Combination therapy in *Plasmodium falciparum* malaria in Sudan and characterization of molecular markers of chloroquine and sulfadoxine-pyrimethamine resistance

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A thesis submitted for fulfillment of the requirements for the degree of
Doctor of Philosophy in medical Biochemistry
June 2007

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Acknowledgments

I am greatly indebted to my supervisor Professor Mustafa Idris Elbashir, Dean Faculty of Medicine, University of Khartoum for his guidance throughout my postgraduate studies, and whose expertise, understanding, and patience, added considerably to my experience. I gratefully acknowledge the time devoted to and the valuable insights into this work. Without his stimulating suggestions, encouragement and constant guidance this research would not have been possible.

Thanks also go to my co supervisor Dr. Hayder A. Giha, for his contribution in designing the proposal for the studies in Gedaref and Khartoum areas and for supervising the field work in these areas.

I would like to express my gratitude to my external supervisor Dr. Frank P. Mockenhaupt, head of the malaria group, Institute of Tropical Medicine, Charité, Humboldt University, Berlin, Germany, who extended all kinds of support and assistance during my stay in Germany and I had the pleasure to work with. Thanks go to the staff members of the Institute who made the Institute a wonderful workplace, Susane Roewer and Baerbel Jacob, deserves special mention for providing skillful technical assistance.

My appreciation extended to the staff members of the Centre for Medical Parasitology, Institute for Medical Microbiology and Immunology, University of Copenhagen. Sincerest gratitude to Dr. Insaf F. Khalil and Dr. Michael Alifrangis, for their unlimited help in performing PCR –ELISA assays and for their comments on the result, technical assistance from Ulla Abildstrup is appreciated.

My thanks are also extended to the staff members of the Department of Biochemistry, Faculty of Medicine, University of Khartoum for their support.

Deepest thanks to Dr. Ishag Adam for his great help in providing the parasite samples from New Halfa and Kassala and for his continuous encouragement.

I also owe many thanks to the patients and patient’s guardians who participated in the study for their cooperation and patience during the long follow up period, and to the members of the health team for their interest shown in the study, efforts and patience during the hard days of the field work.
Thanks to my family for the support they provided me through my entire life, and for their sincere encouragement to pursue my interests, even when the interests went beyond boundaries of language and geography.

Sincere and genuine thanks to my friends in and outside Sudan for being around when ever needed and for believing in me.

The study in Gedaref State was sponsored by the Joint Eastern Mediterranean Region (EMRO), Division of Communicable Disease (DCD) and WHO Special Programme for Research and Training in Tropical Diseases (TDR): The EMRO DCD/TDR Small Grants Scheme for Operational Research in Tropical and other Communicable Disease, project number SGS02/110. A scholarship from German Academic Exchange Service (DAAD) code number A/03/21656 was received in October 2003. I gratefully acknowledge support from these agencies. The local fund from the National Center for Research, Ministry of Science and Technology and from Malaria Administration, Ministry of Health which partially supported the study in Khartoum State is highly appreciated.
**Abbreviations**

A. = *Anopheles*

ACPR = Adequate clinical and parasitological response

ACTs = Artemisinin based combination therapies

ART = Artemisinin

AS = Artesunate

ATP = adenosine triphosphate

bp = base pair

Cl⁻ = Chloride ions

CQ = Chloroquine

CQR = CQ resistant

CT = Combination therapy

Cyt B = Cytochrome b

DHA = Dihydroartemisinin

Dhfr = Dihydrofolate reductase

Dhps = Dihydropteroate synthase

DNA = Deoxyribonucleic acid

dNTPs = deoxynucleoside triphosphate

DV = Digestive vacuole

EDTA = Ethylenediaminetetraacetic acid

ELISA = Enzyme linked immunosorbent assay

ETF = Early treatment failure

G6PD = glucose-6-phosphate dehydrogenase

GTP = Guanosine triphosphate

LCF = Late clinical failure

LPF = Late parasitological failure

*Msp-1* = Merozoite surface proteins 1

*Msp-2* = Merozoite surface proteins 1

Na⁺-H⁺ = sodium-hydrogen ions

P. = *Plasmodium*

P/µl = Parasites / micro liter blood
PBS = Phosphate buffered saline
PCR = Polymerase chain reaction
Pfcrt = *Plasmodium falciparum* chloroquine resistance transporter
Pfdhfr = *Plasmodium falciparum* dihydrofolate reductase
Pfdhps = *Plasmodium falciparum* dihydropteroate synthetase
PfEMP1 = *Plasmodium falciparum* erythrocyte membrane protein 1
Pfmdr1 = *Plasmodium falciparum* multidrug resistance gene 1
Pgh1 = P glycoprotein homologue 1
PNG = Papua New Guinea
RFLP = Restriction fragment length polymorphism
rpm = Round per minute
SE = Southeast
SERCA-PfATPase 6 = *Plasmodium falciparum* sarco/endoplasmic reticulum calcium-dependent *Plasmodium falciparum* adenosine triphosphatase 6
SNPs = Single nucleotide polymorphisms
SP = Sulfadoxine-pyrimethamine
SSOPs = Sequence specific oligonucleotide probes
Stevor = Subtelomeric variant open reading frame
WBC = White blood cells
WHO = World Health Organization
Abstract

This work aimed at contributing data to help policy makers in planning malaria control and adopt policies that might delay the emergence of drug resistance in Sudan.

