) compared to early treatment failures (ETF), and among late treatment failure in late parasitological failures (87.5%) compared to late clinical failures (LCF) **(Table 5.6)**.

	No. tested (%)	No. infected with IC/3D7 parasite clone (%)	*p.* value
Age	36	22(61)	.683*
<5 years	8 (22)	4 (18)	
≥5 years	28 (78)	18 (82)	
Sex	36	22 (61)	.171
Male	18 (50)	9 (41)	
Female	18 (50)	13 (59)	
Fever	36	22 (61)	.221
≥37°C	16 (44)	8 (36)	
<37°C	20 (56)	14 (64)	
Parasite density	35	21 (60)	1.000*
<25,000 parasite/µl	27 (77)	16 (76)	
≥25,000 parasite/µl	8 (23)	5 (24)	
Treatment failure (TF)	28	18 (64)	.601*
ETF	4(17)	2 (11)	
LTF	24 (83)	16 (89)	
Late treatment failure (LTF)	24	16 (67)	.288*
LCF	5 (12)	2 (12.5)	
LPF	19 (88)	14 (87.5)	

Table 5.4 Association of MSP2 allelic genotype present in pre-treatment samples with a number of factors.

**p.* values according to Fisher's Exact test.

To avoid any bias that might be caused by the presence of fever, the analysis was repeated using only samples of asymptomatic (afebrile) patients at presentation. The analysis showed that the prevalence of IC/3D7 infection was significantly higher in children ≥ 5years of age compared to FC27 infection, which was higher in young children < 5 years (*Fisher Exact p-value < 0.02*) (**Figure 5.6)**

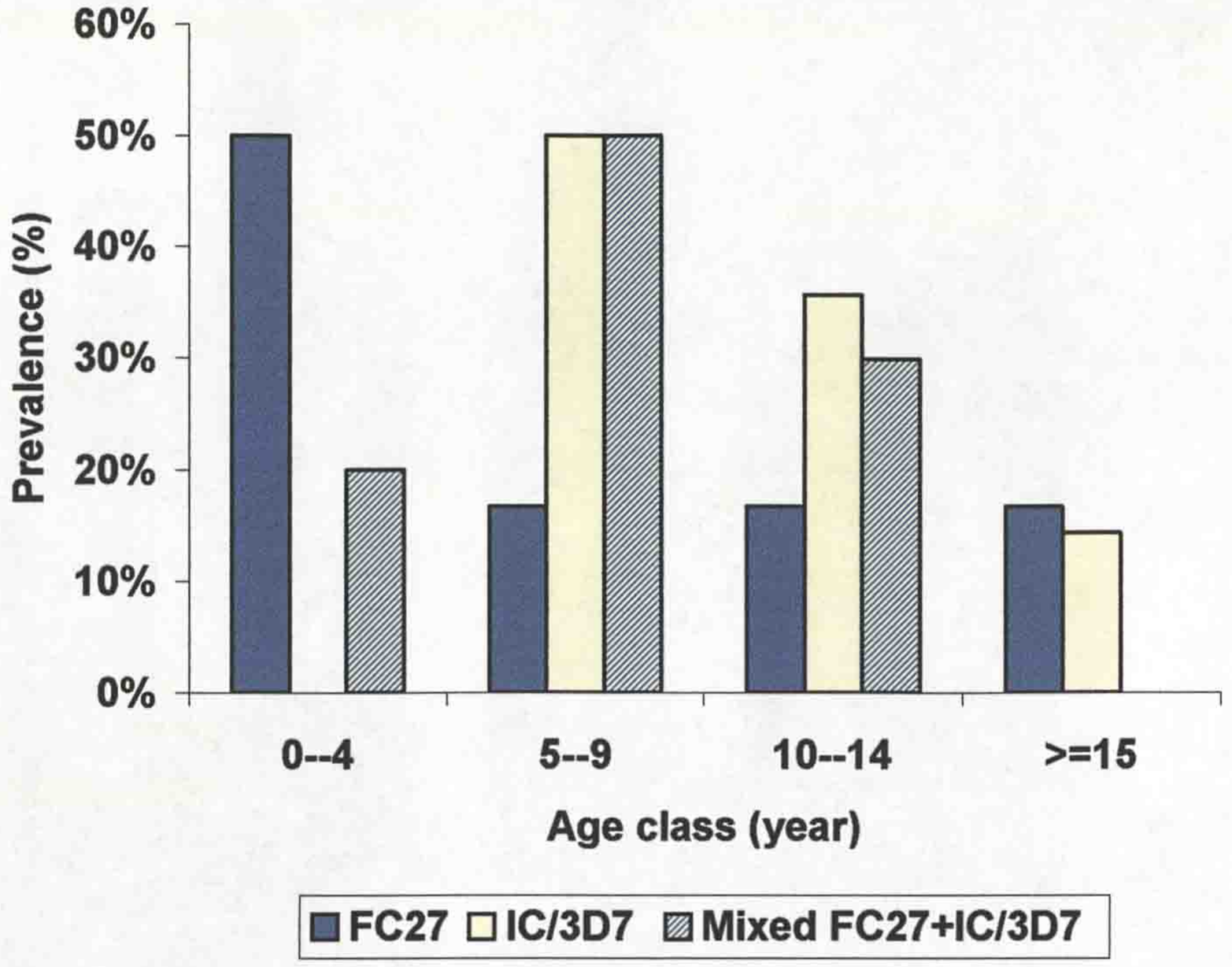


Figure 5.6 Prevalence of infection with FC27, IC/3D7 and mixed FC27and IC/3D7 parasite clones in pre-treatment samples of asymptomatic patients.

Multiplicity of infection

Multiplicity of infection was calculated as the maximum number of alleles (bands) detected per sample. Multiple infections (presence of >1 band/sample) were detected in 56% (27/48) of pre-treatment samples and in 50% (20/40) of post-treatment samples. In pre-treatment samples the number of alleles detected/sample ranged from 1 to 6 different sized alleles with a mean of 1.92 (SEM= .163), while in post treatment samples the number of alleles detected/sample ranged from 1 to 3 with a mean of 1.63 (SEM= .111). This difference in the mean of multiple clones per sample when comparing pre-treatment and post-treatment samples was statistically significant (*Wilcoxon Signed Ranks Test, p-value=0.028*). A comparison of the prevalence of multiple clone infection in pre-treatment and post-treatment samples is illustrated as the number of detectable bands in **figure 5.7**.

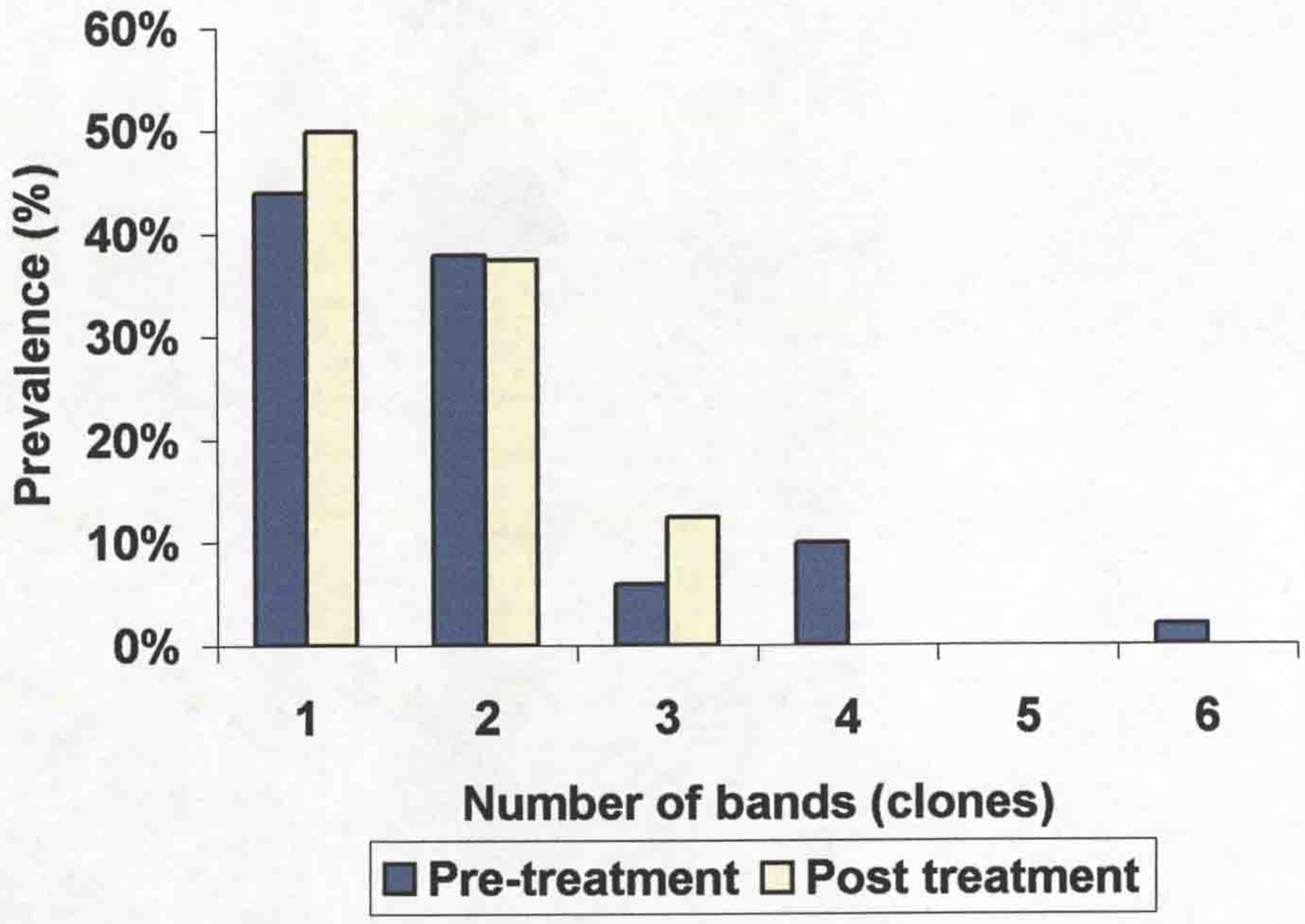


Figure 5.7 Prevalence of multiple genetically distinct parasite clones (bands) identified in pre-treatment and post-treatment samples.

Multiple clone infection was calculated also as the number of alleles (bands) per individual patient by adding all the alleles observed in both pre-treatment and post-treatment samples for each patient, this number ranged from 1 to 8 alleles/patient with a mean of 2.74 (SEM= .216).

The association of the presence of multiple clone infection per sample (in pre-treatment samples) with a number of factors including age, sex, fever (≥37° C), parasite density at presentation (in the pre-treatment sample), treatment failure outcome and the presence of either FC27 or IC/3D7 family was tested using Chi-square or Fisher's Exact tests. No statistically significant association was detected between any of these factors and the presence of more than one clone in the samples (**Table 5.5**). The small sample sizes of comparison groups used in this analysis were the most likely cause for the failure to detect any significant association. However, it was noticed that multiple clone infection (more than one clone infection/sample in pre-treatment samples) was more prevalent in children ≥5 years (85%), in non-febrile patients (70%), in infections with parasite density <25,000 parasite/µl (77%), in late treatment failures (90.5%) compared to early treatment failures (ETF), and among

late treatment failures multiple clone infection was more common in late parasitological failures (68%) compared to late clinical failures (LCF) (**Table 5.5**).

	No. tested (%)	No. infected with >1 parasite clone (%)	*p.* value
Age	48	27 (56)	.174*
<5 years	11 (23)	4 (15)	
≥5 years	37 (77)	23 (85)	
Sex	48	27 (56)	.383
Male	24 (50)	12 (44)	
Female	24 (50)	15 (56)	
Fever	48	27 (56)	.202
≥37°C	18 (37.5)	8 (30)	
<37°C	30 (62.5)	19 (70)	
Parasite density	47	26 (55)	.668
<25,000 parasite/µl	35 (74.5)	20 (77)	
≥25,000 parasite/µl	12 (25.5)	6 (23)	
MSP2 families	36	15 (42)	.908
IC/3D7	22 (61)	9 (60)	
FC27	14 (39)	6 (40)	
Treatment failure (TF)	39	21 (54)	.387*
ETF	6 (15)	2 (9.5)	
LTF	33 (85)	19 (90.5)	
Late treatment failure (LTF)	33	19 (58)	.698*
LCF	9 (27)	6 (32)	
LPF	24 (73)	13 (68)	

Table 5.5 Association of multiplicity of infection in pre-treatment samples with a number of factors.

* *p.* values according to Fisher's Exact test.

Further analysis of the multiplicity of infection per sample in pre-treatment samples was performed comparing febrile and non-febrile patients at presentation (symptomatic versus asymptomatic infections) stratified by age groups (0-4, 5-9, 10-14, ≥15 years). Generally the multiplicity of infection was higher in asymptomatic infections for all ages (**Figure 5.8**). In asymptomatic infections (afebrile), the multiplicity of infection was highest in the age group 5-9 (mean 2.4). There was a trend of decreasing multiplicity with increasing age in those who are above 5 years (not significant). The least multiplicity was observed in adults ≥15 years and in children less than 5 years.

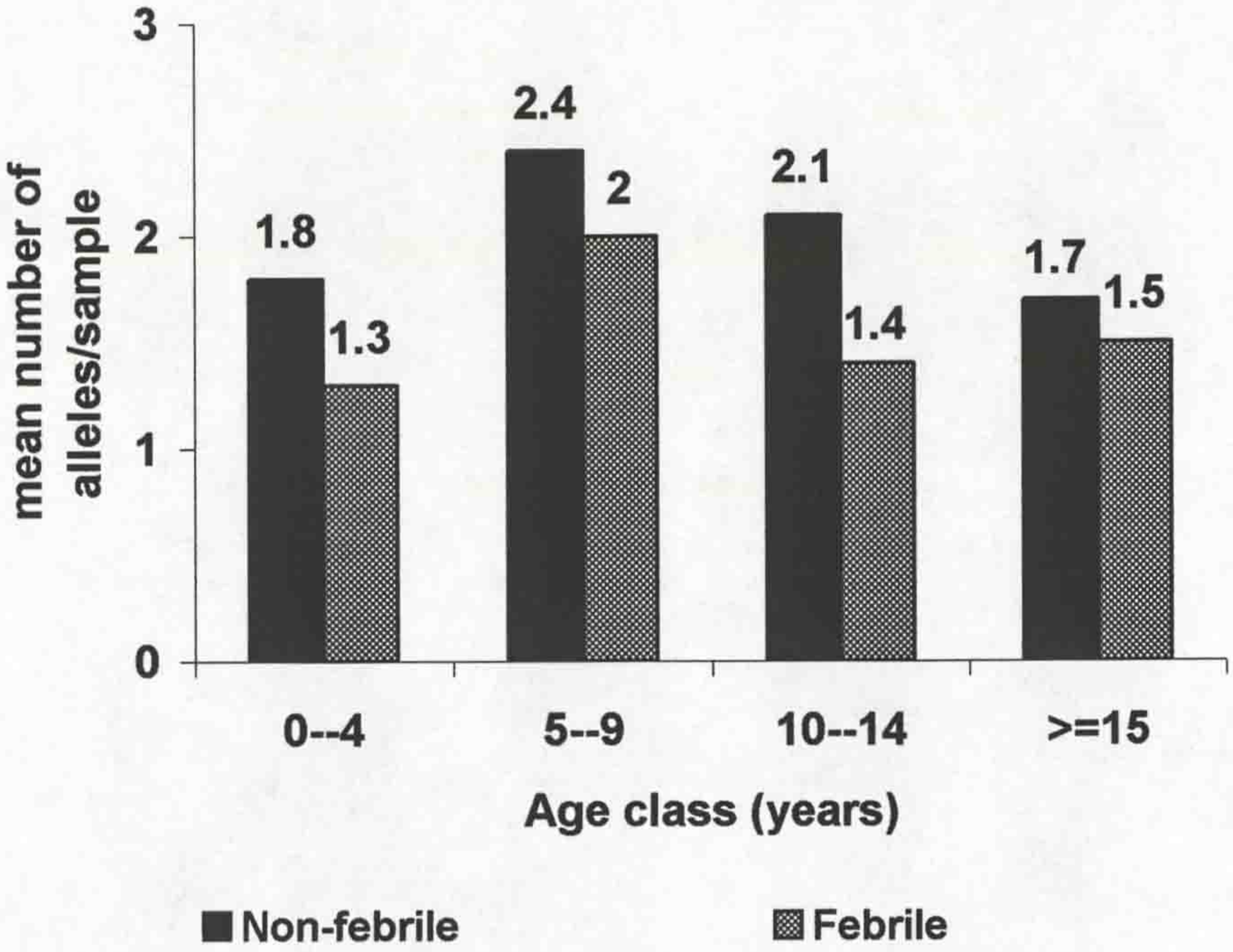


Figure 5.8 Multiplicity of infection per sample in pre-treatment samples in asymptomatic (afebrile) and symptomatic (febrile) infections.

The mean number of alleles per sample within each allelic family of MSP2 was calculated in pre-treatment and post-treatment samples (**Table 5.4**). The mean number of clones/sample in post-treatment samples was less than that in pre-treatment samples for both families (**Table 5.4**), however, using *Wilcoxon Signed Ranks* test, this difference in the means of the number of clones per sample between pre-treatment and post-treatment samples was only statistically significant in FC27 family (*p-value*=.016) but not in IC/3D7 family (*p-value*=.157)

	Pre-treatment samples		**Post-treatment samples**	
	FC27	**IC/3D7**	**FC27**	**IC/3D7**
Mean (range)	1.65 (1-4)	1.38 (1-3)	1.12(1-2)	1.26 (1-2)
SEM	.192	.095	.066	.080

Table 5.6 The mean number of multiple clone infection within FC27 and IC/3D7 in pre-treatment and post-treatment samples. SEM=standard error of the mean.

When the average number of clones of both FC27 and IC/3D7 per sample in pre-treatment and post-treatment samples was stratified by age, the decrease in multiple

clone infection by FC27 parasites in pos-treatment samples was clear in children above the age of 5 years and in adults. In contrast, in children less than 5 years of age, the average number of FC27 alleles per sample was higher in post-treatment samples than pre-treatment samples (**Figure 5.9**). With regard to IC/3D7 family, the average number of infecting parasites/sample was decreased in post-treatment samples only in those above the age of 15 years (**Figure 5.9**). This analysis was performed on pure infections with either FC27 or IC/3D7 family alleles, the mixed FC27 + IC/3D7 infections were excluded.

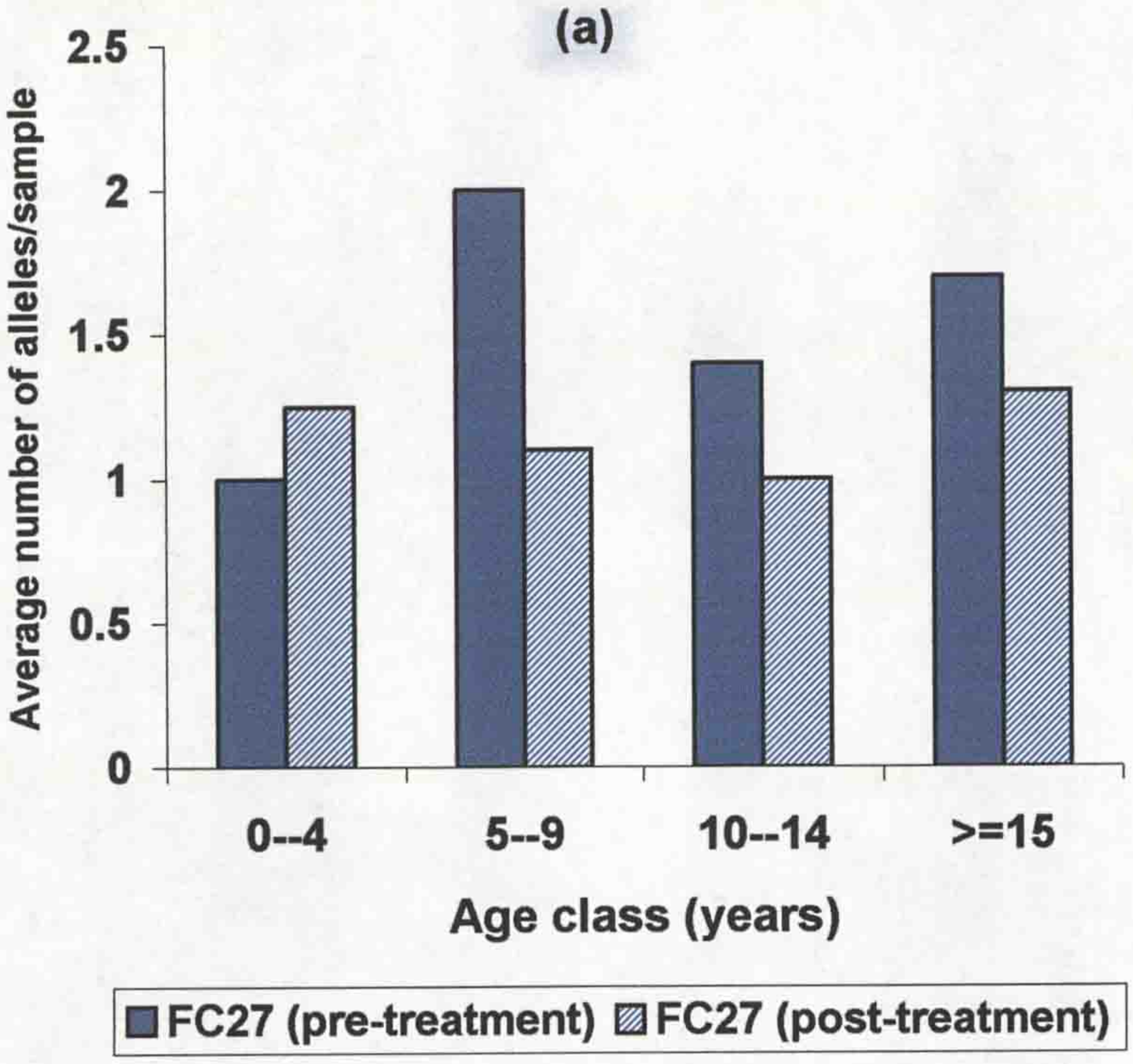


Figure 5. 9 Multiplicity of infection with FC27**(a)** and IC/3D7**(b)** allelic families in pre-treatment and post-treatment samples.

5.4 DISCUSSION

The in-vivo assessment of the efficacy of CQ in Al-Musaimeer district, Lahj governorate in Yemen (**Chapter 3**) showed a high level of treatment failures (61%). Sixty-two percent of those treatment failures occurred between day 7 and day 14 after CQ treatment (late treatment failures). Reoccurrence of parasite after treatment could be caused by either inherent inefficacy of the drug in-vivo or, by the presence of parasites that are genetically resistant to the drug, the latter was documented in chapter 4 by the finding of a high prevalence of *pfcrt*-T76 mutation (98%) that has been established as a molecular marker for CQ resistance. However, the incubation period of *P. falciparum* can be as short as seven days and in high endemicity areas new inoculations of parasites can not be excluded and it is difficult to distinguish parasites that have escaped drug action (recrudescence) from those parasites arising from new inoculation (reinfection) by conventional in-vivo tests (microscope).

The main objective of this part of the study was to distinguish recrudescence caused by persistent parasites that escaped CQ treatment from new infections caused by possible new inoculations of parasites from infected mosquitoes after CQ treatment using a PCR method. Cases that failed to respond to CQ and demonstrated reappearing parasites in blood samples between day 7 and day 14 after CQ treatment, which include late clinical failures and late parasitological failures were studied. Some difficulties were encountered due to a non-standardized methodology and interpretation of results, particularly the definitions used to classify outcomes as recrudescence or reinfection. Some of these difficulties are discussed below.

The *msp-2* marker has been found to be the most polymorphic and the most reliable genetic marker to distinguish recrudescence from reinfection and to evaluate the diversity and complexity of *P. falciparum* infections in a number of studies (Happi *et al*, 2004; Cattamanchi *et al*, 2003; Felger *et al*, 1999).

Using the *msp-2* marker to distinguish recrudescence from reinfection, the analysis showed that in 71% of the studied paired samples (pre-treatment and post-treatment samples) from patients who failed CQ treatment, identical parasite genotypes were present before and after treatment suggesting that all these parasites were resistant to the curative dose of CQ. However, although infrequent, it should be noted that the

appearance of identical parasite genotypes in pre-treatment and post-treatment samples could be explained by two different inoculations with the same parasite line. Snounou and Beck, (1998) suggested that confidence in determining identical parasite infections increases with the number of loci analyzed, as different parasites might be identical at one locus but might differ in other loci, thus, increasing the number of marker genes to be analysed enhances the probability of detecting genetically different *P. falciparum* infections. However, Cattamanchi *et al*, (2003) concluded that in areas of high transmission like in Kampala, Uganda, evaluation of recrudescence is best achieved by detailed analysis of a single, highly polymorphic locus like *msp-2*, which resulted in an acceptably low probability of outcome misclassification.

The use of a single, high-resolution locus gives rise directly to patterns of appearance and disappearance of individual infections. In contrast multi-locus genotypes of individual parasite clones cannot be identified in multiple infections, since the marker genes are unlinked (Felger *et al*, 1999) and this makes longitudinal studies using multilocus genotyping in areas of high parasite multiplicity difficult to interpret. Cattamanchi *et al*, (2003) concluded also that using the three genetic markers *msp-1*, *msp-2* and *glurb* together increased the level of diversity but at the cost of a considerably larger proportion of outcomes being classified as indeterminate (presence in post-treatment sample of new alleles together with previously detected alleles in pre-treatment sample). This demonstrates one of the difficulties encountered with the interpretation of the results.

Moreover, because the polymorphism of malaria genes is often the result of repetitive units, these units can differ slightly in their individual sequence and/or order. Thus, two alleles can have the same size but differ in sequence and/or arrangement of the repeat units (Snounou and Beck, 1998). Direct sequencing of the amplified DNA may solve this problem but this method is not practicable under field conditions, and would fail in the frequent cases of multiple infections. Subtle differences within gene sequence may also be missed during amplification resulting in misclassification of possible new infections as recrudescence. One of the strategies to improve the resolution of detection is the use of restriction fragment length polymorphism (RFLP) analysis (Felger *et al.*, 1994), which classifies *msp-2* alleles

on the basis of their distinct digestion patterns. However, using this method, alleles resulting from recombination of the allelic families remain undetected if no obvious alteration in the restriction pattern results. PCR amplification with mixed family-specific primers provided evidence for high frequency of recombination (Ntoumi *et al.*, 1997). A further limitation of genotyping by RFLP is the sequence diversity located outside the restriction site. There is also an obvious inherent limitation in typing only one locus since 2 infections sharing the same *msp-2* allele will be determined as one (Felger *et al*, 1999).

Another strategy used to improve the resolution of detection is the single-stranded conformational polymorphism (SSCP) method (Kain *et al.*, 1996), which can detect differences of as little as one base substitution between alleles, and depends on the altered electrophoretic mobility of the denatured DNA. However, the SSCP technique is technically demanding, and is only effective when fragment sizes are small (optimally <150 bp) (Snounou and Beck, 1998).

A possible confounding factor of the analysis and classification of recrudescence is the presence of gametocytes rather than asexual parasites in the post-treatment samples. Gametocytes in post-treatment samples but originating from parasites in pre-treatment samples would be mistaken as evidence of recrudescence. Gametocytes can be detected differentially by reverse transcription- PCR (RT-PCR), as genomic DNA is amplified from all stages (Snounou and Beck, 1998). However, this is not possible to perform on old samples collected on filter papers.

In cases where only a subset of the original parasites in the pre-treatment isolates were detected in the post-treatment samples (**Figure 5.1b**), it could be concluded that a proportion of the original parasite populations were sensitive to CQ hence cleared by the drug while those remaining represented resistant populations. An alternative explanation is that some of the parasites in the pre-treatment sample were not detected in the post-treatment sample due to limitation in the sensitivity of detection or due to sequestration of the parasite in deep blood vessels as discussed below.

The presence of new alleles in the post-treatment samples that were not detected in the pre-treatment samples was detected in 7 of 24-paired samples tested. However, 5 of these samples (21%) contained also some variants detected in the pre-treatment sample. Those were initially classified as indeterminate infections. The classification of this group as either recrudescence or reinfection is not consistent in different studies, some studies recommended the classification of this outcome as reinfection (Cattamanchi *et al*, 2003), others defined this group as recrudescent due to the persistence of one genotype at enrollment (Irion *et al*, 1998), and some others omitted this group from analysis (Brockman *et al.*, 1999). Generally, the definition of recrudescence depends on the objective of the study, and in drug efficacy studies, like the present study, where the objective is the elimination of all parasites, the most stringent definition is adopted and a post-treatment sample containing a mixture of new and original parasite lines are defined as a recrudescence. Accordingly, the indeterminate group was considered as recrudescence in this study and the prevalence of the total recrudescence was 92%.

According to this classification the presence of only new alleles in post-treatment samples that were not detected in pre-treatment samples (reinfection) was detected in 2 (8%) of the 24-paired samples tested. One of these two samples that were classified as reinfection belonged to a 4 years aged child who failed CQ treatment and was classified in-vivo as late clinical failure on day 7 after CQ treatment. However, the failure to detect a particular genotype pattern by PCR dose not exclude its presence in the sample (Snounou and Beck, 1998). In this patient the new parasite clone that was detected on day 7 might have been present as liver stages at the time when the drug was administered. Chloroquine is not believed to act on pre-erythrocytic stages, however, the blood levels would be expected to be high at the time of post-treatment sampling, so it is likely that the new appearing parasites on day 7 were CQ resistant. Unfortunately, DNA of day 3 of this patient was not available to test whether the newly arising allele on day 7 was originally present before treatment and escaped detection on day 0.

Other scenarios might be responsible for the failure to detect parasite clones in some samples and could be explained by a number of factors including the sensitivity of the PCR and efficiency of the amplification, which are affected by the volume of

blood used in both pre-treatment and post-treatment samples. Furthermore, different alleles of the same genetic marker are amplified with varying efficiency, probably because of different length and/or sequence composition (Snounou and Beck, 1998). In multiple concurrent infections, template competition can affect the yield of individual products adding to the variability of sensitivity (Contamin *et al.*, 1995). Parasites may have been present in different proportions so that the minority clones were not amplified successfully in the PCR. The use of allele family-specific primers can reduce this problem because a smaller subset of variants is amplified (Snounou and Beck, 1998).

Other explanations for undetected parasite clones in some samples are sequestration and asynchrony. *P.falciparum* may be sequestered in the deep vasculature and therefore might not be present in the peripheral blood sample. In a synchronous infection parasite densities might temporarily fall below the level of detection of microscopy or even of that of PCR and in asynchronous infections the paroxysms can occur at any time and two or more can be recorded in quick succession leading to the appearance of different parasite genotypes on successive days (Farnert *et al.*, 1997), this may lead to misclassification of those parasite as new infections. Analysing multiple samples collected in quick succession (for example analysing an additional sample obtained on day 3 after treatment) could be a potential solution to this biological problem (Snounou and Beck, 1998). The presence of a strain-specific acquired immunity can also influence the course of an infection following treatment. Infections with resistant parasite to which a degree of immunity exists, may persist at low levels and their sporadic appearance may be classified falsely as new infection. Similarly, an inoculation with parasites to which some degree of immunity exists may not immediately reach detectable levels and their appearance later would be defined as new infections. The same may occur due to the effect of the pharmacokinetics of the drug together with the different degrees of drug resistance between different parasites, a weakly resistant parasite line might be partially controlled in the early phase of treatment, and fall below the threshold of detection only to reappear when the drug levels fall and be misclassified as a new infection (Snounou and Beck, 1998).

Despite these difficulties the results obtained indicated that most of the treatment failure cases (92% of the 61% in-vivo CQ treatment failure) were due to recrudescence of parasites resistant parasites to the curative dose of chloroquine. Accordingly the corrected treatment failure prevalence would be 56%.

This study is also the first to describe the genetic diversity and complexity of *P. falciparum* infections in Yemen. Up to 21 differently sized PCR product bands representing different *msp-2* allelic families IC/3D7 and FC27 with molecular size variation, which ranged from 300 bp to 1100 bp were detected in pre-treatment and post-treatment samples. However, it is possible that the sampling strategy underestimated the number of distinct clones since the clonal composition of parasites of a single patient may change from one day to another, possibly because of sequestration of parasites in deep blood vessels (Farnert *et al*, 1997). Furthermore, this part of the study was not originally designed to detect the diversity of the parasite and the complexity of infection, thus the low sample size used in this part of the study could be an additional factor for the underestimation of the diversity and complexity of infection. Moreover, the samples analysed belonged to symptomatic and asymptomatic patients 67% of which were afebrile during sample collection but had a history of fever during the last 24 hours. Including samples of febrile patients might be another factor resulting in the underestimation of the diversity and complexity of infection; the relationship between fever and the diversity and complexity of infection is discussed below.

The IC/3D7 family produced up to 11 bands with molecular size ranging from 400 bp to1000 bp (**Figure 5.2a**), and the FC27 family produced up to 10 bands, which ranged in size from 300 bp to1100 bp (**Figure 5.2a**). Unusual PCR products of 1000 bp and 1100 bp were detected when amplifying the FC27 family in pre-treatment samples. Bands were faint, some of them were present together with other bands in the sample as part of multiple clone infection (**Figure 5.2b, samples 3,6,7,10**) and others were present alone in the sample (**Figure 5.2b, sample 9**). It was suggested initially that these bands might be an artefact of the PCR, however, PCRs were repeated for these samples with less DNA but the same results were obtained. The FC27 alleles of 1000 bp and 1100 bp comprised 14% of all FC27 alleles detected in pre-treatment samples. They were only detected in pre-treatment samples and they

all disappeared in the post-treatment samples. This may suggest that these parasites were CQ sensitive and were cleared by the drug. The inclusion of this group of alleles in the analysis did not affect the classification of infections to recrudescent or reinfections (because all these alleles disappeared in post-treatment samples) and it did not affect the analysis of the multiplicity of infection.

The almost equal proportion of FC27 (48%) and IC/3D7 (52%) alleles documented in this study is similar to results of most other studies in other malaria endemic areas like Kilombero, Tanzania (Felger *et al.*, 1999), Ndipo in Senegal (Konate *et al.*, 1999), the Gambia (Conway *et al.*, 1991), Papua New guinea (Felger *et al.*, 1994) and in Kassena-Nankana District (KND) in northern Ghana (Owusu-Agyei *et al.*, 2002). This is consistent with the suggestion by (Felger *et al.*, 1999) that although the genetic diversity is extensive, it is limited by structural constraints or immune selection. However, different proportions of the two allelic families have been observed in studies in Dielmo in Senegal (Ntoumi *et al.*, 1995; 1997) and in Sao Tomé (Muller *et al.*, 2001). Ntoumi *et al,* 1997 attributed this difference to the high recombination frequency in Dielmo, however, Owusu-Agyei *et al*, 2002 argued against this explanation since the levels of transmission are similar in Dielmo and in KND in northern Ghana, so there is no reason why recombination should be more frequent in Dielmo than in KND (Owusu-Agyei *et al*, 2002).

The prevalence of IC/3D7 in pre-treatment samples was higher (70%) in asymptomatic patients (axillary temperature <37^{o}C), this was not significant (p=.202). Some studies found that the IC/3D7 family alleles were predictive of clinical symptoms (Magesa *et al*, 2002). Others claimed that children carrying FC27 were more likely to fall ill than those carrying the IC/3D7 type (Engelbrecht *et al.*, 1995); (Al-Yaman *et al.*, 1997). Thus different studies in different locations have associated different allele types with clinical symptoms possibly as a result of variations in both parasite genotype composition and human host immunological experience (Magesa *et al*, 2002).

The prevalence of IC/3D7 in samples of asymptomatic infections was significantly higher in children ≥ 5 years of age compared to FC27 infection, which was higher in young children < 5 years (*Fisher Exact p-value < 0.02*). Despite the small numbers

in the comparison groups the effect of age on the prevalence of IC/3D7 and FC27 infections was clear (**Figure 5.6**). A possible explanation for this observation is that antiparasite specific immune response against FC27 might have been developed early in life and become effective by the age of 5 so that children above the age of 5 years were more able to clear FC27. In contrast, antiparasite specific immune response against IC/3D7 needed more time to develop so that children above 5 years still had high prevalence of IC/3D7 but this frequency decreased with age until the antiparasite specific immune response became effective by the age of 15 years where the prevalence of IC/3D7 is low (**Figure 5.6**). This difference in the time of development and maturity of the specific immune response might be attributed to more diversity of IC/3D7 than FC27, the diversity might be underestimated in this study, so that the immune system needs more exposure to diverse antigens in order to develop an effective immune response against IC/3D7.

Multiple clone infection (>1 distinct clone per sample) was detected in 56% of pre-treatment samples and in 50% of post-treatment samples. Malaria parasites are haploid during their life cycle in human hosts (Walliker *et al.*, 1987), and the *msp-2* gene exists only as a single copy in the parasite genome (Kemp *et al.*, 1990). Thus, the presence of two or more alleles of the gene or the gene family in a single blood sample denotes the presence of a mixed infection with parasites of different genotypes. The number of clones/sample, or multiplicity, of such infections in any one infected host may be related to the endemicity of malaria (Konate *et al*, 1999) and to the degree of immunity against *P. falciparum*, and the risk of clinical malaria (AL-Yaman *et al*, 1997; Beck *et al*, 1997).

In this study, the number of alleles detected/sample in pre-treatment samples ranged from 1 to 6 different sized alleles with a mean of 1.92. Data on multiclonal infection of *P. falciparum* in some countries with different malaria endemicities have shown a positive relationship between the mean number of genotypes in people and the entomological inoculation rate (EIR) (**Table. 5.5**) (reviewed in (Babiker *et al.*, 1997). However, comparison of data from Africa and from some other countries like Papua New Guinea concluded that this correlation between the numbers of clones and the EIR did not hold (reviewed in Babiker *et al*, 1997) (**Table. 5.5**). This was attributed to variations in the mosquito and human host behaviour in different areas (Babiker *et*

al, 1997). Furthermore, factors that limit the malaria parasite load in the host must limit opportunities for outcrossing. Thus, sequestration of the parasite, strain-specific immunity in older children and adults and the possibility that not all clones carried by infected individuals produce infectious gametocytes simultaneously may limit the number of clones detectable at any one time (Babiker *et al*, 1997). An understanding of the mechanisms involved and its relation to intensity of transmission has important implications for malaria control. Unfortunately, information about the entomological inoculation rate in Yemen is not yet available.

Country	**EIR**	**Mean no. clones (range)**
Tanzania	>500	3.29 (1-6)
The Gambia	2-24	2.34 (1-4)
Sudan	0.6	1.3 (1-3)
PNG	40-400	1.8 (1-3)
Ghana	300	3.4 (1-8)
Senegal		
(Dielmo)	100-200	3.7 (1.8)
(Ndipo)	17	1.6 (1-4)
Kenya	0.1-27	2.1 (1-4)

Table 5.7 Relationship between multiclonal infection with *P. falciparum* and entomological inoculation rate (EIR) in some countries. PNG= Papua New Guinea. Source: Babiker *et al*, 1997; Owusu-Agyei *et al*., 2002)

Underestimation of the number of alleles detected/sample in this study is possible as discussed above. Though the use of only one genetic marker for detection might add to the underestimation of the number of alleles, it has the advantage of making the analysis of the association of the multiplicity of infection with some host factors, such as age and fever, more easy to interpret (Felger *et al*, 1999).

The findings that multiple clone infections per sample in pre-treatment samples was more prevalent in children ≥5 years (85%), in non-febrile (asymptomatic) patients (70%), in infections with parasite density <25,000 parasite/µl (77%), in late treatment failures (90.5%) compared to early treatment failures (ETF), and that among late treatment failures multiple clone infection was more common in late parasitological failures (68%) compared to late clinical failures (LCF), though not

statistically significant, are consistent with the expectation of the strain-specific immunity phenomenon (Marsh and Howard, 1986; Gupta and Day, 1994). A child with high multiplicity is expected to have a broader spectrum of immunological memory than one with low multiplicity, thus being more protected. The observed low multiplicity in symptomatic infections could be due to the anti-parasite effect of fever (Kwiatkowski and Nowak, 1991), however, other scenarios might be involved, for example most fever episodes appear to be associated with parasitaemia peaks (Magesa *et al*, 2002), which might result from expansion of just a few or even a single genotype and minority clones might not be detectable by PCR. It is also possible that symptomatic children are more predisposed to clinical malaria, thus more likely to be taking treatment, thus eliminating a considerable number of genotypes from their parasite pool (Magesa *et al*, 2002).

Further stratification by age showed that the highest multiplicity occurred in asymptomatic children aged 5-9 years followed by that in children aged 10-14 years. The least multiplicity was observed in adults ≥15 years and in children less than 5 years. This influence of age on the multiplicity of infection is similar to the findings in many other malarious areas with high transmission (Ntoumi *et al.*, 1995; Konate *et al.*, 1999; Smith *et al.*, 1999; Owusu-Agyei *et al*, 2002). However, studies in areas of low endemicity such as Ndipo in Senegal (Konate *et al.*, 1999), Riboque in Sao Tomé (Muller *et al*, 2001), the Gambia (Conway *et al*, 1991) and Sudan (Babiker, 1998) reported little or no influence of age on the multiplicity of infection.

The low multiplicity in children <5 years is explained by poor immunity in this age group. This age group is also expected to be the most ill and febrile hence receiving the most treatment, which could be another explanation for low number of clones per infection. Similarly, the low multiplicity in adults ≥ 15 years could be explained by strain-specific immunity acquired after 10-15 years of age in holoendemic areas (Ntoumi *et al*, 1997), which leads to the clearance of a number of parasites in this age group. The 10-14 years old children still had a multiplicity higher than the older age group. Thus the 5-14 years old children, in which clinical immunity is in place and antiparasite immunity is being developed, is the group with the highest number of different genotypes, consistent with the notion that cumulative exposure to numerous *P. falciparum* types is required to achieve effective parasite clearance.

The low parasite density usually observed in older children and adults ≥ 15 years might lead to failure of detection of some parasite genotypes accounting for the low multiplicity in older age groups, however, in this analysis the parasite densities in samples of those above the age of 15 years ranged from 2640 to 69040 asexual parasite/µl with a geometric mean of 12589 asexual parasite/µl, nevertheless, it is still possible that even when parasite density is sufficiently high for detection, some genotypes in the parasite pool may exist in low undetectable densities.

These immunity related findings are also consistent with previous findings of the significant association between young age <10 years and CQ treatment failure (**Chapter 3**) and the finding of significant association between age ≥10 years and the clearance of resistant parasite (**Chapter 4**). The failure to detect any significant effect is mainly due to the small sample sizes of comparison groups used in this analysis.

The multiplicity of infection may also have important implications for the epidemiology of drug-resistant *P. falciparum* malaria and the outcome of treatment in patients. The initial presence of several parasite populations with different drug response profiles would result in the elimination of the drug sensitive populations and selection of resistant parasites. This was confirmed in the present study by the significant decrease of the mean number of alleles per sample in pre-treatment samples of both symptomatic and asymptomatic patients from 1.92 to 1.63 in post-treatment samples (*Wilcoxon Signed Ranks Test, p-value=0.028*). This is similar to results obtained from the analysis of samples collected from patients treated with chloroquine or sulfadoxine-pyrimetamine in other malaria endemic areas like Uganda (Jelinek *et al.*, 1999) and Nigeria (Happi *et al*, 2004).

Moreover, this difference in the means of the number of clones per sample between pre-treatment and post-treatment samples was only statistically significant in the FC27 family (*p-value*= .016) but not in IC/3D7 family (*p-value*= .157). A possible explanation for this observation is that most of the alleles of the FC27 family parasites found in pre-treatment samples might be CQ-sensitive and hence cleared by the drug. It might be explained also by the possibility that the drug boosted the

immune system to develop more strain-specific antibodies against alleles of the FC27 family parasites leading to the clearance of these alleles after treatment more than the clearance of alleles of the IC/3D7 family parasites. The finding that the multiplicity of infection with FC27 clones was lowered more than the multiplicity of infection with IC/3D7 clones in post-treatment samples of patients above the age of 5 years but not in younger children (**Figure 5.9a and b**) may support this conclusion and is consistent with the finding of lower prevalence of FC27 infection in children ≥ 5 years (**Figure 5.7**). In contrast, the multiplicity of infection with IC/3D7 clones in post-treatment samples was only lowered in samples of patients ≥15 years of age (**Figure 5.9b**), which might be also explained by a degree of strain-specific antiparasite immunity in this age group and this might be due to the underestimated diversity of IC/3D7 clones compared to FC27 clones as discussed above. A population study with large sample size and sampling procedure based on collection of successive samples would be needed to give more accurate estimation of the diversity and complexity of infection in the area. However, despite the limitations related to small sample size and sampling procedure in this part of the study, it can be concluded that malaria parasites in the area are highly diverse and multiplicity of infection tends to be age-dependent comparable to many areas with high malaria transmission. Implications of the findings for malaria control in Yemen will be discussed in Chapter 7.