A study conducted in a rural area, Gedaref State, eastern Sudan, has examined therapeutic efficacies of chloroquine (CQ) Plus dihydroartemisinin (DHA) and CQ plus sulfadoxine-pyrimethamine (SP) in uncomplicated falciparum malaria. *Plasmodium falciparum* isolates of this area were genotyped for detection of mutations in *P. falciparum* chloroquine transporter (*Pfcrt*), multidrug resistance (*Pfmdr1*), dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) genes. Polymerase chain reaction (PCR) corrected cure rate was comparable in the two treatment arms (CQ + SP, 62.5% [15/24]; CQ + DHA, 68.2% [15/22]). The frequency of mutant alleles, *Pfdhfr* 51I, *Pfdhfr* 108N, *Pfdhps* 540E, *Pfdhps* 581G and *Pfcrt* 76T, *Pfmdr1* 86Y were; 0.84, 0.84, 0.80 and 0.20, 0.90, 0.86, respectively. No mutations were detected in positions A16V, C59R and I164L, and for *Pfdhps* loci S436A, A437G and A613S. There was statistically significant association between *Pfcrt* 76T and *Pfmdr1* 86Y alleles associated with CQ resistance (P ≤ 0.001), and between *Pfdhfr* 51I, *Pfdhfr* 108N and *Pfdhps* 540E alleles associated with SP resistance (P ≤ 0.001–0.04) and all the detected mutations in both CQ and SP resistance genes (P = 0.001) except *Pfdhps* 581G.

Another study in suburban area in Khartoum State has examined therapeutic efficacy of SP plus artesunate (AS) in treatment of uncomplicated falciparum malaria. Furthermore, polymorphisms associated with SP resistance in *Pfdhfr* and *Pfdhps* genes were examined. PCR corrected cure rate was 92.6%. Single nucleotide polymorphisms (SNP) were detected in positions 51 and 108 *Pfdhfr*, and positions 436/437, 540 and 581 of *Pfdhps*. The most frequent haplotype was *Pfdhfr* double mutant haplotype CJCNI detected in 91.9% (N = 79) of the infections, only one infection (1.2%) expressed the wild type *Pfdhfr* haplotype (CNCSI). In contrast *Pfdhps* mutant haplotypes were found at low rate (9.6%), and only two infections (2.2%) had more than one mutation. No significant association was observed between the presence of *Pfdhfr* and/or *Pfdhps* mutant haplotypes and SP plus AS treatment failure.
A study in New Halfa and Kassala, eastern Sudan has investigated the distribution of \textit{Pf}dhfr, \textit{Pf}dhps SP resistance determinant haplotypes. Quintuple mutations at \textit{Pf}dhfr 51/59/108 and \textit{Pf}dhps 437/540 positions were detected for the first time in Sudan (2.9% in New Halfa and 5.9% in Kassala). In both study sites the predominant \textit{Pf}dhfr mutant haplotype was the double mutant haplotype (CI\textsc{CN}I). \textit{Plasmodium falciparum} dhfr triple mutant haplotype (CIR\textsc{NI}) was identified in New Halfa (2.5%) and Kassala (4.1%). \textit{Plasmodium falciparum} dhps haplotype; harboring 437G and 540E mutations was the most frequent mutant \textit{Pf}dhps haplotype in both sites yielding a percentage of 17.7 in New Halfa and 62.5 in Kassala.

Failure to AS plus SP, high frequency of SP resistance marker alleles in different parts of the country, in addition to the presence of quintuple mutations in \textit{Pf}dhfr/\textit{Pf}dhps genes is alarming.
خلاصة الدراسة:

هدف هذه الدراسة تحديد معدلات انتقال المرض في مساحة متخصصة في تطبيق طريقة مبتكرة لقيادة الطيور على الملاحة.

أجريت دراسة في ريفي شرق السودان لاختبار فعالية خليط عقاري كلوروكوكين و الدابايدروأرتيسيمين (CQ+SP) (CQ+DHA) في علاج الملاحة. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1 والعملاء المتغير في الجينات في نطاقات متراوحDé de 86% و 90% في الخلايا. كما تم استخدام جينات مختوطة للعوالق بجامع العوالق التي جمعت قبل بداية العلاج. كان مستوى الاستجابة لنوع العوالق بعد التحصين بنسبة التفاعل السلبي المتغير (PCR correction cure rate) Pfmdr1
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The study results include the treatment failure due to resistance to artemisinin and the falciparum. The study results also include resistance to the antifolate and the falciparum. The study results were analyzed by grouping cases in different regions with different resistance patterns.
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1. Introduction and Literature Review

1.1. General introduction

Malaria is a complex public health problem in the African continent. It presents an enormous obstacle to national development because of its high human and economic costs. Malaria is a mosquito borne disease caused by the protozoan falciparum, five species of which infect humans. These are *Plasmodium falciparum* (*P. falciparum*), *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Malaria is an important cause of morbidity and mortality in children and adults in tropical countries, almost all deaths and severe disease are caused by *P. falciparum* (1). The vector responsible for malaria transmission is the female Anophelene mosquito. There are over 40 species of anopheline mosquitoes that transmit human malaria, which differ in their transmission potential. The most competent and efficient malaria vector, *Anopheles gambiae* (*A. gambiae*), occurs exclusively in Africa (2). However, despite enormous and diverse efforts to control the disease, malaria is among the top three most deadly communicable diseases and the most deadly tropical parasitic disease today (3).