CHAPTER 6

Sequencing The Plasmodium falciparum Chloroquine Resistance Transporter Gene *pfcrt* In Yemeni Isolates

6.1 INTRODUCTION

The identification of *pfcrt* gene, which encodes a putative transporter or channel protein (Fidock *et al.*, 2000) was a major breakthrough in the search for the genetic basis of chloroquine resistance in *P. falciparum.* The *pfcrt* gene is a highly interrupted gene, with 13 exons (**Figure 6.9**) spanning 3.1 kb and encoding a 424 amino acid (48.6 kDa) protein localized to the digestive vacuole membrane of the parasite (Fidock *et al.*, 2000a; (Cooper *et al.*, 2002). Fifteen polymorphic amino acid positions in PfCRT protein are associated with chloroquine resistance in field isolates. These vary greatly depending on geographic location and selection history, while chloroquine sensitive strains maintain an invariably wild-type allele (Wootton *et al.*, 2002; Chen *et al.*, 2003; Best Plummer *et al.*, 2004; Durrand *et al.*, 2004). A K76T change appears necessary for the resistance phenotype, and is the most reliable molecular marker of resistance among the various *pfcrt* mutations (Djimde *et al.*, 2001a; Plowe, 2003).

Types of mutations at the level of the DNA sequence, described in Brown, (1992), could be classified as: 1) point mutations: resulting from the replacement of one nucleotide by another. This can be further classified into: a) transition if it is a purine to purine (A↔G) or a pyrimidine to pyrimidine (T↔C) change, b) transversion if the alteration is purine to pyrimidine or vice versa (A or G ↔T or C). 2) insertion or deletion: resulting from the addition or removal of anything from one base pair up to quite extensive pieces of DNA, and 3) inversion resulting from the excision of a portion of the double helix followed by its reinsertion at the same position but in the reverse orientation.

Chloroquine resistance appeared in the early 1950s in Southeast Asia and in the late 1960s in South America, leading to the suggestion that resistance arose from independent founder events (Su *et al.*, 1997). Recent studies analysing a large number of geographically diverse *pfcrt* alleles and microsatellite genotypes from parasite isolates have identified at least three additional independent foci of resistance (Wootton *et al.*, 2002; Chen et al., 2003). The origins of chloroquine resistance, which have so far been discovered are: the Thai-Cambodian border region (eventually spreading westward into Africa), Papua New Guinea, the Philippines, and Colombia and Peru (Hayton and Su, 2004). Currently, 21 unique Chloroquine

resistance PfCRT protein sequences are identified from field isolates and two additional haplotypes have been generated through the laboratory experiments using CQ selective pressure on the 106/1 parasite line (Cooper *et al.*, 2002). Four unique chloroquine sensitive haplotypes have been generated by laboratory drug selection experiments (Johnson *et al.*, 2004;Cooper *et al.*, 2005).

Three pfcrt haplotypes have been reported from multiple, geographically distinct locations. The Old World resistant haplotype CVIETIHSESII (amino acids72-73-74-75-76-77-97-220-271-326-356-371), represented in the FCB line of Southeast Asia, is also commonly found in African isolates such as RB8, consistent with the spread of chloroquine resistance from Asia to Africa (Su *et al.*, 1997; Wootton *et al.*, 2002). The CVIETIHSESTI haplotype, found in the 102/1 Sudan strain, is also in other well-characterized isolates such as Dd2 from Thailand, and in the newly described PH4 from Morong, Philippines (Fidock *et al.*, 2000a; Chen *et al.*, 2003). The Old World CVIET *pfcrt* haplotype was reported in South America (Vieira *et al.*, 2004), suggesting that a traveller may have recently introduced this parasite. The SVMNTIHSQDLR haplotype has been detected in the INDO19 isolate line from Thailand, the FCQ22 found in Papua New Guinea, and the 7G8 line from Brazil (Fidock *et al.*, 2000; Chen *et al.*, 2003). Proper analysis of field isolates may reveal new haplotypes, and new foci of chloroquine resistance.

Sequencing the full length of *pfcrt* was performed in three representative isolates of which two carried the pfcrt-T76 (by PCR) and the third carried the pfcrt-K76 (by PCR). One of the two isolates (that carried the pfcrt-T76 by PCR) was CQ-resistant in-vivo (belonged to a case of early treatment failure ETF) and the other was a CQ-sensitive in-vivo (belonged to a case of adequate clinical and parasitological response ACPR).

Note: the word isolate is used in this chapter to represent studied DNA of parasites from blood samples collected in filter papers without any implication that they have been cultured.

Sequencing was performed with the following objectives:

- To confirm the presence of pfcrt-K76T in CQ- resistant isolates.
- To describe the possible CQ-resistance haplotype of a Yemeni parasite.
- To find if there is any difference in PfCRT sequence between isolates carrying the *pfcrt*-T76 but having different in-vivo CQ treatment response.

6.2 MATERIALS AND METHODS

6.2.1 PCR Amplification Of The *pfcrt* Gene

The full length of the *pfcrt* gene was amplified using a number of primers that flank the 13 exons of the gene (*Johnson D.J, personal communication*). The DNA used for amplification was extracted using either the methanol-fixation heat-extraction method (**Chapter 2, section 2.2.2.1**) or using the Qiagen kit (**Chapter 2, section 2.2.2.2**). The 13 exons were amplified using nested PCR in 3 different reactions; i) amplification of exons1 and 2 (**Chapter 2, section 2.4.1**), ii) amplification of exons 3 to 8 (**Chapter 2, section 2.4.2**), and iii) amplification of exons 9 to 13 (**Chapter 2, section 2.4.2**). Products of amplification were purified either directly from PCR (**Chapter 2, section 2.4.3.1**) or after gel extraction (**Chapter 2, section 2.4.3.2**) before sending to Lark Technologies for sequencing. The primer sequences used to amplify and to sequence the 13 exons of *pfcrt* were designed by D.J.Johnson and were synthesized by Sigma-Genosys (Pampisford, UK; http://www.sigma-genosys.co.uk). Primer names and sequences are listed in **chapter 2**, **Table 2.9** and the PCR cycling conditions used to amplify different exons of *pfcrt* are shown in **chapter 2**, **Table 2.10**.

6.2.2 Contig Formation And Assembly Of The pfcrt DNA Sequence

Direct DNA sequencing of the *pfcrt* gene from three representative field isolates was carried out by Lark Technologies, Inc (Saffron Walden, UK). DNA sequence files received from Lark Technologies contained the raw DNA sequence, as well as, the sequence chromatograms. The computer software SeqMan (DNA*star*, Lasergene, USA) was used to build the contigs for each parasite isolate and MegAlign was utilised to align and compare the DNA sequences of *pfcrt* from each parasite isolate.

6.3 RESULTS

6.3.1 PCR Amplification Of Exons 1and 2

The amplification of exon1-2 was performed on methanol-extracted DNA (**Chapter 2, section 2.2.2.1**) and yielded the target product of an approximate molecular size of 670 base pairs plus 2 additional unspecific bands with lower molecular sizes (approximately 450 bp and 280 bp) (**Figure 6.1**). Therefore, the target band (700 bp) was excised from the gel and then purified using QIAquick Gel Extraction Kit (*QIAGEN*) (**Chapter 2, section 2.4.3.2**). The amount of purified DNA was then measured using 1µl in a ND-1000 Spectrophotometer (Nanodrop Technologies) before sending to Lark Technologies for sequencing.

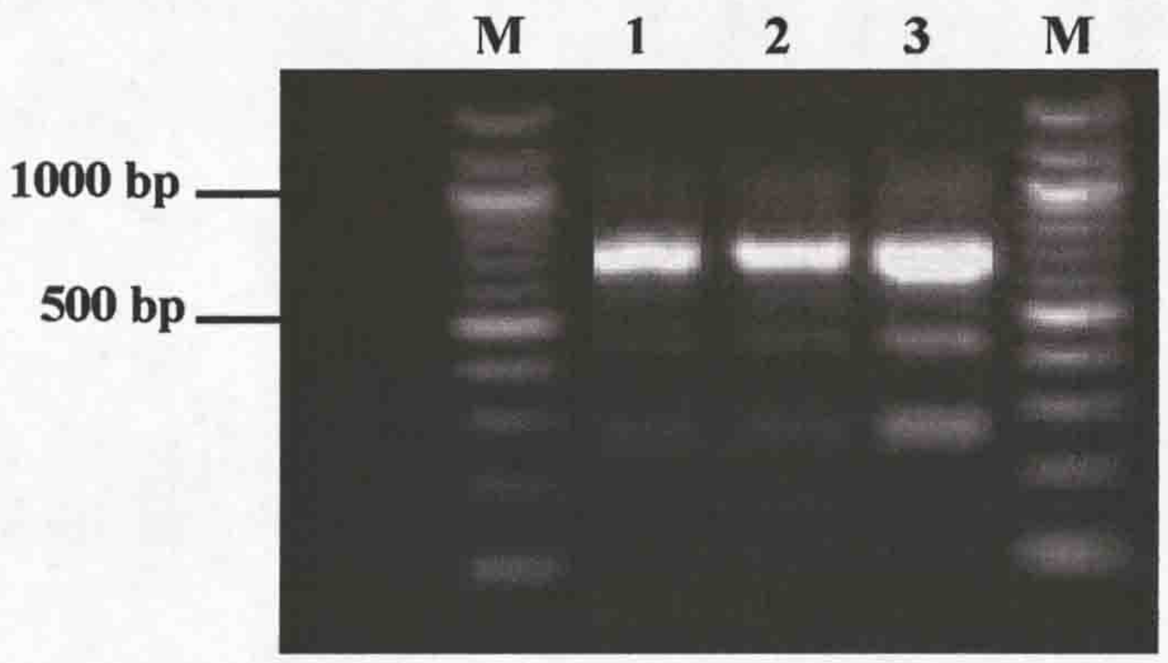


Figure 6.1 PCR amplification of exons 1 and 2 of the *pfcrt* gene for subsequent direct sequencing. Lanes 1,2, and 3 represent three representative Yemeni field isolates. Lanes M on both sides represent a 100 bp molecular ladder marker.

6.3.2 PCR Amplification Of Exons 3, 4, 5, 6, 7 And 8

Using the DNA extracted by the methanol-fixation heat-extraction method, the PCR amplification of exons 3, 4, 5, 6, 7 and 8 yielded very faint bands even after several trials to optimise the PCR and cycling conditions (**Figure 6.2, Lanes 1 and 2**). The PCR amplification was then repeated using DNA extracted with the Qiagen kit (**Chapter 2, section 2.2.2.2**), this resulted in successful amplification of the target product of an approximate molecular size of 1500 base pairs plus additional unspecific bands with lower molecular sizes (**Figure 6.2**). Therefore, the target band (1500 bp) was excised from the gel and then purified using QIAquick Gel Extraction Kit (*QIAGEN*) as described in **chapter 2, section 2.4.3.2**.

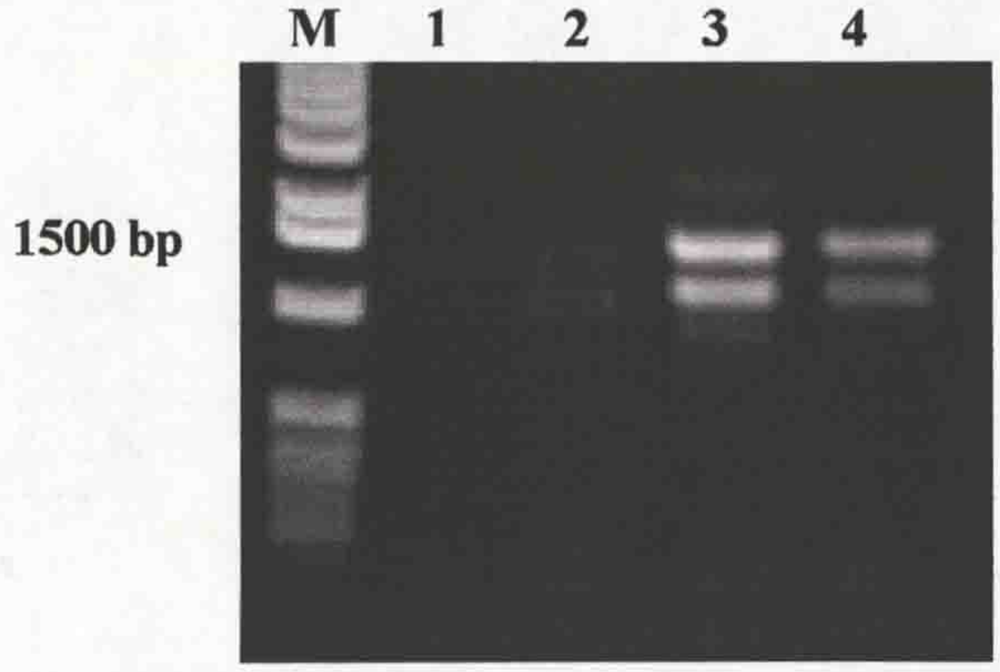


Figure 6.2 PCR amplification of exon 3,5.6,7 and 8 of the *pfcrt* gene for subsequent direct sequencing. Lanes 1, and 2 represent the amplification of methanol-extracted DNA of 2 field isolates. Lanes 3 and 4 represent the amplification of Qiagen kit extracted of the same samples. Lane M is a 1 Kb bp molecular ladder marker.

The amount of the purified DNA prepared as measured using a ND-1000 Spectrophotometer (Nanodrop Technologies) was not satisfactory to perform the required sequencing reaction (4-9 ng/µl). This problem of low DNA concentration was solved by repeating the nested amplification reaction of exon 3-8 (using the primers of the secondary reaction listed in **chapter 2, Table 2.9**) and using the resultant purified, gel excised DNA as a template for this reaction. The objective was to obtain a strong, clean band of the right size and to purify the resulting DNA directly from the PCR product. This was successful as shown in **Figure 6.3** and after PCR product purification, the amount of DNA was found to be 35-100 ng/µl. DNA was then sent to Lark Technologies for sequencing.

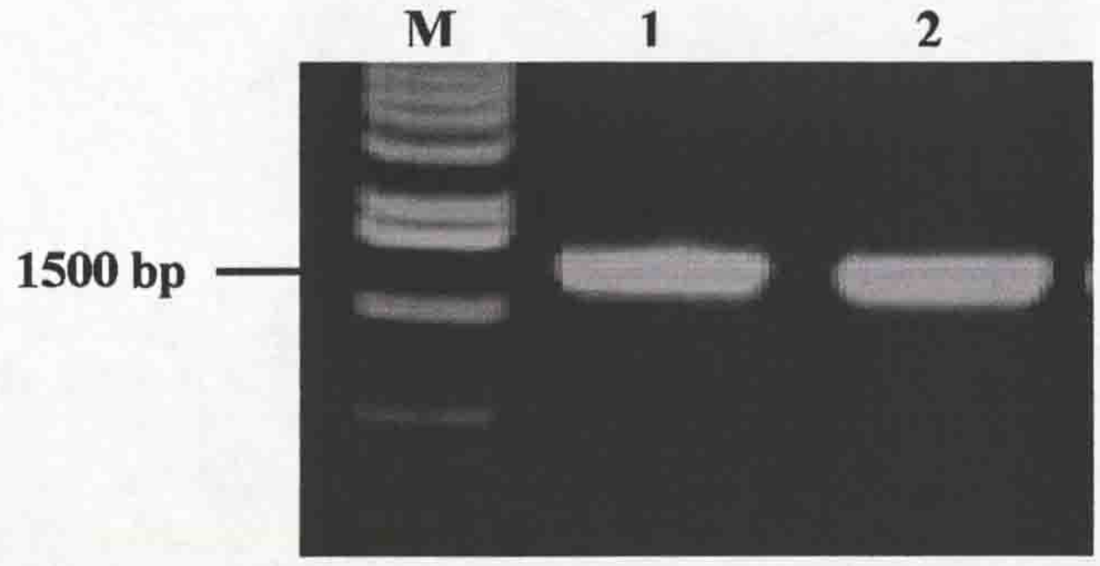


Figure 6.3 Repeated PCR amplification of exon 3,5.6,7 and 8. Lanes 1, and 2 represent the amplification of the purified, gel excised DNA resulted from the PCR shown in Lanes 3 and 4 of figure 6.3. Lane M is a 1 Kb bp molecular ladder marker.

6.3.3 PCR Amplification Of Exon 9, 10, 11, 12 And 13

PCR amplification of exon 9-13 was performed using the DNA extracted by the Qiagen kit (**Chapter 2, section 2.2.2.2**). This resulted in successful amplification of the target product of an approximate molecular size of 1200 base pairs plus an additional unspecific band with a molecular size of approximately 1000 bp (**Figure 6.4**). The target band (1200 bp) was excised from the gel and then purified using QIAquick Gel Extraction Kit (*QIAGEN*) as described in **chapter 2, section 2.4.3.2**. The amount of the resultant purified DNA was optimum for sequencing as measured by a ND-1000 Spectrophotometer (Nanodrop Technologies) and DNA was sent to Lark Technologies for sequencing.

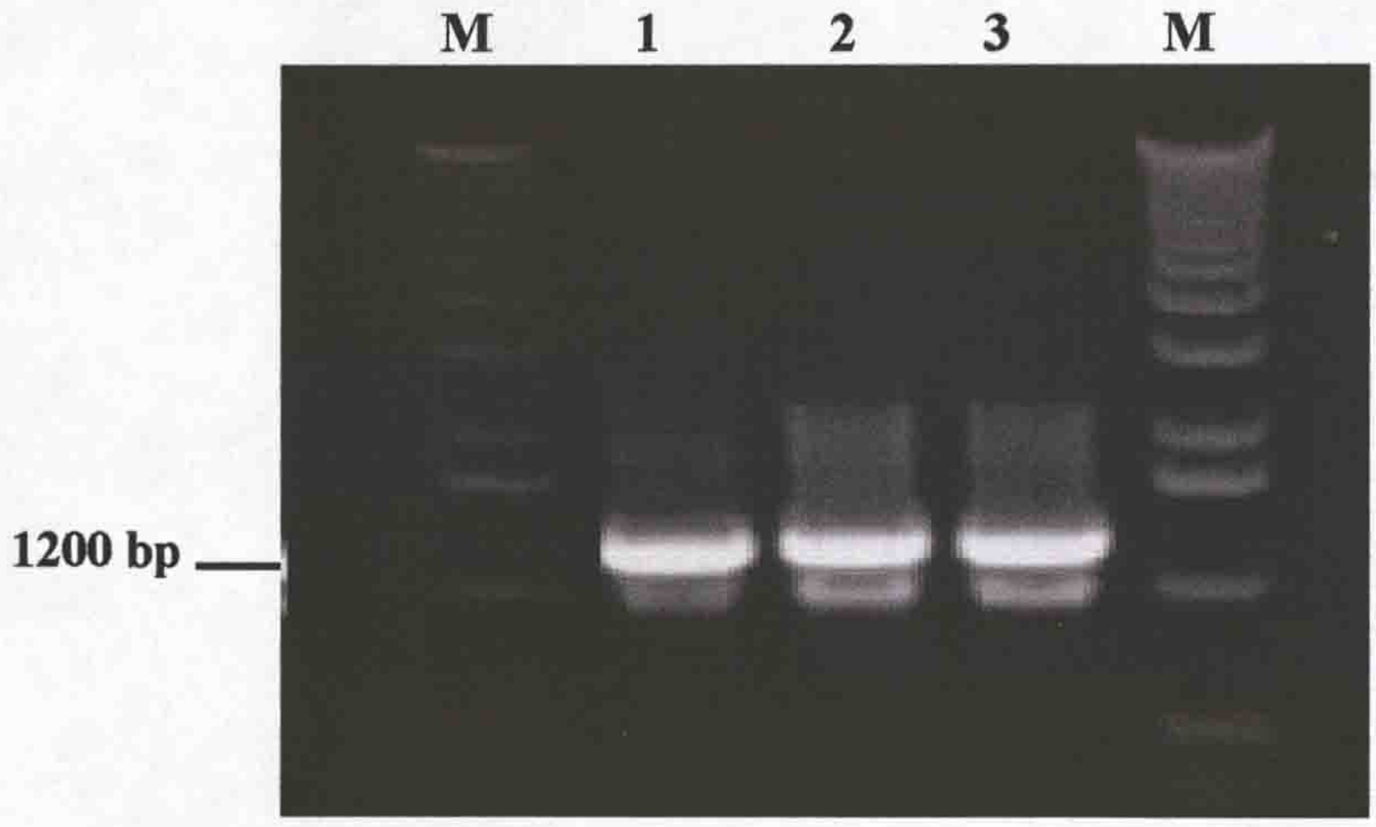


Figure 6.4 PCR amplification of exon 9,10,11,12 and 13 of the *pfcrt* gene for subsequent direct sequencing. Lanes 1, 2 and 3 represent the amplification of Qiagen kit extracted DNA of the three representative Yemeni field isolates. Lanes M on both sides represent a 1 Kb bp molecular ladder marker.

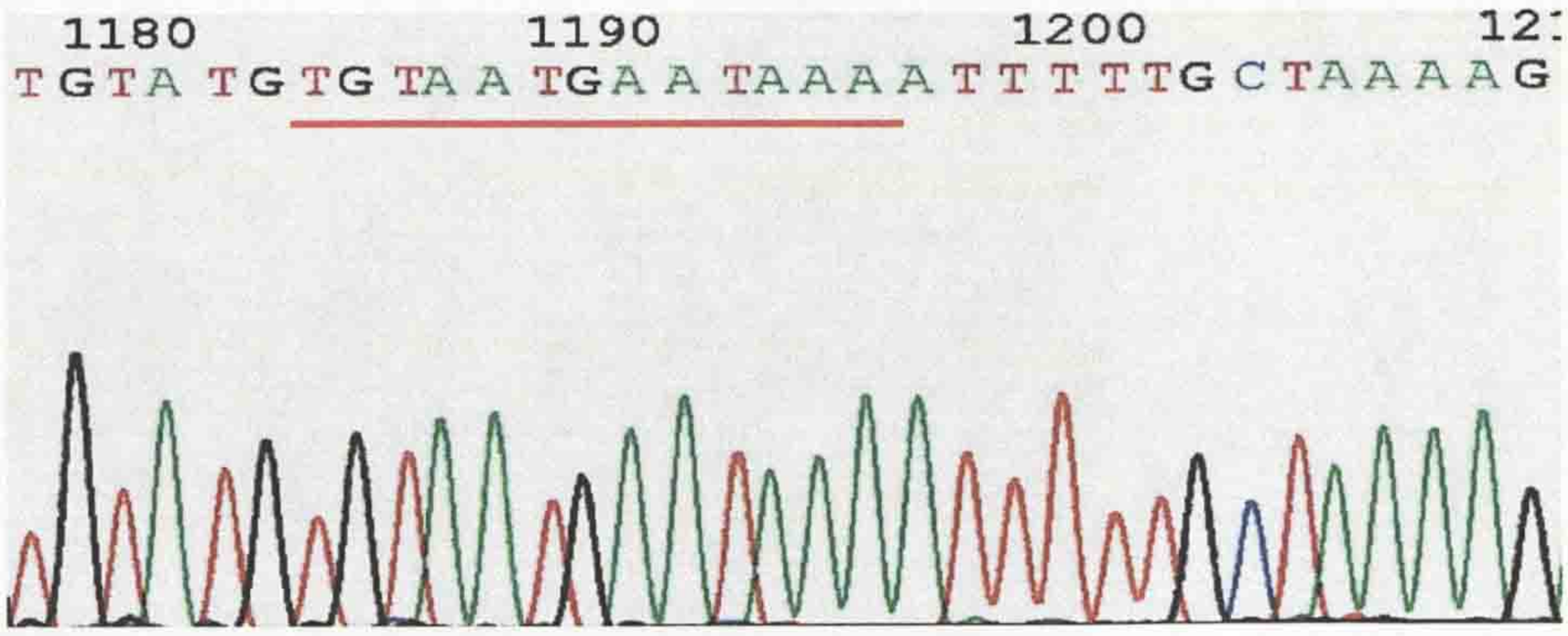
6.3.4 Results Of Sequencing Of Three Field Isolates:

The amplified and purified DNA of the 13 exons was sent together with the sequencing primers to Lark Technologies, Inc (Saffron Walden, UK) for direct DNA sequencing of the *pfcrt* gene.

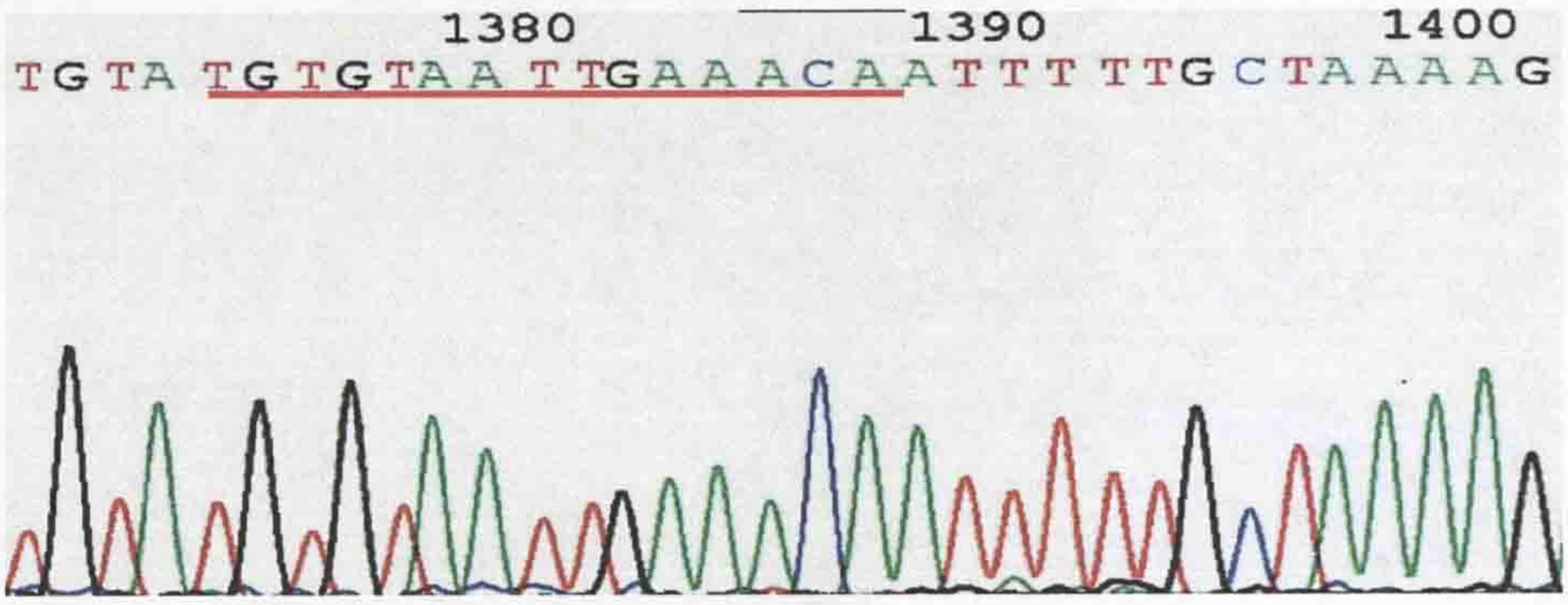
The three representative field isolates were chosen according to the *pfcrt*-K76T polymorphism detected by PCR and to their sensitivity to CQ in-vivo. They were subsequently named as follows: **Ye02-70W** is a wild isolate (carrying K76 by PCR) and CQ-sensitive in-vivo (belonging to a case of in-vivo adequate clinical and parasitological response ACPR), **Ye02-4M** is a mutant isolate (carrying T76 by PCR) and CQ-resistant in-vivo (belonging to a case of in-vivo early treatment failure ETF), and **Ye02-54M** is a mutant isolate (carrying T76 by PCR) but was CQ-sensitive in-vivo (belonging to a case of in-vivo adequate clinical and parasitological response ACPR). The **Ye** in the name of each isolate represents the international code of Yemen, the **02** refers to the year when the isolates were collected (2002), the numbers **(70, 4, 54)** indicates the laboratory number of each isolate through which we can also refer back to the patient number, and finally the letters **(W** and **M)** indicates whether the isolate is wild or mutant compared to the HB3 clone of *P. falciparum.*

The computer software SeqMan (DNA*star*, Lasergene, USA) was used to build the contigs for each parasite isolate **(Figure 6.5 and Figure 6.6)**.

Ye02-70W



Ye02-4M



Ye02-54M

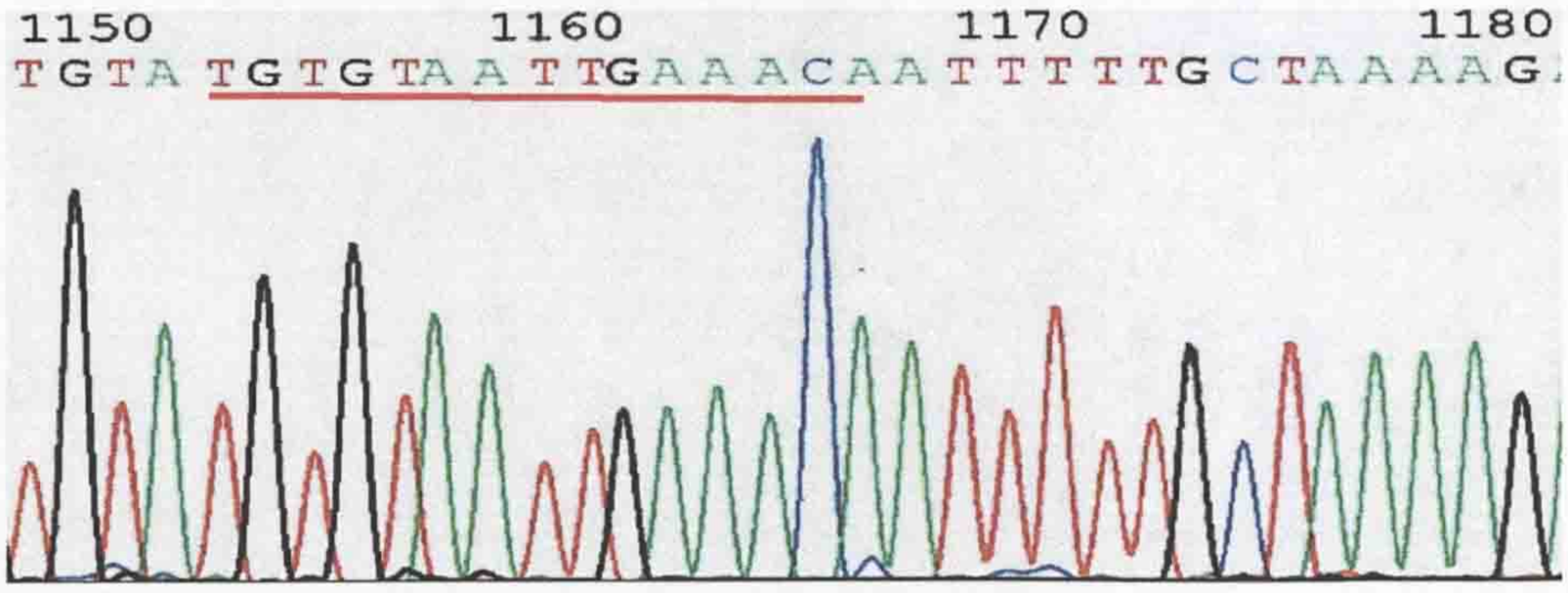
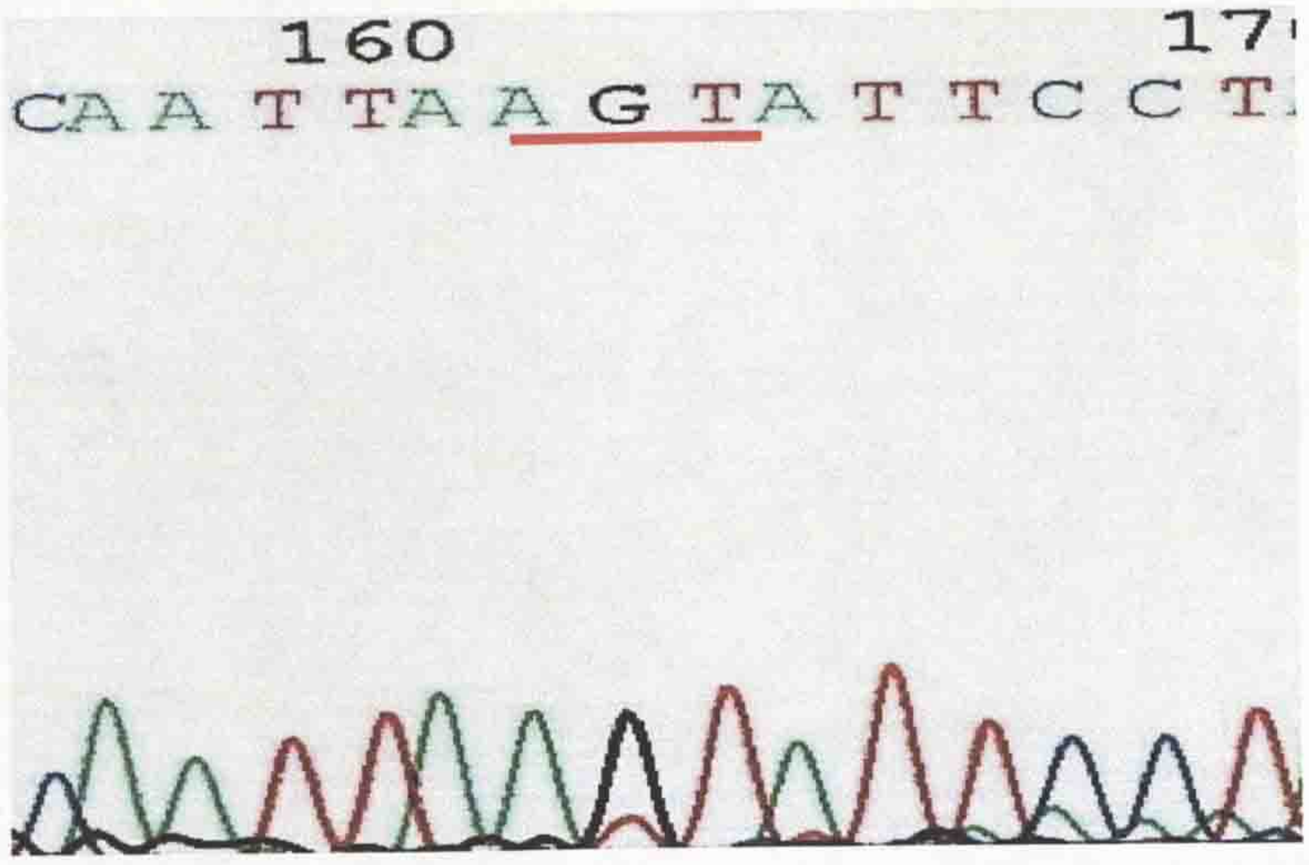
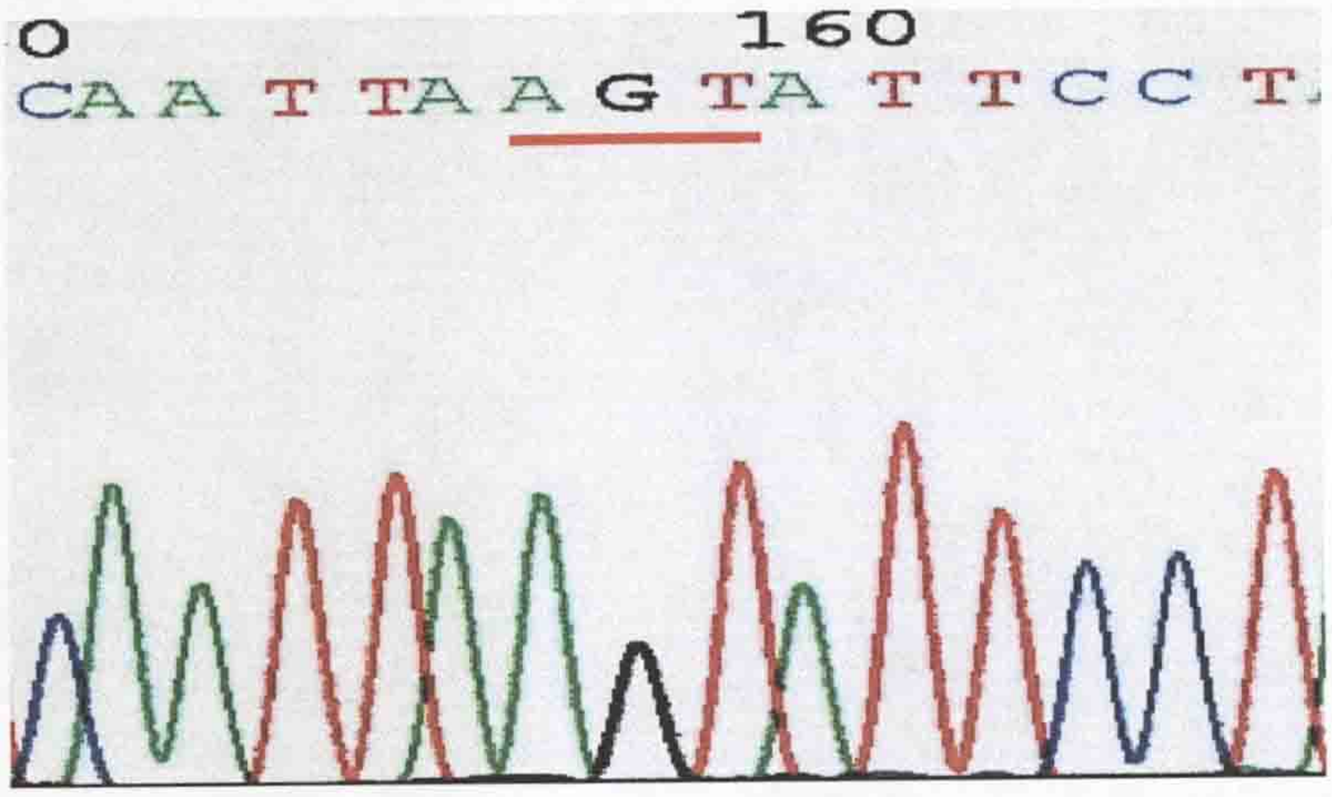


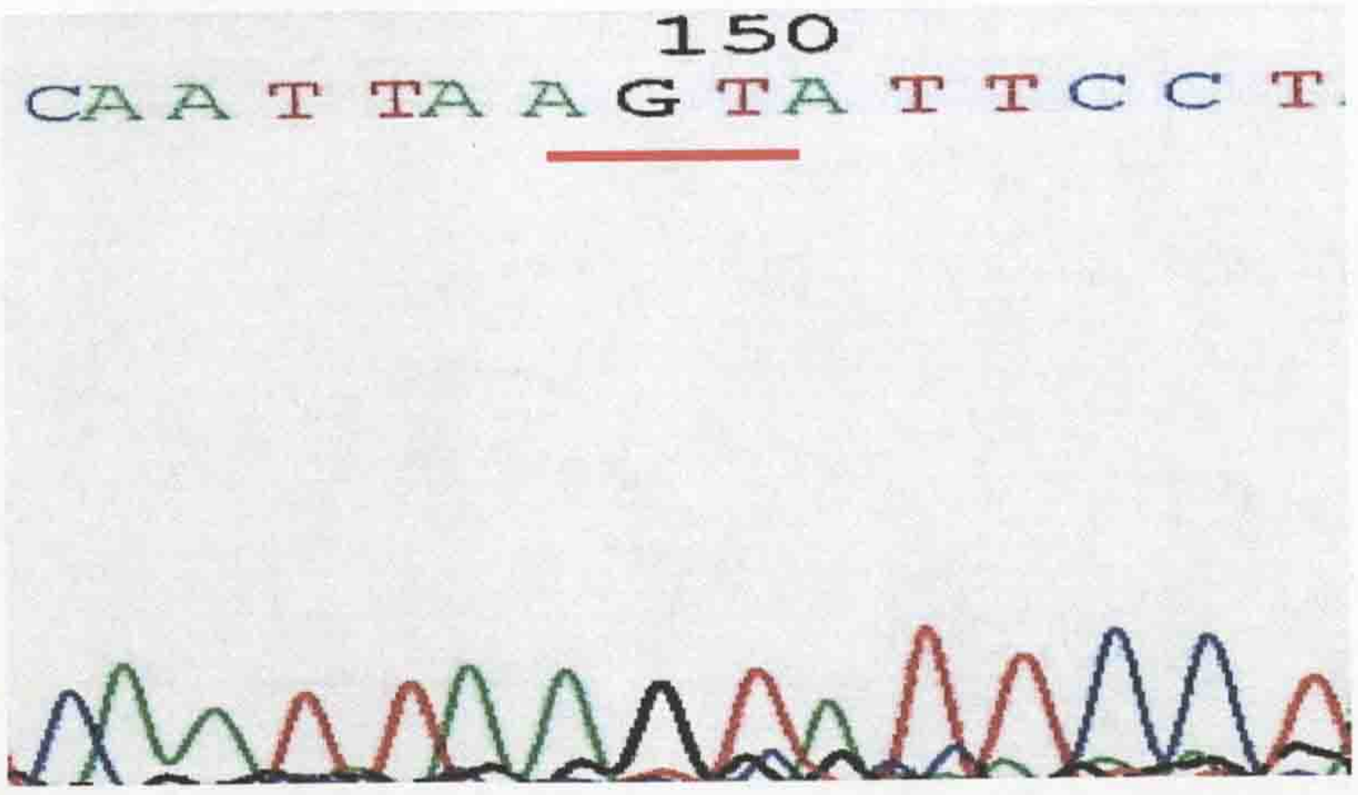
Figure 6.5 Chromatogram of part of exon 2 of the 3 isolates. Codons 72-76 are underlined with a red line. Note the mutant codons 74 (ATT), 75 (GAA) and 76 (ACA) in both Ye02-4M and Ye02-54M compared to the wild codons 74 (ATG), 75 (AAT) and 76 (AAA) in Ye02-70W



Ye02-70W



Ye02-4M



Ye02-54M

Figure 6.6 Chromatogram of part of exon 3 of the 3 isolates. Codon 163 is underlined with a red line showing a wild-type sequence (AGT) encoding for the amino acid serine in this position in the 3 isolates.

6.3.5 Alignment Of PfCRT Sequence And Identification Of The Mutations In The Three Isolates:

Alignment of the PfCRT sequences from the three field isolates Ye02-70W, Ye02-4M and Ye02-54M, and the comparison of their sequence with the HB3 clone of *P. falciparum* was performed using the MegAlign (Lasergene) computer program utilising the Jotun Hein method for alignment (**Figure 6.7**). Amino acid sequence alignment showed similarity of the Ye02-70W sequence to the sequence of HB3. The alignment showed also similarity in the amino acid sequences of the Ye02-4M and Ye02-54M.

Compared to the sequence of HB3, 7 mutations were identified in each of the two isolates Ye02-4M and Ye02-54M (**Figure 6.7**). The 7 mutations were point mutations resulted from the substitution of a single nucleotide (**Table 6.1**) and were as follows: 1) a substitution of methionine with isoleucine at codon 74 (M74I), which resulted from a transversion (G→T) point mutation, 2) a substitution of asparagine with glutamic acid at codon 75 (N75E), which resulted from 2 separate point mutations, which are a transition (A→G) and a transversion (T→A), 3) a substitution of lysine with thereonine at codon 76 (K74T), which resulted from a transversion (A→C) point mutation, 4) a substitution of alanine with serine at codon 220 (A220S), which resulted from a transversion (G→T) point mutation, 5) a substitution of glutamine with glutamic acid at codon 271 (Q271E), which resulted from a transversion (C→A) point mutation, 6) a substitution of asparagine with serine at codon 326 (N326S), which resulted from a transition (A→G) point mutation, and 7) a substitution of arginine with isoleucine at codon 371 (R371I), which resulted from a transversion (G→T) point mutation. **Table 6.1** shows the PfCRT haplotypes of the three isolates and the corresponding codon (the triplet of nucleotides that code for a single amino acid) of each amino acid.

The intron/exon boundaries were determined by aligning the consensus sequence against the published Dd2 mRNA (Accession number AF030694) sequence of PfCRT (**Figure 6.8**). The intron/exon boundaries were identical for the three isolates studied. Alignment of the PfCRT sequences from the three isolates against all available PfCRT sequences from GeneBank (for accession numbers please see

Appendix 6) indicated the absence of any novel mutation in the sequences of the 3 field isolates.

Figure 6.7 Alignment of amino acid sequence of PfCRT from the three sequenced parasites (Ye02-70W, Ye02-4M and Ye02-54M) and the HB3 clone. Amino acid residues that differ from HB3 are shaded in solid black. The haplotype for codons 72-76 is underlined.

```
HB3         M K F A S K K N N Q K N S S K N D E R Y R E L D N L V Q E G N G S R L G G G S C L G K C A H V F K L I F K E I K D N I F
Ye02-70W    M K F A S K K N N Q K N S S K N D E R Y R E L D N L V Q E G N G S R L G G G S C L G K C A H V F K L I F K E I K D N I F
Ye02-4M     M K F A S K K N N Q K N S S K N D E R Y R E L D N L V Q E G N G S R L G G G S C L G K C A H V F K L I F K E I K D N I F
Ye02-54M    M K F A S K K N N Q K N S S K N D E R Y R E L D N L V Q E G N G S R L G G G S C L G K C A H V F K L I F K E I K D N I F

HB3         I Y I L S I I Y L S V C V M N K I F A K R T L N K I G N Y S F V T S E T H N F I C M I M F F I V Y S L F G N K K G N S K
Ye02-70W    I Y I L S I I Y L S V C V M N K I F A K R T L N K I G N Y S F V T S E T H N F I C M I M F F I V Y S L F G N K K G N S K
Ye02-4M     I Y I L S I I Y L S V C V I E T I F A K R T L N K I G N Y S F V T S E T H N F I C M I M F F I V Y S L F G N K K G N S K
Ye02-54M    I Y I L S I I Y L S V C V I E T I F A K R T L N K I G N Y S F V T S E T H N F I C M I M F F I V Y S L F G N K K G N S K

HB3         E R H R S F N L Q F F A I S M L D A C S V I L A F I G L T R T T G N I Q S F V L Q L S I P I N M F F C F L I L R Y R Y H
Ye02-70W    E R H R S F N L Q F F A I S M L D A C S V I L A F I G L T R T T G N I Q S F V L Q L S I P I N M F F C F L I L R Y R Y H
Ye02-4M     E R H R S F N L Q F F A I S M L D A C S V I L A F I G L T R T T G N I Q S F V L Q L S I P I N M F F C F L I L R Y R Y H
Ye02-54M    E R H R S F N L Q F F A I S M L D A C S V I L A F I G L T R T T G N I Q S F V L Q L S I P I N M F F C F L I L R Y R Y H

HB3         L Y N Y L G A V I I V V T I A L V E M K L S F E T Q E E N S I I F N L V L I S A L I P V C F S N M T R E I V F K K Y K I
Ye02-70W    L Y N Y L G A V I I V V T I A L V E M K L S F E T Q E E N S I I F N L V L I S A L I P V C F S N M T R E I V F K K Y K I
Ye02-4M     L Y N Y L G A V I I V V T I A L V E M K L S F E T Q E E N S I I F N L V L I S S L I P V C F S N M T R E I V F K K Y K I
Ye02-54M    L Y N Y L G A V I I V V T I A L V E M K L S F E T Q E E N S I I F N L V L I S S L I P V C F S N M T R E I V F K K Y K I

HB3         D I L R L N A M V S F F Q L F T S C L I L P V Y T L P F L K Q L H L P Y N E I W T N I K N G F A C L F L G R N T V V E N
Ye02-70W    D I L R L N A M V S F F Q L F T S C L I L P V Y T L P F L K Q L H L P Y N E I W T N I K N G F A C L F L G R N T V V E N
Ye02-4M     D I L R L N A M V S F F Q L F T S C L I L P V Y T L P F L K E L H L P Y N E I W T N I K N G F A C L F L G R N T V V E N
Ye02-54M    D I L R L N A M V S F F Q L F T S C L I L P V Y T L P F L K E L H L P Y N E I W T N I K N G F A C L F L G R N T V V E N

HB3         C G L G M A K L C D D C D G A W K T F A L F S F F N I C D N L I T S Y I I D K F S T M T Y T I V S C I Q G P A I A I A Y
Ye02-70W    C G L G M A K L C D D C D G A W K T F A L F S F F N I C D N L I T S Y I I D K F S T M T Y T I V S C I Q G P A I A I A Y
Ye02-4M     C G L G M A K L C D D C D G A W K T F A L F S F F S I C D N L I T S Y I I D K F S T M T Y T I V S C I Q G P A I A I A Y
Ye02-54M    C G L G M A K L C D D C D G A W K T F A L F S F F S I C D N L I T S Y I I D K F S T M T Y T I V S C I Q G P A I A I A Y

HB3         Y F K F L A G D V V R E P R L L D F V T L F G Y L F G S I I Y R V G N I I L E R K K M R N E E N E D S E G E L T N V D S
Ye02-70W    Y F K F L A G D V V R E P R L L D F V T L F G Y L F G S I I Y R V G N I I L E R K K M R N E E N E D S E G E L T N V D S
Ye02-4M     Y F K F L A G D V V I E P R L L D F V T L F G Y L F G S I I Y R V G N I I L E R K K M R N E E N E D S E G E L T N V D S
Ye02-54M    Y F K F L A G D V V I E P R L L D F V T L F G Y L F G S I I Y R V G N I I L E R K K M R N E E N E D S E G E L T N V D S

HB3         I I T Q
Ye02-70W    I I T Q
Ye02-4M     I I T Q
Ye02-54M    I I T Q
```

Decoration: residues that differ from HB3 clone, shades (with solid black) indicate residues that differ from HB3 clone

Isolate	PfCRT amino acid positions																		
	72	74	75	76	97	144	148	152	160	163	194	220	271	275	326	333	352	356	371
Ye02-70W	C	M	N	K	H	A	L	T	L	S	I	A	Q	P	N	T	Q	I	R
	(tgt)	(atg)	(aat)	(aaa)	(cac)	(gcc)	(ctt)	(act)	(ctt)	(agt)	(ata)	(gcc)	(caa)	(caa)	(aac)	(acc)	(caa)	(ata)	(aga)
Ye02-4M	C	I	E	T	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	I
	(tgt)	(att)	(gaa)	(aca)	(cac)	(gcc)	(ctt)	(act)	(ctt)	(agt)	(ata)	(tcc)	(gaa)	(caa)	(agc)	(acc)	(caa)	(ata)	(ata)
Ye02-54M	C	I	E	T	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	I
	(tgt)	(att)	(gaa)	(aca)	(cac)	(gcc)	(ctt)	(act)	(ctt)	(agt)	(ata)	(tcc)	(gaa)	(caa)	(agc)	(acc)	(caa)	(ata)	(ata)

Table 6.1 PfCRT haplotypes of three sequenced Yemeni *P. falciparum* parasites. Shaded boxes represent polymorphisms (mutations) compared to the CQ-sensitive wild-type sequence of the HB3 clone of *P. falciparum*. Codons are presented between brackets under each amino acid.
Ye02-70W is wild (carried K76 by PCR), CQ- sensitive in-vivo, **Ye02-4M** is mutant (carried T76 by PCR), CQ- resistant in-vivo, and **Ye02-54M** is mutant (carried T76 by PCR) but CQ-sensitive in-vivo.

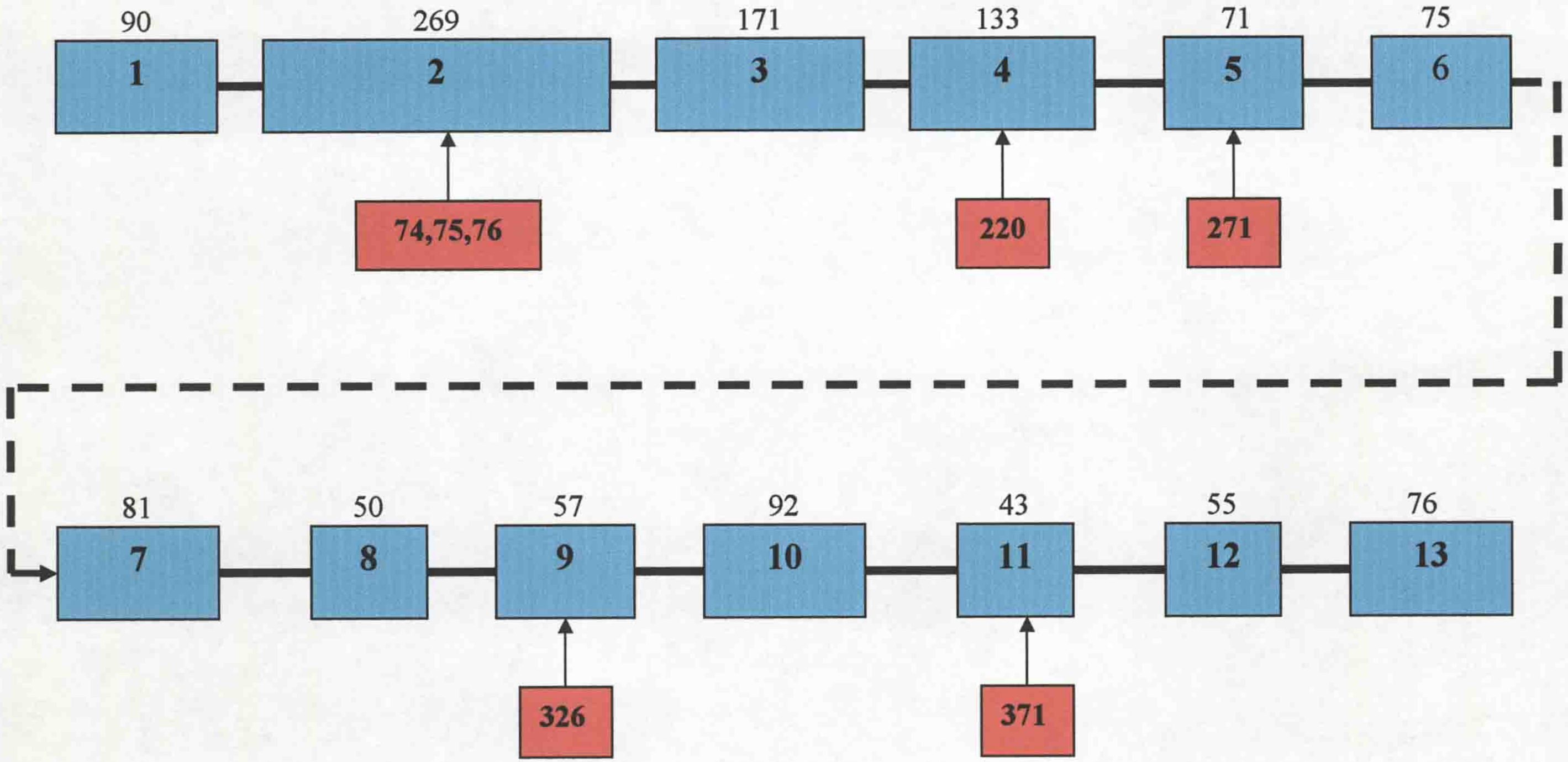


Figure 6.8 Intron/exon boundaries of *pfcrt*. The 13 exons are represented by blue boxes with the number above indicating the size of the exon (bp). The introns are represented by the lines joining the exons. The introns vary in length with the smallest being 107 bp and the longest 191 bp. The red boxes indicate the exons where the 7 detected mutations are situated in. (Adapted from Johnson, 2003)

6.3.6 Nucleotide Sequence Of The PfCRT (Putative Chloroquine Resistance Transporter) Of Ye02-70W

atgaaattcg caagtaaaaa aataatcaa aaaaattcaa gcaaaaatga cgagcgttat
agagaattag ataatttagt acaagaagga aatggctcac gtttaggtgg aggttcttgt
cttggtaaat gtgctcatgt gtttaaactt atttttaaag agattaagga taatattttt
atttatattt taagtattat ttatttaagt gtatgtgtaa tgaataaaat ttttgctaaa
agaactttaa acaaaattgg taactatagt tttgtaacat ccgaaactca caactttatt
tgtatgatta tgttctttat tgtttattcc ttatttggaa ataaaaaggg aaattcaaaa
gaacgacacc gaagctttaa tttacaattt tttgctatat ccatgttaga tgcctgttca
gtcattttgg ccttcatagg tcttacaaga actactggaa atatccaatc atttgttctt
caattaagta ttcctattaa tatgttcttc tgctttttaa tattaagata tagatatcac
ttatacaatt atctcggagc agttattatt gttgtaacaa tagctcttgt agaaatgaaa
ttatcttttg aaacacaaga agaaaattct atcatattta atcttgtgtt aattagtgcc
ttaattcctg tatgcttttc aaacatgaca agggaaatag tttttaaaaa atataagatt
gacattttaa gattaaatgc tatggtatcc tttttccaat tgttcacttc ttgtcttata
ttacctgtat acacccttcc atttttaaaa caacttcatt taccatataa tgaaatatgg
acaaatataa aaaatggttt cgcatgttta ttcttgggaa gaaacacagt cgtagagaat
tgtggtcttg gtatggctaa gttatgtgat gattgtgacg gagcatggaa aaccttcgca
ttgttttcct tctttaacat ttgtgataat ttaataacca gctatattat cgacaaattt
tctaccatga catatactat tgttagttgt atacaaggtc cagcaatagc aattgcttat
tactttaaat tcttagccgg tgatgttgta agagaaccaa gattattaga tttcgtaact
ttgtttggct acctatttgg ttctataatt taccgtgtag gaaatattat cttagaaaga
aaaaaaatga gaaatgaaga aaatgaagat tccgaaggag aattaaccaa cgtcgattca
attattacac aataa

6.3.7 Amino Acid Sequence Of The PfCRT Of Ye02-70W

“MKFASKKNNQKNSSKNDERYRELDNLVQEGNGSRLGGGSCLGKCAHVFK
LIFKEIKDNIFIYILSIIYLSVCVMNKIFAKRTLNKIGNYSFVTSETHNFICMIMF
FIVYSLFGNKKGNSKERHRSFNLQFFAISMLDACSVILAFIGLTRTTGNIQSFV
LQLSIPINMFFCFLILRYRYHLYNYLGAVIIVVTIALVEMKLSFETQEENSIIFN
LVLISALIPVCFSNMTREIVFKKYKIDILRLNAMVSFFQLFTSCLILPVYTLPFL
KQLHLPYNEIWTNIKNGFACLFLGRNTVVENCGLGMAKLCDDCDGAWKTF
ALFSFFNICDNLITSYIIDKFSTMTYTIVSCIQGPAIAIAYYFKFLAGDVVREPR
LLDFVTLFGYLFGSIIYRVGNIILERKKMRNEENEDSEGELTNVDSIITQ”

6.3.8 Nucleotide And Sequence Of The PfCRT (Putative Chloroquine Resistance Transporter) Of Ye02-4M And Ye02-54M

atgaaattcg caagtaaaaa aataatcaa aaaaattcaa gcaaaaatga cgagcgttat
agagaattag ataatttagt acaagaagga aatggctcac gtttaggtgg aggttcttgt
cttggtaaat gtgctcatgt gtttaaactt atttttaaag agattaagga taatattttt
atttatattt taagtattat ttatttaagt gtatgtgtaa ttgaaacaat ttttgctaaa
agaactttaa acaaaattgg taactatagt tttgtaacat ccgaaactca caactttatt
tgtatgatta tgttctttat tgtttattcc ttatttggaa ataaaaaggg aaattcaaaa
gaacgacacc gaagctttaa tttacaattt tttgctatat ccatgttaga tgcctgttca
gtcattttgg ccttcatagg tcttacaaga actactggaa atatccaatc atttgttctt
caattaagta ttcctattaa tatgttcttc tgctttttaa tattaagata tagatatcac
ttatacaatt atctcggagc agttattatt gttgtaacaa tagctcttgt agaaatgaaa
ttatcttttg aaacacaaga agaaaattct atcatattta atcttgtgtt aattagttcc
ttaattcctg tatgcttttc aaacatgaca agggaaatag tttttaaaaa atataagatt
gacattttaa gattaaatgc tatggtatcc tttttccaat tgttcacttc ttgtcttata
ttacctgtat acacccttcc atttttaaaa gaacttcatt taccatataa tgaaatatgg
acaaatataa aaaatggttt cgcatgttta ttcttgggaa gaaacacagt cgtagagaat
tgtggtcttg gtatggctaa gttatgtgat gattgtgacg gagcatggaa aaccttcgca
ttgttttcct tctttagcat ttgtgataat ttaataacca gctatattat cgacaaattt
tctaccatga catatactat tgttagttgt atacaaggtc cagcaatagc aattgcttat
tactttaaat tcttagccgg tgatgttgta atagaaccaa gattattaga tttcgtaact
ttgtttggct acctatttgg ttctataatt taccgtgtag gaaatattat cttagaaaga
aaaaaaatga gaaatgaaga aaatgaagat tccgaaggag aattaaccaa cgtcgattca
attattacac aataa

6.3.9 Amino Acid Sequence Of The PfCRT Of Ye02-4M And Ye02-54M.

“MKFASKKNNQKNSSKNDERYRELDNLVQEGNGSRLGGGSCLGKCAHVFK
LIFKEIKDNIFIYILSIIYLSVCVIETIFAKRTLNKIGNYSFVTSETHNFICMIMFFI
VYSLFGNKKGNSKERHRSFNLQFFAISMLDACSVILAFIGLTRTTGNIQSFVL
QLSIPINMFFCFLILRYRYHLYNYLGAVIIVVTIALVEMKLSFETQEENSIIFNL
VLISSLIPVCFSNMTREIVFKKYKIDILRLNAMVSFFQLFTSCLILPVYTLPFLK
ELHLPYNEIWTNIKNGFACLFLGRNTVVENCGLGMAKLCDDCDGAWKTFA
LFSFFSICDNLITSYIIDKFSTMTYTIVSCIQGPAIAIAYYFKFLAGDVVIEPRLL
DFVTLFGYLFGSIIYRVGNIILERKKMRNEENEDSEGELTNVDSIITQ”

6.3.10 Additional Observations

Studying the chromatogram of exon 4 of the wild isolate Ye02-70W carefully showed the possible presence of the mutant sequence at codon 220 (tcc) that encodes for the amino acid serine in addition to the wild sequence (gcc) that encodes for the amino acid alanine (**Figure 6.9**).

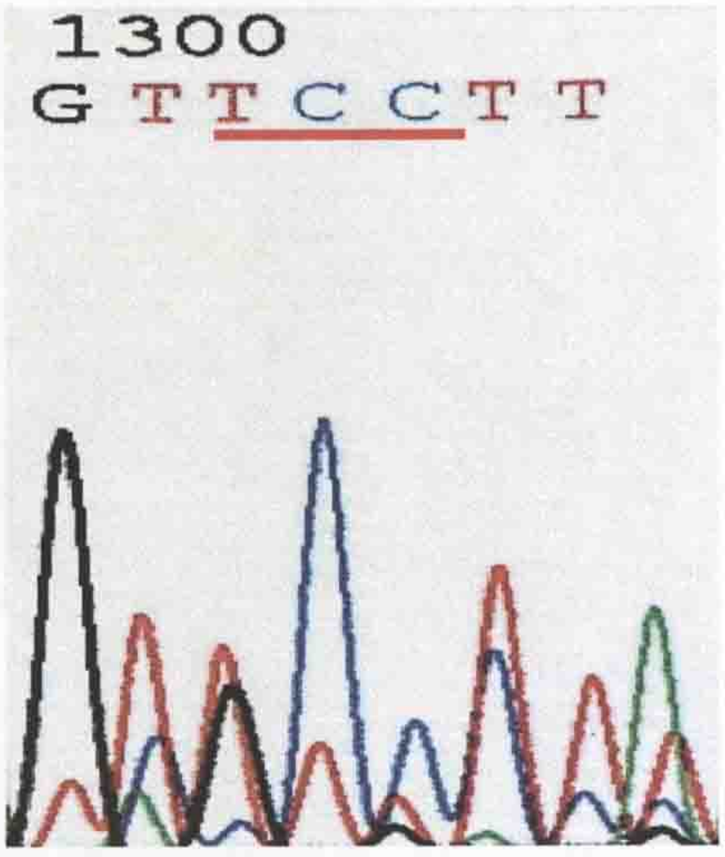


Figure 6.9 Chromatogram of part of exon 4 of the 3 Ye02-70W. Codon 220 is underlined with a red line. Note the possible presence of (GCC) and (TCC) sequences at this codon.

This suggested the presence of mixed infection with 2 isolates in the sample both of which carried the wild-type K76 because this isolate was chosen after confirmation that it lacked the T76 by PCR and because it was clear from the chromatogarm of exon 2 that only the sequence (aaa) that encodes for the amino acid lysine is present (**Figure 6.6**).

To test the possibility of the presence of mixed infection with 2 wild-type K76 isolates but with different polymrphisms at codon 220, new DNA from the same sample harbouring the isolate Ye02-70W was extracted again to rule out any possible contamination of the sample sequenced. The newly extracted DNA was subjected to PCR amplification followed by restriction enzyme digestion to detect the polymorphisms at codons 76 as described in **Chapter 2, section 2.2.5.1** and at codon 220 (available at *http://medschool.umaryland.edu/CVD/plowe.html*). This indicated the presence of only the wild-type K76 (**Figure 6.10**) and the presence of both the wild-type A220 and the mutant-type S220 (**Figure 6.11**).

Figure 6.10 Detection of polymorphism at positions 76 in PfCRT by PCR and restriction digestion with *Apo* 1. Samples before and after digestion were run in 2% agarose gel in adjacent wells to allow for comparison. Odd numbered lanes represent the amplified samples before digestion, while even numbered lanes represent the samples after digestion. Lanes M is a 100 bp step DNA ladder with the numbers indicating the approximate fragment size (bp). Lanes 1 and 2 represent the results of the amplification and digestion of the sample that contained the Ye02-70W isolate. Lanes 3 and 4 are the results of the amplification and digestion of the sample that contained the Ye02-54M isolate. Isolates containing the wild sequence (K76) contain the restriction site with 45 bp being cleaved from the PCR amplicon (Ye02-70W in lane 2).

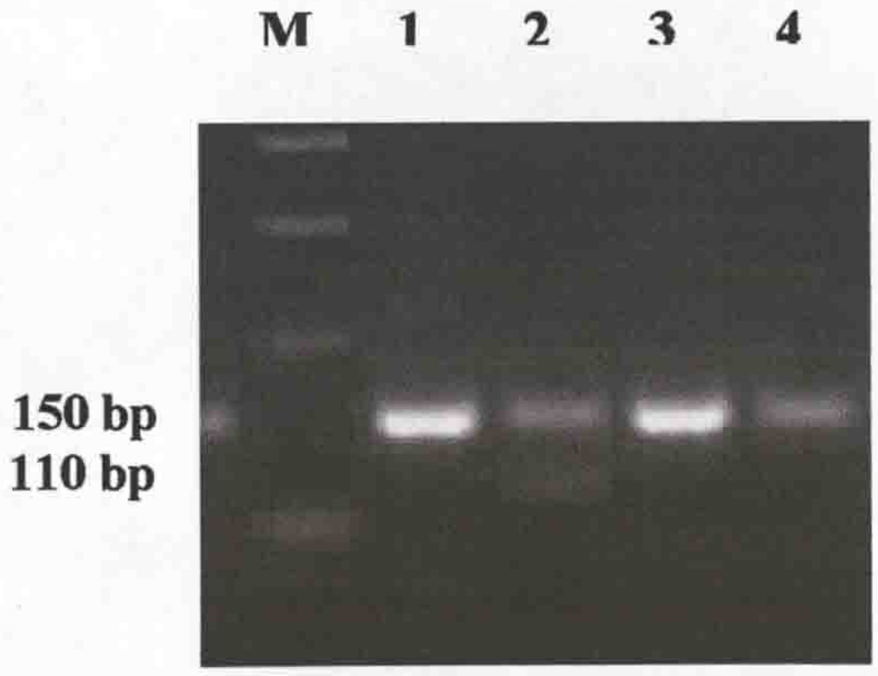


Figure 6.11 Detection of polymorphism at positions 220 in PfCRT by PCR and restriction digestion with *Bgl*1. Samples before and after digestion were run in 2% agarose gel in adjacent wells to allow for comparison. Odd numbered lanes represent the amplified samples before digestion, while even numbered lanes represent the samples after digestion. Lanes M is a 100 bp step DNA ladder with the numbers indicating the approximate fragment size (bp). Lanes 1 and 2 represent the results of the amplification and digestion of the sample that contained the Ye02-70W isolate. Lanes 3 and 4 are the results of the amplification and digestion of the sample that contained the Ye02-54M isolate. Isolates containing the wild sequence (A220) contain the restriction site with 40 bp being cleaved from the PCR amplicon (Ye02-70W in lane 2). However, lane 2, contained another band (150 bp) not cleaved by the enzyme indicating the presence of another isolate in the sample that lacked

the wild sequence suggesting the presence of a mixed infection with 2 isolates with wild and mutant sequence at position 220 in the sample containing the Ye02-70W isolate.

The newly extracted DNA from the sample harbouring the isolate Ye02-70W was then subjected to PCR amplification followed by restriction enzyme digestion to detect the polymorphisms at the other codons where mutations were detected (271, 326, 371 (available at *http://medschool.umaryland.edu/CVD/plowe.html*). This showed the presence of both the wild-type and the mutant-type alleles at codon 371 (R371 and I371) (**Figure 6.12)**

Figure 6.12 Detection of polymorphism at position 371 in PfCRT by PCR and restriction digestion with *Afl* II. Samples before and after digestion were run in 2% agarose gel in adjacent wells to allow for comparison. Odd numbered lanes represent the amplified samples before digestion, while even numbered lanes represent the samples after digestion. Lane M is a 50 bp step DNA ladder with the numbers indicating the approximate fragment size (bp). Lanes 1 and 2 represent the results of the amplification and digestion of the sample that contained the Ye02-70W isolate. Lanes 3 and 4 are the results of the amplification and digestion of the sample that contained the Ye02-54M isolate. Isolates containing the wild sequence (R371) contain the restriction site with 40 bp being cleaved from the PCR amplicon (Ye02-70W in lane 2). However, in lane 2, there is another band (80 bp) not cleaved by the enzyme indicating the presence of another isolate in the sample that lacked the wild sequence suggesting the presence of a mixed infection with 2 isolates with wild and mutant sequence at position 371 in the sample containing the Ye02-70W isolate.

The same work was repeated using newly extracted DNA from the samples that contained the 2 other isolates Ye02-4M and Ye02-54M to detect the possible polymorphisms present at the 7 codons. The sample Ye02-4M was found to be a

single pure infection with a mutant parasite carrying the 7 described mutations, while sample Ye02-54M was found to be a mixed infection with 2 parasites both of which carried the mutant-type T76 (**Figure 6. 10**) and one of the isolates carried all the other six mutations, while the other isolate carried only 5 of the six other mutations and lacked the mutation at codon 271 (**Figure 6.13, lane 6**).

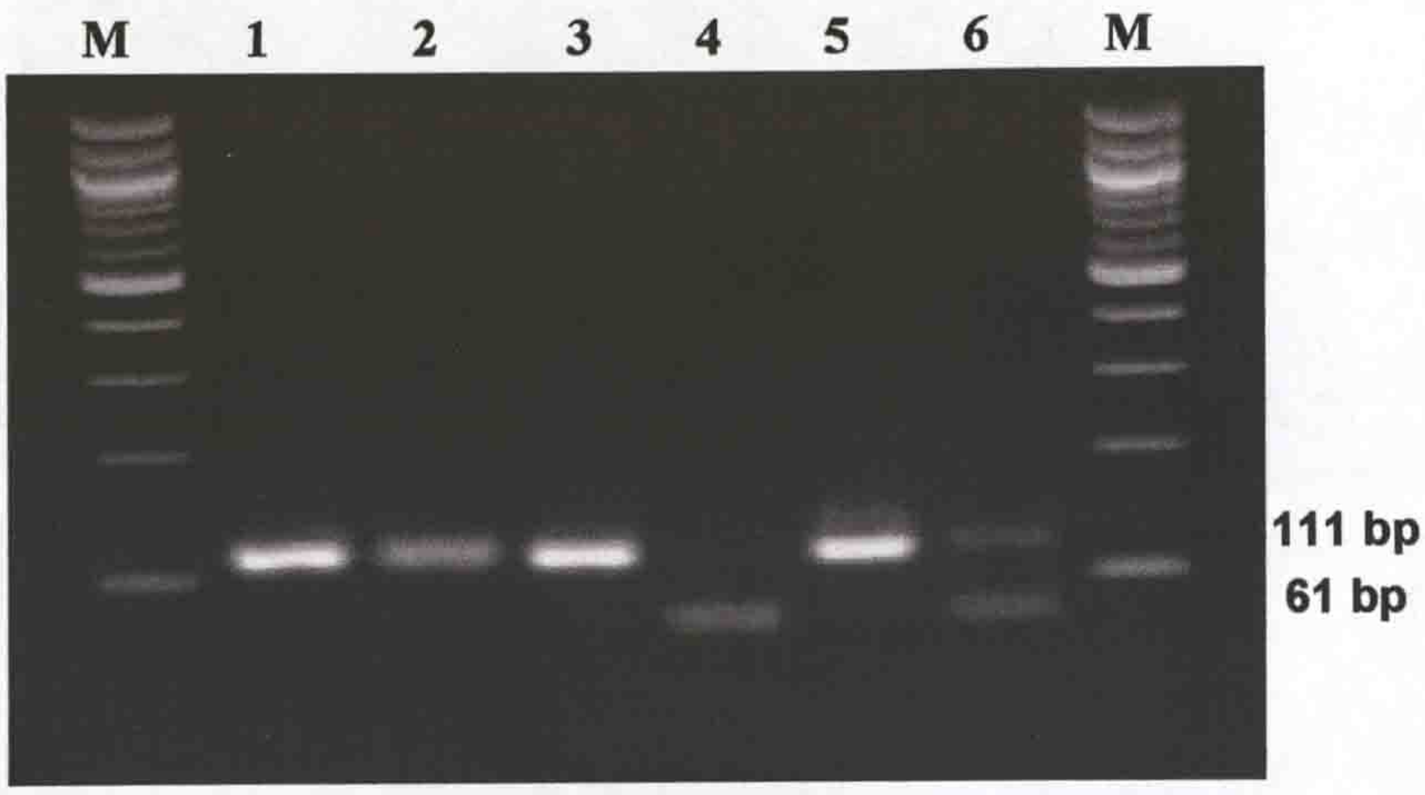


Figure 6.13 Detection of polymorphism at position 271 in PfCRT by PCR and restriction digestion with *Xmn*1. Samples before and after digestion were run in 2% agarose gel in adjacent wells to allow for comparison. Odd numbered lanes represent the amplified samples before digestion, while even numbered lanes represent the samples after digestion. Lanes M on both sides represent a 100 bp step DNA ladder with the numbers indicating the approximate fragment size (bp). Lanes 1 and 2 represent the results of the amplification and digestion of the sample that contained the Ye02-70W isolate. Lanes 3 and 4 are the results of the amplification and digestion of the sample that contained the Ye02-4M isolate, and lanes 5 and 6 are the results of the amplification and digestion of the sample that contained the Ye02-54M isolate. Isolates containing the mutant sequence (Q271E) contain the restriction site with 50 bp being cleaved from the PCR amplicon (Ye02-4M in lane 4 and Ye02-54M in lane 6). However, in lane 6, there is another band (111 bp) not cleaved by the enzyme indicating the presence of another isolate in the sample that lacked the mutant sequence suggesting the presence of a mixed infection with 2 isolates with wild and mutant sequence at position 271.

PCR genotyping using *msp-2* (**Chapter 2, section 2.3.1**) was performed to detect the possibility of mixed allele infection in the samples containing the isolates Ye02-54M and Ye02-70W. The PCR genotyping (using only *msp-2*) confirmed the presence of mixed infection (FC27 +IC/3D7) in the sample containing the isolate Ye02-54M but not in the sample containing the isolate Ye02-70W as shown in (**Figure 6.14**)

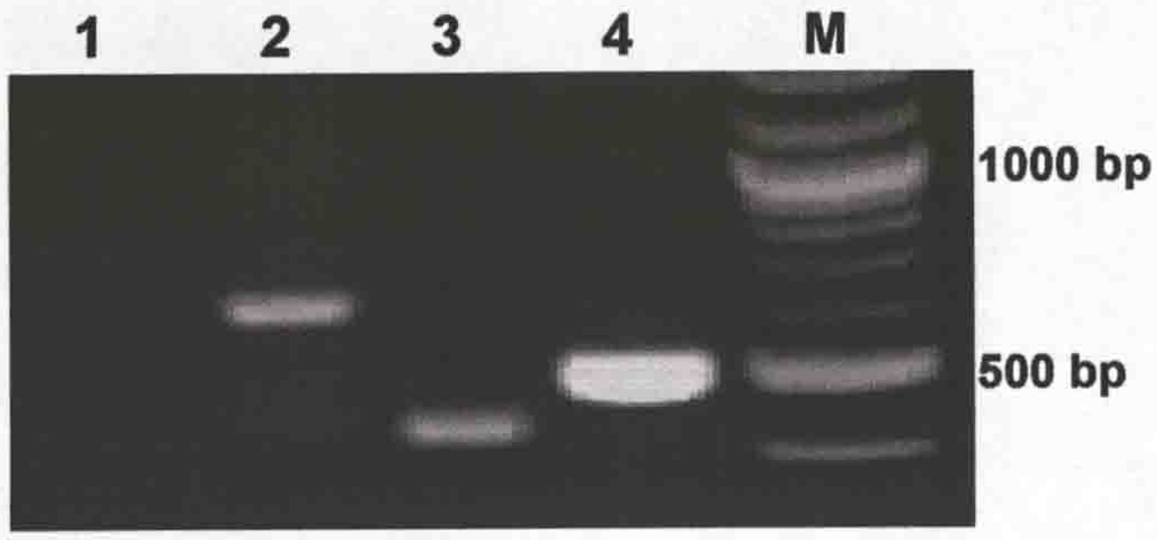


Figure 6.14 PCR genotyping of the samples containing the Ye02-54M and Ye02-70W. Odd numbered lanes represent the amplification of
FC27 family, while even numbered lanes represent the amplification of IC/3D7 family. Lane M is a 100 bp molecular ladder marker. Lanes 1 and 2 represent the results of the amplification of the sample that contained the Ye02-70W isolate, showing the appearance of only one band (700 bp) of IC/3D7 (lane 2). Lanes 3 and 4 are the results of the amplification of the sample that contained the Ye02-54M isolate, with the appearance of one band (450 bp) of FC27 (lane 3) and another band (500 bp) of IC/3D7 (lane 4).

In conclusion, the results suggested the possible presence of different haplotypes, such as, CVMNKAQN**R**, CVMNK**S**QN**I,** CVIETS***E***SI and CVIETS***Q***SI in the *pfcrt* of in-vivo CQ-sensitive Yemeni field isolates.

6.4 DISCUSSION

In the previous chapters, in-vivo chloroquine resistance was found to be high (61%) in the study area (**Chapter 3, figure 3.1**), and the mutation K76T in the *pfcrt* gene (located on chromosome 7) was found to be highly prevalent (98%) in the samples collected and found to occur in all cases of chloroquine treatment failure and in 95.5% of cases who responded adequately to chloroquine (**Chapter 4, figure 4.5**).

Chloroquine resistance (or failure to respond to chloroquine clinically) was observed in Yemen in the early 1980s. However, the first formal report of CQR was between 1986-1987 according to a 28-day in-vivo test (Roll Back Malaria Program, Yemen, 2000). This coincided with the influx of refugees from some African countries (Somalis, Ethiopians, Eritrians) due to political conflicts, which led to the suggestion that drug-resistant strains of *P. falciparum* might have been introduced in to the country from Africa (Roll Back Malaria Program, Yemen, 2000).

The aim of this chapter was to confirm the presence of *pfcrt*-K76T in in-vivo CQR isolates by sequencing of representative isolates, and to take the first step in answering the question of the origin of CQR in Yemen by sequencing the full length of the *pfcrt* gene and describing the possible haplotypes associated with CQR and to compare it with different haplotypes identified around the world. Sequencing the full length of two isolates carrying the *pfcrt*-K76T (identified by PCR) but having different in-vivo response to CQ (CQ treatment failure in isolate Ye02-4M versus adequate CQ response in isolate Ye02-54M) was also carried out to find whether any difference in the sequence of the two isolates might be a possible explanation for the difference in the in-vivo CQ response.

As shown in the chromatogram of the 3 sequenced isolates (**Figure 6.5**), the substitution of the amino acid lysine (K) with thereonine (T) at position 76 of isolates Ye02-4M and Ye02-54 resulted from a point mutation (transversion- aaa→aca), while isolate Ye02-70W carried the wild sequence (aaa) encoding the amino acid lysine as expected from its PCR analysis. Alignment of the isolates with the wild-type isolate HB3 of Honduras showed that the *pfcrt* sequence of the wild-type isolates, Ye02-70W, and HB3 were similar (**Figure 6.7**). However, in the 2 mutant-

types Ye02-4M and Ye02-54, six additional mutations together with the critical K76T were recovered (**Figure 6.7 and Table 6.1**). These six mutations are a transversion (atg→att) leading to a substitution of methionine with isoleucine at position 74, a transition a→g and a transversion t→a at position 75 (aat→gaa) leading to a substitution of asparagine with glutamic acid, a transversion (gcc→tcc) resulting in the substitution of alanine with serine at position 220 (A220S), a transversion (caa→gaa) resulting in the substitution of glutamine with glutamic acid at position 271 (Q271E), a transition (aac→agc) leading to a substitution of asparagine with serine at position 326 (N326S), and a transversion (aga→ata) leading to a substitution of arginine with isoleucine at position 371 (R371I). The seven identified mutations were distributed within the 13 *pfcrt* exons as shown in **Figure 6.8.** A minimum of three changes, in addition to the critical K76T, in the *pfcrt* gene were thought to be required for CQR phenotype in *P. falciparum* (Wootton *et al.*, 2002). These additional substitutions might be required to compensate deleterious effect of K76T and/or modulate the level of CQ response.

Comparing the CVIETSESI haplotype found in positions (72, 73, 74, 57, 76, 220, 271, 326, and 371) of the in-vivo CQR isolate with the identified haplotypes worldwide revealed the similarity of the Yemeni CQR haplotype to the old world (African and Southeast Asian origin) haplotypes (**Table 6.2**). Moreover, the absence of I356T in the tested isolates makes them similar to most African isolates compared to Asian isolates (Wootton et al., 2002). These data support the idea that chloroquine resistance might have been introduced from Africa into the country. This is highly probable because there is a large African refugee settlement, especially for Somalis, in Yemen. They are now incorporated with the local Yemeni communities as workers, mainly as helpers in household activities. However, it should be noticed that this is not a complete and final answer to the question of the origin of CQR in Yemen because this analysis has been done in only 2 representative isolates and it is possible that other haplotypes might be circulating in the area and in other areas. For example, in Philippines, where two novel allelic types of the *pfcrt* gene were identified representing the fifth founder event for CQR worldwide (Chen *et al.*, 2003), Southeast Asian haplotypes (type E1a) were also identified together with the novel haplotypes and it was suggested that this was introduced by refugees from Southeast

Asia (Chen *et al.*, 2003). Similarly in the Indonesian Papua, different haplotypes (African, South American, Southeast Asian), and two new chloroquine resistant haplotypes were identified (Nagesha *et al.*, 2003), which raised the question of the evolution of these different haplotypes in the region that might be due to sequential acquisition of the mutations or due to population movements within the area.

The observation of the possible occurrence of K76 with A220S (**Figure 6.9**) was also reported from two Italian imported *P.falciparum* isolates from Africa (Severini *et al.*, 2005). Similarly the possible occurrence of K76 with R371I (**Figure 6.12**) and of K76T with Q271 highlights the possible presence of intermediate *P. falciparum* CQS and CQR forms. This, however, needs further clarification and confirmation. Recombination after crossing between CQS and CQR forms of the parasite is an unlikely explanation for the occurrence of such intermediate forms because the chance of recombination occurring among closely linked loci is small and in resistant parasites alleles at loci closely linked to *pfcrt*-T76 would tend to be inherited as a group under continued CQ usage (Walliker, 2005).

A possible explanation for the evolution of these intermediate forms is the sequential acquisition of mutations at the *pfcrt* gene. This, however, is an area of current debate. For example, (Lim *et al.*, 2003), had observed that in Cambodian isolates, CQR is strongly associated with the presence of *pfcrt* mutations and had detected an intermediate haplotype CVMNTIF//ISA in which K76T was associated with A220 and which presented an increased IC_{50} compared to the wild-type CVMNKIF//ISA. This led the authors to suggest that acquisition of CQR is a stepwise process, during which the accumulation of point mutations might modulate the response to CQ (Lim *et al.*, 2003), and that the mutation at position 76 seems to be the last in the long process leading to CQ clinical failure (Djimde *et al.*, 2001)a; (Hastings *et al.*, 2002). This is in agreement with the slow genesis of CQ resistance in the field as well as the difficulties that have been experienced with attempts to select CQ resistance in the laboratory. Conversely, (Daily *et al.*, 2003), observed that the presence or absence of polymorphisms at codons K76T, A220S, Q271E, N326S, and R371I were almost completely linked, therefore, they did not find any correlation between these additional mutations and CQ IC_{50} in Senegalese isolates nor any stepwise accumulation of these mutations.

Sequencing was performed on uncloned PCR products of isolates carrying either *pfcrt*-K76 or *pfcrt*-T76 (by PCR). The possibility of the presence of mixed infection was always there. Therefore, to detect different genotypes of cloned isolates, it is necessary to sequence a large number of these cloned isolates. Alternatively, the isolates were chosen after PCR genotyping of codon 76 to ensure they are not mixed (with respect to codon 76 and sensitivity to CQ) and sequenced directly from PCR products, which led to a homogenous sequences with no superimposition of signals at the polymorphic positions except at codon 220 in the isolate Ye02-70W. This suspicious tracing at codon 220 in Ye02-70W led to additional work involving restriction enzyme digestion at position 220 (followed by other polymorphic positions) to rule out possible errors in signalling. This was also followed by *msp*-2 genotyping to confirm the presence of mixed infection. The absence of evidence of mixed infection in the sample containing Ye02-70W (**Figure 6.13**) did not exclude its possible presence since only one marker (*msp*-2) was used. However, the presence of mixed infection was confirmed in the sample containing Ye02-54M (**Figure 6.13**). Isolate Ye02-4M was a pure mutant-type using all PCR and restriction digests, as well as, genotyping and sequencing procedures.

It is of interest to recall here that the isolate Ye02-4M belonged to a case of early treatment failure (ETF), while the isolate Ye02-54M belonged to a case of adequate clinical and parasitological response. As shown in **Figure 6.6**, the Ye02-54M isolate, as was the case of other tested isolates, lacked the S163R that was implicated recently in the restoration of the sensitivity of *P. falciparum* to CQ (Johnson et al., 2004). Whether the presence of the intermediate CQR haplotype CVIETS***Q***SI in the blood of that patient played a role in his response to CQ needs also to be clarified since in-vivo response is confounded by the host factors, most importantly, immunity.

In conclusion, the results of this chapter are important in that it 1) reports the sequence of the *pfcrt* gene and the predicted amino acid sequence of the putative *P. falciparum* chloroquine resistance transporter of the first sequenced CQS and CQR Yemeni isolates, 2) Confirms the presence of *pfcrt*- K76T mutation in Yemen, 3) reports the haplotype associated with CQR in a Yemeni isolate suggesting the

possible origin of chloroquine resistance in Yemen, and finally, 4) highlights the possibility of the presence of other (intermediate) CQS and CQR haplotypes and recommends further studies to clarify this observation.

Geographic distribution	Parasite clone/isolate	PfCRT amino acid positions																			
		72	74	75	76	77	97	144	148	152	160	163	194	220	271	275	326	333	352	356	371
CQS parasites																					
Honduras	HB3[a]	C	M	N	K	I	H	A	L	T	L	S	I	A	Q	P	N	T	Q	I	R
Sudan	106/1[a]	C	I	E	K	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	I
Lab clones	106/1-IR[b]	R	I	E	I	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	I
	106/1-IK[b]	C	I	E	I	I	H	A	L	T	L	S	I	S	E	P	S	T	K	I	I
(Thailand)	K1AM[c]	C	I	E	T	I	H	A	L	T	L	R	I	S	E	P	S	T	Q	V	I
(Thailand)	K1Hf[c]	C	I	E	T	I	H	A	L	A	L	R	I	S	E	L	S	T	Q	I	I
CQR parasites Africa																					
Mali	S35CQ[d]	C	I	E	T		H	A	L	T	L	S	I	S	E	P	N	T	Q	I	I
South Africa	RB8[a]	C	I	E	T	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	I
Lab clones	106/1-N[e]	C	I	E	N	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	I
	106/1-I[e]	C	I	E	I	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	I	I
Southeast Asia																					
Thailand	Dd2[a]	C	I	E	T	I	H	A	L	T	L	S	I	S	E	P	S	T	Q	T	I
Thailand	TM93-C1088[f,g]	C	I	E	T		L	A			L			S	E		S			T	I
Cambodia	783[h]	C	I	E	T		H	A	L				I	S	E		N	T		T	I

Geographic distribution	Parasite clone/isolate	PfCRT amino acid positions																			
		72	74	75	76	77	97	144	148	152	160	163	194	220	271	275	326	333	352	356	371
Cambodia	738[h]	C	I	D	T		H	A	I				T	S	E		N	S		I	R
Cambodia	734[h]	C	I	D	T		H	F	I				T	S	E		N	S		I	R
Cambodia	176[i]	C	I	E	T	T								S							
Cambodia	108[i]	C	I	D	T	I								S							
Cambodia	36[i]	C	T	N	T	I								S							
Philippines																					
Morong	PH1[g]	C	M	N	T		H	T			Y			A	Q		D			I	R
Morong	PH2[g]	S	M	N	T		H	T			Y			A	Q		D			I	R
Indonesia																					
Lombok	Field isolate[j]	C	M	N	N																
Indonesian Papua																					
Tamika	2300[k]	C	I	K	T																
Armopa	CQ076[k]	S	I	E	T																
Papua New Guinea																					
Solomon	PNG4[b]	S	M	N	T	I	H	A	L	T	L	S	I	A	Q	P	D	T	Q	L	R

Geographic distribution	Parasite clone/isolate	PfCRT amino acid positions																			
		72	74	75	76	77	97	144	148	152	160	163	194	220	271	275	326	333	352	356	371
South America																					
Ecuador	Ecu1110[a]	C	M	N	T	I	H	A	L	T	L	S	I	S	Q	P	D	T	Q	L	R
Colombia	Jav[a]	C	M	E	T	I		A	L	T	L	S	I	S	Q	P	N	T	Q	I	T
Brazil	7G8[a]	S	M	N	T	I	H	A	L	T	L	S	I	S	Q	P	D	T	Q	L	R
Guyana	Field isolate[l]	S	M	I	T																
Guyana	Field isolate[l]	R	M	N	T																

Table 6.2 Unique PfCRT haplotypes associated with CQS and CQR in *P. falciparum.* Amino acids in shaded grey represent polymorphisms compared to the CQS wild-type sequence, derived from the HB3 clone of *P. falciparum*. Empty positions indicate unavailable sequence information. (a) Fidock *et al*. (2000a), (b) R. Cooper, unpublished data. (c) Johnson *et al*. (2004), (d) Wootton *et al*. (2002), (e) Cooper *et al*. (2002), (f) Chen *et al*. (2001), (g) Chen *et al*. (2003), (h) Durrand et al. (2004), (i) Lim *et al*. (2003), (j) (Huaman *et al*., 2004), (k) Nagesha *et al*. (2003), (l) Best Plummer *et al*. (2004). (Source: Cooper *et al*., 2005).

CHAPTER 7

GENERAL DISCUSSION

7.1 OVERVIEW

Malaria continues to be a leading cause of death in the tropics. In Yemen it is a top health priority problem accounting for 1% mortality, mainly among children and pregnant women. The National Malaria Control Program in Yemen was started in 2001 and the "National Strategic Plan for Roll Back Malaria in The Republic of Yemen" was prepared and issued in 2002. It contained the following 8 strategic directions: 1) human resource development to have competent national cadres in all the areas of malariology, 2) early and correct diagnosis followed by prompt and effective treatment of malaria cases including severe cases of malaria through a national anti-malaria drug policy which should be standardized and adhered to in all the health institutions country-wide, 3) selective integrated vector control through multiple and sustainable preventive measures, 4) prevention of malaria in pregnancy, 5) epidemic preparedness and response, 6) strengthening the information system and surveillance, 7) Increasing the capability of the community to recognize, prevent and control malaria. This includes the initiation of "Home management" in selective remote areas where there is a problem with accessibility to disease management, 8) developing the capacity to plan and implement operational field research and to utilize results in program intervention.

As a response to the increasing observations of the ineffectiveness of the first-line treatment and to the urgent need to establish a practical and sustainable system for continuous monitoring of therapeutic efficacy of antimalarial drugs in order to provide essential data for formulation and updating of antimalarial drug policy in Yemen, this study was conducted in 2002 in collaboration with the Ministry of Public Health and WHO/EMRO.

When this work was initiated (in 2002), the degree and the extent of resistance against the first-line malaria treatment in Yemen (chloroquine) was not known, there was no system for monitoring antimalarial drug resistance in the country, the prevalence of the molecular marker of chloroquine resistance *pfcrt-K76T* was not known and its use as a molecular marker for chloroquine resistance had not been validated. In addition, there were uncertainties with regard to the level of malaria transmission and the level of SP resistance (SP is the second-line treatment) and the absence of data about the origin of CQ-resistance in Yemen. This thesis tries to

answer these questions in one of the first sentinel sites established in Yemen in October 2002.

The results of this study were presented and discussed recently in the national seminar on malaria and other vector borne diseases, held in Sana'a, in Yemen, from the 20th to 22nd of November 2005. With regard to the in-vivo efficacy of CQ the result of this study (high level of CQ-resistance) was consistent with the results of studies from 2 other sentinel sites, which led to agreement of the participants of the need to change the first-line treatment and to withdraw CQ from the market gradually.

The choice of a possible successor to CQ (**Section 7.2**) was discussed with the participation of national and international representatives of different areas involved in the process of change (Ministry of Public Health, National Malaria Control Program, Supreme Board of Drugs and Medical Appliances, Universities of Aden and Sana'a, WHO/EMRO, and WHO/HQ). The results of the discussion groups are presented in **section 7.2.**

7.2 THE CHOICE OF A SUCCESSOR TO CHLOROQUINE

The decision of which drug regimen to change to, and how to implement the change in a way that maximizes potential benefit, are confronted by many practical difficulties, but delaying a decision to switch because of these difficulties can only result in increased morbidity and mortality. The longer the decision is delayed, the more widespread will become the use of the available anitmalarial drugs as monotherapies, compromising their future use in combinations with other effective antimalarials, such as artimisinins.

Combination therapy is recommended now by WHO (WHO, 2001) for its potential effect in delaying the development of resistance against antimalarial drugs. The artimisinin-based combination therapy (ACT) has the advantages of reducing the parasite densities and clinical symptoms rapidly, reducing gametocyte carriage, and effective action against multidrug resistant *P. falciparum*. However, concerns regarding its future supply are rising because the artimisinin compounds are derived

from plant extracts, and at least 2 years are needed for its cultivation, which may make the supply of the raw material a potential problem, slowing the deployment of ACT (WHO, 2001a).

The choice of antimalarial combination drugs is governed by a number of criteria including: 1) therapeutic efficacy of the combination, 2) safety of the drugs, especially amongst the high risk groups, 3) potential for the widespread use of the combination at all levels of the health care system, 4) consumer compliance, 5) cost effectiveness, 6) potential to delay or prevent development of resistance, 7) other factors including availability and production capacity. A scoring system was generated from these criteria to provide a means of guiding comparisons of different combination therapies (WHO, 2001a).

In the national seminar on malaria and other vector borne diseases held in Sana'a, in Yemen between the 20th and 22nd of November 2005, the two proposed ACTs (artesunate+SP and artemether/lumefantrine) as possible successors to chloroquine were compared and scored (**Table 7.1**). The discussion groups concluded that artesunate+SP in a blister co-administration (**Figure 7.1A and B**) is a preferable choice as a first-line treatment, while artemether/lumefantrine fixed-dose formulation (**Figure 7.2**) is an option as a second-line treatment.

The choice of artesunate+SP as a first-line treatment was based on the findings that artesunate+SP is an effective option in countries where SP efficacy is >80% and based on evidence from field results of 3 in-vivo SP efficacy studies conducted in Yemen during 2004 in 3 sentinel sites (Harad, Al-Musaimeer and Al-Odein) (National Malaria Control Program, Yemen, 2005), which indicated 95%-100% efficacy of SP. However, it should be noticed that one of these 3 SP efficacy studies that indicated 100% SP efficacy (in Harad) was a 14-day follow-up test, which would lead to underestimation of the true rate of drug failure. In the other 2 studies the number of cases who completed the 28-days of follow-up did not exceed 57 (51 in Al-Odein and 57 in Al-Musaimeer). The prevalence of young children <5 years of age in the 3 studies, which would affect the rate of treatment failure, is not known. This is primarily because of lack of national expertise in the proper analysis of the results of the efficacy studies and lack of communication and efficient dissemination

of important details of the results of the studies (e.g. lack of a web site for the Malaria Control Program to disseminate necessary information to interested researchers).

The limitations of the SP efficacy studies mentioned above, the finding in this study of 5% *dhfr*-C59R mutation as indicative of the *dhfr*-triple mutations, the finding that the area is a high transmission area and the pharmacokinetic properties (half-lives) of the combination drugs should be all taken in consideration in the choice of the partner for ACT. The prevalence of *dhfr*-C59R is expected to increase from 2002 to 2006 as SP is available and frequently used in the area due to the high percentage of CQ treatment failure. Recently developed models for ACT by (Watkins *et al.*, 2005) indicated that if the *dhfr* triple mutation exceeds 5%, it is unlikely that any useful extension of the useful therapeutic life (UTL) will be achieved by adopting artesunate+SP combination due to the inability of a 3-day artesunate regimen to cure patients infected with parasites having the *dhfr* triple mutation (Dorsey *et al*, 2002). Moreover, SP is eliminated slowly from the body, while the artimisinin compound will be eliminated rapidly, leaving the SP unprotected and exposing sub-therapeutic levels of the drug to new infections (with *dhfr* mutations) especially in a high transmission area.

However, the situation is difficult and we do not have many choices. A 28-day amodiaquine efficacy test was conducted during 2004 in Al-Musaimeer sentinel site. Sixty-one patients were included and completed the follow-up period and the test resulted in 43% treatment failure (National Malaria Control Program, Yemen, 2005). Another 28- day study tested the efficacy of artesunate+ amodiaquine was conducted in Al-Odein sentinel site during 2004 and found that the efficacy of the combination was 86%.

In general, we need more evidence about the efficacy of the possible combinations irrespective to the efficacy of the individual components, we need to implement comparative efficacy studies of the combinations in different sentinel sites taking in consideration that the follow-up period should be at least 28-days because the rapid action of the artimisinin component may mask the rate of treatment failure in the case of short (14-day) follow-up period. Also children <5 years should be included in

the tests together with other age groups. We, also, need to detect the prevalence of the *dhfr* triple mutations in newly collected samples from different sentinel sites.

In all cases, the health system should be prepared and strengthened for this challenging task of policy change. Implementing a change without addressing underlying problems of the local capacity to deliver health care to the population is likely to result in low rates of coverage, and the inappropriate use of the drugs, risking our most valuable antimalarial (artimisinin), and leading to a potential catastrophic event.

Criteria	Artesunate+SP	Artemether/lumefantrine
Therapeutic efficacy	1 (expected but depends on pre- existing SP resistance)	1 (expected)
Safety in pregnancy	2	3
Safety during lactation	2	3
Dosage schedule	1	2
Good packaging for consumer	2	1 (fixed-dose formulation)
Good packaging for supply management	2	1
Tolerability	2	1
Price/cost	1	2
Reliable production	1	2
Local registration	2	2
Acceptability to consumer	2	3
Acceptability to prescriber	2	3
Potential problems	3 (reported resistance, use of cotrimoxazole, short useful therapeutic life, need for efficacy and safety data)	2 (frequent administrations, varied absorption, need for efficacy and safety data)
PREFERABLE OPTION	**23**	26

Table 7.1 Matrix for comparing antimalarial combination therapies used in the discussion of changing antimalarial drug policy in Yemen, 20-22 November, 2005. A lower score is better for each of the individual categories and therefore for the overall score. (*Adapted from WHO,2001a).*

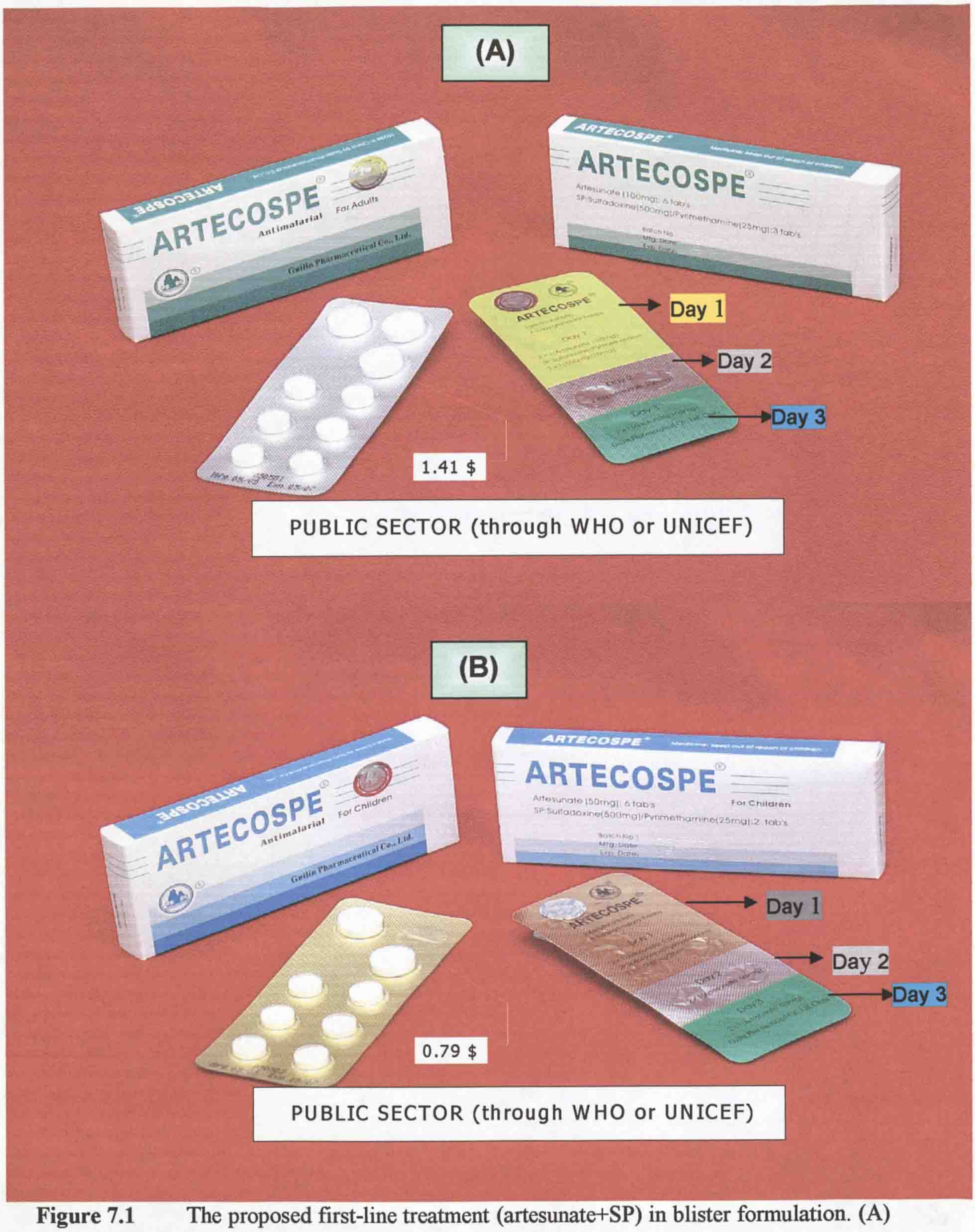


Figure 7.1 The proposed first-line treatment (artesunate+SP) in blister formulation. (A) blister formulation for adults, (B) blister formulation for children. Note illustrative colours on the blister, and the price for each course of treatment when provided through the public sector. (Source: Bosman A, (medical officer, RBM/WHO HQ), presentation in the national seminar on malaria & other vector borne diseases, Sana'a – republic of Yemen 20-22 November 2005).

Figure 7.2 The proposed second-line treatment (arthemeter-lumefantrine, Coartem®) fixed dose formulations for different age groups. (Adapted from: Bosman A, (medical officer, RBM/WHO HQ), presentation in the national seminar on malaria & other vector borne diseases, Sana'a – republic of Yemen 20-22 November 2005).

Since 2001, a total of 56 countries have adopted one of the WHO recommended artemisinin-based combination therapies, several as first-line treatment and a few as second-line (**Table 7.2**).

Continent	Countries	Options	Line
AFRICA	Burundi, Cameroon, Côte d'Ivoire, Democratic Republic of the Congo, Equatorial Guinea, Gabon, Ghana, Guinea, Liberia, Madagascar, Senegal, Sao Tome and Principe, Sierra Leone, Sudan (S), Zanzibar	AS + AQ	1st
	Angola, Benin, Burkina Faso, Comoros, Ethiopia, Gambia, Guinea Bissau, Kenya, Mali, Namibia, Niger, Nigeria, Rwanda, Uganda, South Africa (Kwa Zulu Natal), Tanzania, Togo, Zambia	AL	1st
	Côte d'Ivoire, Gabon, Mozambique, Sudan (N), Sao Tome and Principe, Zanzibar	AL	2nd
	Mozambique, Sudan (N), South Africa (Mpumalanga)	AS + SP	1st
ASIA	Cambodia, Thailand	AS + MQ	1st
	Bangladesh, Bhutan, Laos, Myanmar	AL	1st
	Indonesia	AS + AQ	1st
	Afghanistan, India (5 Provinces), Iran, Tajikistan, Yemen	AS + SP	1st
	Viet Nam	DP	1st
	Papua New Guinea	AS + SP	2nd
	Philippines, Iran	AL	2nd
SOUTH AMERICA	Ecuador, Peru	AS + SP	1st
	Bolivia, Peru, Venezuela	AS + MQ	1st
	Brazil, Guyana, Suriname	AL	1s

AS+AQ = artesunate+amodiaquine; AS+SP = artesunate+sulfadoxine/pyrimethamine;
AS+MQ = artesunate+mefloquine; AL = artemether/lumefantrine; DP = dihydroartemisinin/piperaquine

Table 7.2 List of countries that recently adopted one of the WHO recommended artemisinin-based combination therapies.

(Available at http://www.rbm.who.int/cmc_upload/0/000/015/364/RBMInfosheet_9.htm. Last updated on 1st November 2005).

7.3 CONCLUSIONS

- In-vivo chloroquine resistance is unacceptably high (61%) in Al-Musaimeer district, Lahj governorate, in the south of Yemen.
- The *pfcrt*-K76T mutation is highly prevalent (98%) in the area. The presence of this mutation was confirmed by sequencing.
- Children below the age of 10 years were significantly at greater risk of chloroquine treatment failure than older children and adults.
- Children below the age of 10 years were significantly at greater risk of being late clinical failures (LCF), compared to late parasitological failures (LPF), than older children and adults.
- Febrile patients at presentation and young children (below 5 years of age) were significantly more likely to be early treatment failures.
- Post-treatment asymptomatic failures (LPF) were the most prevalent class of treatment failure (40%) compared to 22% post-treatment symptomatic failures (LCF).
- The association between the presence of K76T and in-vivo CQ treatment failure was not perfect (K76T was not highly predictive of in-vivo treatment failure.
- Clearance of resistant parasites (those carrying the *pfcrt*-K76T) was age dependent.
- Multiplicity of infection with *P. falciparum* followed an age-dependent manner (as the age increased the multiplicity increased).
- Accordingly it was concluded that partial immunity was acquired in older children and adults following frequently repeated exposure to infection, therefore, it is suggested that the study site is a high transmission area. This immunity was the main factor in the dissociation between the presence of K76T and in-vivo CQ treatment failure.
- Prevention of malaria transmission (vector control) is crucial for the control of the disease in the area due to the suggested high intensity of transmission.
- Exclusion of young children from in-vivo drug efficacy studies will severely under estimate the true rate of drug resistance.
- Restriction of in-vivo tests to only children <5 years and febrile patients can introduce selection bias overestimating the rate of treatment failure.

- Young children, who general lack sufficient clinical immunity are placed at a higher risk of morbidity and mortality.
- The *pfdhfr* mutations associated with pyrimethamine resistance are already present in the area (*dhfr*-C59R=5%), suggesting that SP resistance is already present. As discussed in **section 7. 2,** this might compromise the usefulness of SP as a partner for the new combination therapy proposed as a first-line treatment or at least shortens the useful therapeutic life of the drug combination.
- The genotype failure index (GFI) is a valuable measurement that can be used to predict in-vivo treatment failure. Validated in different sentinel sites, it could be used in the future surveillance activities for monitoring drug resistance as a practical, less time consuming, less expensive and effective tool for detecting drug resistance than the laborious in-vivo test. However, it is not a replacement of in-vivo tests, rather it indicates when in-vivo tests should be conducted.
- The Yemeni *P. falciparum* chloroquine resistant strain is similar in its *pfcrt* gene sequence to Asian and African (particularly to African) chloroquine resistant strains. Population movement from African countries might be responsible for the introduction of *P. falciparum* chloroquine resistant strains into the Yemen.
- There is a lack in the national expertise in the analysis of the results of studies implemented by the Malaria Control Program and inadequate networking, and dissemination of the results of these studies to interested malaria researchers.

7.4 RECOMMENDATIONS

- Malaria treatment policy should be urgently reviewed in Yemen.
- Chloroquine, as a first-line drug for the treatment of uncomplicated, *P. falciparum* malaria, should be replaced with a suitable antimalarial combination therapy, preferably artimisinin-based combination because of its gametocytocidal activity.
- Chloroquine should be discontinued from the market. Because it was suggested (in the national seminar on malaria and other vector borne diseases,

in Sana'a, Yemen 20-22 November 2005) that chloroquine will be discontinued gradually, any available supplies of chloroquine should not be given to children below the age of 10 years. This should be explained to the clinicians and health workers (public and private) at different levels of the health system. It should also be explained to the general public through the media. Because chloroquine is affordable, accessible and available over the counter without prescription, it is likely that supplies will be used indiscriminately.

- Detecting the prevalence of *dhfr* triple mutations from newly collected samples from different sentinel sites. Samples are easily collected in filter papers and the molecular studies are rapid depending on the number of samples.
- If SP would be used as a partner for the proposed artimisinin-based combination therapy, then SP (Fansidar as a monotherapy) should be discontinued completely from the market, as soon as possible, to prevent further development of SP resistance. This, however, needs strict rules and monitoring for its application due to the presence of a large private health sector in Yemen.
- Incorporation of the private health sector in all the steps of the process of revising and updating the drug policy to ensure the maximum possible compliance with the process.
- Facilities to confirm laboratory malaria diagnosis should be available at all treatment facilities. This will promote administration of the therapy on the basis of confirmatory laboratory diagnosis to limit unnecessary use.
- Comparative therapeutic efficacy studies of the proposed combination therapies (e.g. artesunate+SP, artesunate+amodiaquine and artemether-lumefantrine) should be implemented in different sentinel sites to test their efficacy and safety in different areas and different groups of people. The tests should adopt at least 28-days of follow-up in order not to underestimate the treatment failure rates because in a 14-day test the combinations may appear effective due to the rapid action of the artesunate component.
- Inclusion of children less than five years is essential in all future in-vivo antimalarial drug efficacy studies otherwise there is a danger of underestimating the true rate of drug resistance, the importance of this should

be explained clearly to the health workers in charge. However, tests should not be restricted to children < 5 years in order not to overestimate treatment failures.

- The replacement therapy should be available, accessible and affordable, especially to the poor remote rural areas, which represent the groups with the highest risk of getting the disease and its complications.
- The replacement therapy should be provided in a simple illustrative (e.g. colour illustration) packaging easily administered by illiterates.
- Education and training of health care providers (public and private) at different levels of health care system is an essential step in the implementation of the process of changing drug policy.
- The dosage schedule and the importance of taking the treatment in combination should be explained simply and clearly, through the media and through the distribution of national, simple and illustrative, treatment guidelines to the general public to ensure maximum possible compliance with the treatment and to minimize the possible development of resistance and to help in extending the useful therapeutic life of the replacement therapy.
- Due to the absence of any component of the media (or even electricity) in some poor rural areas in Yemen, where actually the most burden of malaria lies, it is preferable to arrange national human campaigns (mobile teams) to those areas to inform the people about the new treatment and to explain how it is administered.
- It is important to take into consideration that the health education component of the process of replacement should be directed, in the first instance, to women, who are taking care of children, the most vulnerable group to the disease and complications, and who are themselves vulnerable when pregnant.
- Vector control activities should be strengthened to break the malaria transmission cycle.
- Due to the evidence that suggests that susceptibility of *P. falciparum* to chloroquine may return in the absence of drug pressure, it is advisable to monitor the prevalence of the molecular marker of chloroquine resistance after the implementation of the new drug policy. It is possible to use the

genotype failure index calculated in this study to predict in-vivo chloroquine failure over a period of time.

- Information, education and communication (IEC) campaigns directed at consumers and drug vendors, highlighting the danger of counterfeit drugs and the importance of rational use and prescribing of antimalarial drugs are of special importance since the majority of patients seek first-line treatment in the private sector.
- The quality of the new treatment should be monitored during the process of drug policy change; this should include monitoring the efficacy (see below), the safety (reporting adverse effects), monitoring the quality during registration and/or procurement and post-marketing monitoring (regular testing of samples by a qualified laboratory).
- Activating surveillance to monitor the emerging resistance against the proposed replacement therapy should be one of the priorities of the National Malaria Control Program. Monitoring resistance can be achieved easily by annual PCR surveying for the molecular markers of the combination drugs in different sentinel sites.
- Reviewing the antimalarial drug policy constantly each 3-5 years according to field surveillance monitoring results in order to be able to confront problems of emerging resistance before reaching an unacceptably high level of drug resistance.
- Development of adequate networking and communication between researchers and the malaria control program and the provision of properly analysed information to researchers, which will facilitate the translation of research findings into intervention tools for the control of malaria.
- Finally, this difficult and challenging process of changing drug policy would not attain its benefits unless it is monitored and its impact is evaluated at all levels. It is the responsibility of the Ministry of Public Health, and the National Malaria Control Program, to monitor the effective implementation of the process and to evaluate its public health impact at different levels of health care system. However, it is our responsibility together as decision makers, researchers, health providers, drug suppliers and community residents to raise the health awareness and to ensure proper implementation

of the treatment policy in order to kill malaria in Yemen rather than leaving it killing our children.

7.5 OUTCOMES OF THE STUDY RESULTS AND ITS IMPLICATIONS ON MALARIA CONTROL IN YEMEN

What does this study add to the existing knowledge about the current situation of malaria and antimalarial drug resistance in Yemen? And what are the expected benefits of its results and recommendations to the malaria control program and to the general social and economical situation in the country?

Some answers to these questions are listed below.

This study:

- Provided the drug policy makers with base-line information about the current situation regarding malaria drug resistance in Yemen.
- Reported for the first time (in 2003) an unacceptably high resistance against the first-line treatment based on a standardized WHO in-vivo protocol, highlighting the urgency of revising the antimalarial drug policy in Yemen.
- Provided an evidence-based need for the replacement of chloroquine, as a first-line drug for treatment of uncomplicated, *P. falciparum* malaria in Yemen.
- Introduced, for the first time, the PCR technique as a possible practical, quick and effective tool for monitoring antimalarial drug resistance in surveillance activities of the National Malaria Control Program in Yemen.
- Provided training to some of the health workers in the MoPH in clinical, epidemiological and laboratory methods for monitoring antimalarial drug resistance in Yemen. Al-Musaimeer sentinel site is now one of the most successful sentinel sites in monitoring antimalarial drug resistance in the country.
- Provided health workers and clinicians with a simple method of stratifying patients into those for whom chloroquine use is acceptable, and those for whom alternative treatment should be used (in the period before the total implementation of the replacement treatment).

- Validated, for the first time, the use of the molecular marker for chloroquine resistance (*pfcrt*-K76T) in Yemen.
- Suggested the study site (Al-Musaimeer district, Lahj governorate) as a high transmission area, based primarily, on the apparent clinical and parasitological acquired immunity in old children (>10 years) and adults and on the age-dependent manner of multiplicity of *P. falciparum* infection.
- Highlighted the importance of reconsidering the previous WHO classification of Yemen as a low to moderate intensity transmission area because of its important implications on strengthening the vector control in the area.
- Helped in exploring the origin of chloroquine resistance in Yemen by characterizing the first sequenced *P. falciparum pfcrt* gene of a Yemeni chloroquine resistant isolate.
- Following the recommendations listed above would help in confronting the challenges associated with the difficult process of drug policy change.
- In light of the results of this study, replacement of an ineffective treatment with an effective therapy will help in controlling the spread of chloroquine resistance and of malaria, thereby, reducing malaria related morbidity, its progression to severe disease, and its associated mortality, as well as, the socio-economic losses provoked by the disease.
- Effective therapy will also reduce the burden on the health system by reducing the number of malaria cases seeking medical care (out-patient load) and the number of admissions of malaria cases in hospitals (inpatient load), reducing the cost of treatment due to failure cases and repeated courses of treatment. This will allow available resources to be mobilized towards strengthening the health system in order to be able to confront the challenges of the implementation of the drug policy change.

The main outcome of this study is that it established a practical and sustainable system for continuous monitoring of therapeutic efficacy of antimalarial drugs in order to provide essential data for formulation and updating of antimalarial drug policy in Yemen, thereby, addressing the 1st, 2nd, and the 8th strategic directions of the "National Strategic Plan For Roll Back Malaria In The Republic Of Yemen" (see Section 7.1) that was issued in 2002, when this study was started.

7.6 FUTURE PROSPECTS

The data presented in this thesis provided base-line information about the prevalence of the chloroquine molecular marker *pfcrt-K76T.* Trend analyses of the declining prevalence of this mutation after the discontinuation of CQ like those reported by (Kublin *et al.*, 2003) and the possible reemmergence of the sensitivity of the *P. falciparum* to CQ would have important public health implications because they offer hope that concerted efforts to withdraw a failing drug could be associated with a retrieved sensitivity. This could allow the reintroduction of a modified CQ in combination with efficacious drugs like the artemisinin derivatives.

The possibility of the presence of different *pfcrt* CQ-resistant and CQ-sensitive haplotypes in Yemeni *P. falciparum* parasites highlighted in **Chapter 6,** is interesting and needs further clarification. This would explore the possible presence of intermediate-CQ sensitive and CQ-resistant forms and would help in clarifying the current debate with regard to the possibility of sequential acquisition of *pfcrt* mutations, thus, increasing our understanding of the mechanisms of chloroquine resistance.

A comprehensive study of the diversity and multiplicity of *P. falciparum* infections in the different sentinel sites would contribute to mapping of Yemen with regard to the intensity of malaria transmission, which will help in evidence-based planning of malaria control in different endemic areas in Yemen.

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APPENDICES

Appendix 1

ETHICAL APPROVALS

A) Approval From The Ministry of Public Health, Yemen

Republic of Yemen
Ministry of Public Health & Population
Planning & Development Sector
General Department of Infromatics & Research

بسم الله الرحمن الرحيم

الجمهورية اليمنية
وزارة الصحة العامة والسكان
قطاع التخطيط والتنمية
الإدارة العامة للمعلومات والبحوث

No. :.....
Date :.....

الرقم :.....
التاريخ :.....

صادر
الرقم: 153-26
التاريخ: 14.11.02

(إلى من يهمه الأمر)

تفيد الإدارة العامة للمعلومات و البحوث بوزارة الصحة العامة و السكان بأن الأخت / د. ريم عبد القادر مبجر تقوم بعمل دراسة حول مقاومة الملاريا لبعض مضادات الملاريا في مديرية المسيمير . م. لحج ، بعلم و موافقة وزارة الصحة العامة و السكان و بإشراف مشروع الملاريا و منظمة الصحة العالمية و مكتب الصحة و السكان م. لحج .

و هذه إفادة بحسب طلبها .

و الله الموفق ،،،،

مدير عام المعلومات و البحوث
د. عبد الله علي الأشول
14

Tel.: 967-1- 220590 - 252192 - P.O. Box : 23117 - Sana'a - Yemen - Web Site:www.moh.gov.ye
تليفون : ٢٥٢١٩٢ - ٢٢٠٥٩٠ -١-٩٦٧ - ص.ب : ٢٣١١٧ - صنعاء - الجمهورية اليمنية - الموقع الإلكتروني : www.moh.gov.ye

B) Approval From The Faculty of Medicine, Aden University, Yemen

بسم الله الرحمن الرحيم

Republic of Yemen
University of Aden
Faculty of Medicine &
Health Sciences

الجمهورية اليمنية
جامعة عـــــدن
كلية الطب و العلوم الصحية

Ref:

المرجع :

Date 26/11/2002

التاريخ:

To Whom It May Concern

Re: Ethical approval of the study of drug resistant malaria in Al-Musaimeer, Lahj

This is to certificate that the study of drug resistant malaria conducted by Dr. Reem Mubjer in Al-Musaimeer district, Lahj governorate has been revised and approved by the ethical committee in the Faculty of Medicine and Health Sciences, Aden University.

Associated Prof. Dr. Anisa M Abood

Vice Dean for Higher Education & Scientific Research

كلية الطب و العلوم الصحية – جامعة عدن – ص ب: ٨٧٨ خور مكسر – عدن – الجمهورية اليمنية – تلفون رقم: ٢٣١٧٦٣ / ٢٣٢٢٩٨
Faculty of Medicine & Health Science - University of Aden – P.O.Box : 878 Khor – Makseer – Aden - Y.R Tel: 231763 / 232

C) Approval From The Liverpool School of Tropical Medicine

LIVERPOOL
SCHOOL OF
TROPICAL
MEDICINE (Affiliated to the University of Liverpool)

Pembroke Place
Liverpool L3 5QA
Telephone: 0151·708 9393
Fax: 0151·705 3370
http://www.liv.ac.uk/lstm/lstm.html

26 November 2002

Ms Reem A K Mubjer
C/o Dr M Chance & Dr A Hassan

COPY FOR YOUR INFORMATION

Dear Ms Mubjer

The research protocol **Study of drug resistant malaria in sentinel site in Yemen** Reference No **02.45** was considered by the Research Ethics Committee on **12 September 2002.**

Thank you for your letter of 26 November 2002 with the information requested by the committee. The protocol now has formal Ethical Approval from the LSTM Research Ethics Committee.

This approval should not be seen as a substitute for Local Ethical Approval from the country/institution where the research is to be carried out and that you have undertaken to seek such approval wherever an appropriate mechanism is in place.

The Research Office (RO) maintains a Database of Local Research Committees in the countries where collaborative work is being carried out. Could you, therefore, feed back to me (via Sharda Mistry in the RO) as much information as possible on the local Committees/Review Bodies that will review (or have reviewed) this protocol. The following details would be much appreciated:

- Name
- Address
- Contact numbers or individuals (tel / fax / e-mail)
- A copy of the appropriate form or some details on the submission mechanism
- Any details you are able to obtain on
  - a) number on the committee
  - b) how many lay representatives sit on the committee?

Yours sincerely

Dr D Lalloo
Chair, Research Ethics Committee

Appendix 2

DEFINITION OF SEVERE MALARIA

Severe manifestations of *P.falciparum malaria* in children and adults

Prognostic value[a]			Frequency[a]	
Children	Adults		Children	Adults
Clinical manifestations				
+	(?)[b]	Prostration	+++	+++
+++	+	Impaired consiousness	+++	++
+++	+++	Respiratory distress (acidotic breathing)	+++	+
+	++	Multiple convulsions	+++	+
+++	+++	Circulatory collapse	+	+
+++	+++	Pulmonary oedema (radiological)	+/-	+
+++	++	Abnormal bleeding	+/-	+
++	+	Jaundice	+	+++
+	+	Haemoglobinuria	+/-	+
Laboratory findings				
+	+	Severe anaemia	+++	+
+++	+++	Hypoglycaemia	+++	++
+++	+++	Acidosis	+++	++
+++	+++	Hyperlactataemia	+++	++
+/-	++	Hyperparasitaemia	++	+
++	++	Renal impairment	+	+++

[a] on a scale from + to +++, +/- indicates infrequent occurrence

[b] Data not available

World Health Organization. Severe falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2000,**94(Suppl. 1)**:1-90

Appendix 3

INFORMED CONSENT

The ministry of health is interested in knowing how well the current treatment for malaria is working in our country. To do this, we are carrying out a study in which we are treating a group of patients with malaria and then following them for 14 days to see if their infection is cured. This is not a new treatment formulation as the test drug is Chloroquine.

If you agree to participate in this study, we would like you to come to the clinic 5 more times over the next 2 weeks, so that we can monitor the progress of the treatment. It is very important that we see you on these days, so if you feel you will not be able to return on these days, please let us know now. At each visit you will receive a full medical examination and on 3 of these visits we will take a small amount of blood by finger-prick to make blood smears to see if you still have malaria parasites.

Your participation is completely voluntary. If you do not want to participate in this study, you will receive treatment as usual at this clinic. Participation in this study will not cost you or your family anything. You may also withdraw from the study at any time and for any reason.

You will benefit from participating in this study because you will be closely followed over the next 14 days. If you continue to suffer from malaria, you will receive an alternative treatment that will cure the illness. There will be someone here at the clinic every day so that, even in days over scheduled visits and on week-ends you may come in for check-up if you feel that you are ill.

Do you have any question about the study?

Thank you for your participation and cooperation.

((I have read and understood the forgoing information, or it has been read for me. I have had the opportunity to ask questions about it and they have been answered to my satisfaction. I consent voluntarily to participate as a subject in the study and understand that I have the right to withdraw from the study at any time without affecting in any way my further medical care)).

Name of participant: Date: Signature:

Adapted from WHO, 2001

(Arabic translation of the informed consent)

Translated by Dr. Reem Mubjer

بسم الله الرحمن الرحيم

موافقة المريض للاشتراك في دراسة فعالية الكلوروكوين لعلاج الملاريا المنجلية البسيطة في مديرية المسيميرم / لحج (من ١٧ أكتوبر ٢٠٠٢ إلى ٥ يناير ٢٠٠٣)

تهتم وزارة الصحة العامة بمعرفة مدى فعالية العلاج الحالي للملاريا في بلادنا ، لذلك نحن نقوم بدراسة من خلالها نعالج مرضى الملاريا ونتابعهم لمدة (١٤) يوماً لمعرفة ما إذا كانت العدوى قد عولجت .

العلاج المستخدم في الدراسة ليس علاجاً جديداً و إنما هو عقار الكلوروكوين .إذا وافقتم للاشتراك في هذه الدراسة سنطلب منكم الحضور إلى المركز لـ (٦) زيارات أخرى خلال الأسبوعين القادمين حتى نستطيع متابعة تطور العلاج .انه مهم جداً أن نراكم خلال تلك الأيام ،لذلك إذا كنتم ترون إنكم غير قادرين على العودة الرجاء اطلاعنا بذلك من البداية .في كل زيارة سوف تحصلون على فحص طبي سريري كامل . في أربع زيارات من الزيارات القادمة سوف نأخذ كمية بسيطة من الدم عن طريق وخز الإصبع لعمل عينات في شرائح لنرى إذا ما زلتم تحملون طفيلي الملاريا وكذا عينات في ورق ترشيح لتحليل المورثات المسؤولة عن عدم استجابة طفيلي الملاريا للكلوروكوين .

مشاركتكم طوعيه بالكامل . إذا لم تشاركون في الدراسة سوف تستلمون علاجاً كما هو معتاد في هذا المركز . مشاركتكم لن تكلفكم أو تكلف عائلتكم أي شيء ، وتستطيعون الانسحاب من الدراسة في أي وقت ولأي سبب .

سوف تستفيدون من الاشتراك من الدراسة حيث ستتم متابعتكم بشكل دقيق لمدة (١٤) يوماً . في حال استمرار معاناتكم من الملاريا سوف نعطيكم علاجاً بديلاً والذي سوف يشفي مرضكم بإذن الله . سيكون هناك شخص في المركز كل يوم لتتمكنون من الحضور حتى في الأيام الغير مجدولة وخلال عطلة نهاية الأسبوع في حال شعوركم بأي توعك .

هل عندكم أي سؤال عن الدراسة ؟

شكراً لمشاركتكم في الدراسة .

اسم المشارك : _____ التوقيع : _____

Appendix 4

CASE RECORD FORM
In-vivo test

1- Record Number	2- Study Site Health facility name: Town: District (province)	3- Drug tested:	4- Manufacturer:	5- Batch no.	6-Expiry date:
7- Full Name: OR Guardian's name:	8 -Contact (home) address Tel.:	9- Age: □□ years □□ months	10- Sex: Male □ Female □	11- Weight (Kg): □□.□	
12- History of fever (last 24 hours): Yes □ No □		13- Previous antimalarials: Yes □ No □ Unknown □ If Yes please specify: Drug: Dose:			
14- Total Chloroquine dose (mg base):					

Follow-up parameters	Day of follow-up														
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Date: (day/month/year)															
Parasite count: (asexual parasites/µl)															
Axillary temperature															
Danger signs															
Treatment (no. of tablets)															
Excluded or loss to follow-up															
Concomitant treatment															
Possible side effects															
Observation															

Side effects: Yes □ No □ **If Yes please specify: ----------**	**Reasons for exclusion or loss of follow-up** □ development of danger signs infection □ presence of mixed malaria □ development of complications □ movement outside the study area □ development of concomitant infection □ others (**please specify**):----------
Overall assessment: **□ ETF □ LPF □ LCF □ ACPR □ Excluded □ Loss to follow-up**	**Observations:**

Adapted from WHO, 2001

Appendix 5

PREPARATION OF TAE BUFFER

Preparation of TAE buffer x50 stock

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA 9pH8.0)	100 ml
Distilled water	Up to 1 litre

Source: Noboru Inoue, D.V.M., PhD., E.mail: ircpmi@obihiro.ac.jp

National Research Centre for Protozoan Diseases.

Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

Preparation of working solution x1 TAE buffer

To prepare 1 litre of x1 TAE buffer

x50 TAE buffer	20 ml
Distilled water	Up to 1 litre

Appendix 6

ACCESSION NUMBERS FOR PFCRT SEQUENCE OBTAINED FROM GENBANK

AF030694 Plasmodium falciparum strain Dd2 heat shock protein 86 (HSP86), O1 (o1), O3 (o3), O2 (o2), CG8 (cg8), CG4 (cg4), CG3 (cg3), putative chloroquine resistance transporter (crt), CG9 (cg9), CG1 (cg1), CG6 (cg6), CG2 (cg2), and CG7 (cg7) genes, complete cds
gi|6724279|gb|AF030694.2|[6724279]

DQ156109 Plasmodium falciparum strain TA6182 chloroquine resistance transporter (Crt) mRNA, partial cds
gi|73698141|gb|DQ156109.1|[73698141]

DQ156108 Plasmodium falciparum strain TU741 chloroquine resistance transporter (Crt) mRNA, partial cds
gi|73698139|gb|DQ156108.1|[73698139]

DQ156107 Plasmodium falciparum strain TA7519 chloroquine resistance transporter (Crt) mRNA, partial cds
gi|73698137|gb|DQ156107.1|[73698137]

AY570285 Plasmodium falciparum isolate GUY-PHG021 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458296|gb|AY570285.1|[51458296]

AY570284 Plasmodium falciparum isolate GUY-K15 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458294|gb|AY570284.1|[51458294]

AY570283 Plasmodium falciparum isolate GUY-001 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458292|gb|AY570283.1|[51458292]

AY570282 Plasmodium falciparum isolate GUY-K20 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458290|gb|AY570282.1|[51458290]

AY570281 Plasmodium falciparum isolate GUY-PHG011 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458288|gb|AY570281.1|[51458288]

AY570280 Plasmodium falciparum isolate GUY-PHG005 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458286|gb|AY570280.1|[51458286]

AY570279 Plasmodium falciparum isolate GUY-PHG010 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458284|gb|AY570279.1|[51458284]

AY570278 Plasmodium falciparum isolate GUY-K14 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458282|gb|AY570278.1|[51458282]

AY570277 Plasmodium falciparum isolate GUY-029 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458280|gb|AY570277.1|[51458280]

AY570276 Plasmodium falciparum isolate GUY-K3 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458278|gb|AY570276.1|[51458278]

AY570275 Plasmodium falciparum isolate GUY-PHG024 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458276|gb|AY570275.1|[51458276]

AY570274 Plasmodium falciparum isolate GUY-025 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458274|gb|AY570274.1|[51458274]

AY570273 Plasmodium falciparum isolate GUY-008 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458272|gb|AY570273.1|[51458272]

AY570272 Plasmodium falciparum isolate GUY-016 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458270|gb|AY570272.1|[51458270]

AY570271 Plasmodium falciparum isolate GUY-M23 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458268|gb|AY570271.1|[51458268]

AY570270 Plasmodium falciparum isolate GUY-M3 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458266|gb|AY570270.1|[51458266]

AY570269 Plasmodium falciparum isolate GUY-PHG023 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458264|gb|AY570269.1|[51458264]

AY570268 Plasmodium falciparum isolate GUY-K10 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458262|gb|AY570268.1|[51458262]

AY570267 Plasmodium falciparum isolate GUY-PHG4 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458260|gb|AY570267.1|[51458260]

AY570266 Plasmodium falciparum isolate GUY-PHG9 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458258|gb|AY570266.1|[51458258]

AY570265 Plasmodium falciparum isolate GUY-PHG24 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458256|gb|AY570265.1|[51458256]

AY570264 Plasmodium falciparum isolate GUY-PHG25 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458254|gb|AY570264.1|[51458254]

AY570263 Plasmodium falciparum isolate GUY-PHG28 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458252|gb|AY570263.1|[51458252]

AY570263 Plasmodium falciparum isolate GUY-K8 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458250|gb|AY570262.1|[51458250]

AY570261 Plasmodium falciparum isolate GUY-PHG27 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458248|gb|AY570261.1|[51458248]

AY570260 Plasmodium falciparum isolate GUY-PHG13 digestive vacuole transmembrane protein (CRT) gene, partial cds
gi|51458246|gb|AY570260.1|[51458246]

AF495378 Plasmodium falciparum clone K1Hf putative chloroquine resistance transporter (crt) gene, complete cds
gi|29468336|gb|AF495378.1|[29468336]

AF495377 Plasmodium falciparum clone K1H6/2-AM3.4 putative chloroquine resistance transporter (crt) gene, complete cds
gi|29468334|gb|AF495377.1|[29468334]

AF495376 Plasmodium falciparum clone K1H6/2 putative chloroquine resistance transporter (crt) gene, complete cds
gi|29468332|gb|AF495376.1|[29468332]

AY651315 Plasmodium falciparum isolate 2300 putative chloroquine resistance transporter (crt) mRNA, complete cds
gi|51317937|gb|AY651315.1|[51317937]

AY254700 Plasmodium falciparum digestive vacuole transmembrane protein CRT (crt) mRNA, crt-PH1 allele, partial cds
gi|32307085|gb|AY254700.1|[32307085]

AF233068 Plasmodium falciparum clone HB3 putative chloroquine resistance transporter (crt) mRNA, complete cds
gi|7331104|gb|AF233068.1|AF233068[7331104]

AF233067 Plasmodium falciparum clone 7G8 putative chloroquine resistance transporter (crt) mRNA, complete cds
gi|7331102|gb|AF233067.1|AF233067[7331102]

AF233066 Plasmodium falciparum isolate NF54 putative chloroquine resistance transporter (crt) mRNA, complete cds
gi|7331100|gb|AF233066.1|AF233066[7331100]

AF233065 Plasmodium falciparum clone 106/1 putative chloroquine resistance transporter (crt) mRNA, complete cds
gi|7331098|gb|AF233065.1|AF233065[7331098]

AF233064 Plasmodium falciparum clone DIV30 putative chloroquine resistance transporter (crt) mRNA, complete cds
gi|7331096|gb|AF233064.1|AF233064[7331096]