1.2. Global malaria burden

Malaria has a worldwide distribution, being found in tropical areas, throughout sub Saharan Africa and to a lesser extent in South Africa, Southeast Asia (SE Asia), the Pacific islands, India and Central and South America (1). Falciparum is the predominant species in most endemic countries, and is most prevalent in Africa south of the Sahara and in certain areas of SE Asia and the Western Pacific. The second most common malaria species, *P. vivax*, is rarely fatal and commonly found in most of Asia, and in parts of the Americas, Europe and North Africa (2). *Plasmodium ovale* is mainly found in West Africa. It is found in SE Asia, particularly along the borders of Thailand with Myanmar and Cambodia (1).

Furthermore, the disease burden falls disproportionately on vulnerable groups; including pregnant women and children under five, who have limited access to malaria control interventions, which perpetuates a vicious cycle of poverty and disease.

At the end of 2004, 107 countries and territories had areas at risk of malaria transmission. Some 3.2 billion people lived in areas at risk of malaria transmission (2), estimated incidence totaled 402 million (range 350-500 million) cases (4), resulting in over 1 million deaths. The estimated mortality rate has risen in recent years, probably due to increasing resistance to antimalarial medicines (5). About 59%
of the world’s clinical malaria cases occur in Africa, around 38% in Asia and around 3% in the Americas, for falciparum malaria specifically, the estimated regional distribution is around 74% in Africa, 25% in Asia and around 1% in the Americas (2). About 90% of malaria deaths occur in Africa south of the Sahara, 90% of all malaria deaths in Africa occur in young children (6). In Africa young children and pregnant women are the population groups at highest risk for malaria morbidity and mortality. The disease takes an economic toll as well because of reduced productivity, which is responsible for an estimated average loss of 1.3% of economic growth annually in countries with intense transmission for those countries with the highest burden (7).

Patterns of malaria transmission vary markedly between regions and even within an individual country. This diversity results from variations between malaria parasites and mosquito vectors, ecological conditions that affect malaria transmission and socioeconomic factors, such as poverty and access to effective health care and prevention services. As a result of differing intensities of malaria transmission, the population groups at risk of malaria also differ between world regions.

1.3. Malaria infection

Malaria infection manifests itself in different ways; it can be asymptomatic or present with various uncomplicated (mild) or severe forms.

Asymptomatic malaria occurs in areas of constant and high transmission, where malaria immunity develops rapidly. It is characterized by the presence of asexual blood stage parasites in the peripheral blood without obvious outward signs of infection. Asymptomatic infections contribute to further transmission, and may also affect the overall health status of the individual by contributing to anaemia and/or weakening the individual’s ability to fight off other infections, this is common in intense malaria transmission areas (8,9,10). Uncomplicated malaria (non severe) presenting with vague symptoms before the development of acute paroxysms of high fever and chills, which in a proportion of cases may progresses to severe malaria requiring hospitalization (8). Almost all severe forms of malaria are caused by *P. falciparum* but rarely *P. vivax* or *P. ovale* produce serious complications, debilitating relapses and even death (11).

1.4. Malaria diagnosis

Conventional diagnosis still uses the skilled but laborious and time consuming microscopic examination of thin and thick blood films stained with Giemsa’s or Field’s stain. Newly developed tests include the quantitative buffy coat (QBC)
method for the fluorescent staining of parasites after an enrichment step for the infected erythrocyte; it is reported to be as good as thick films for *P. falciparum* but not for the other species (12); the *ParaSight F* (13) and the Malaquick tests (14) based on the immunological capture of the *P. falciparum* histidine-rich protein 2 in whole blood; and the OptiMal assay, which is antibody based detection of parasite lactate dehydrogenase (15). These antibody based dipstick tests are still being evaluated. Polymerase chain reaction (PCR) based diagnostic tests for human malarias have been developed (16), but these are more applicable to large scale surveys than to clinical diagnosis. Polymerase chain reaction has been especially effective at detecting sub microscopic levels of parasitemia (17,18).

### 1.5. Malaria control and prevention

The world’s malaria situations can be divided into three categories (19). First category represent areas with intense malaria transmission which have largely been unaffected by vector control programs, such as tropical Africa. Second, are areas where large scale vector control programs have been operating, which represent the malaria situation in most malarious countries in Asia and the Americas. Areas of rapid economic development and countries seriously affected by social disruption are placed into the third category. No single strategy is applicable for all situations because characteristics of the malaria disease differ from country to country and even within the same country.

Malaria control efforts in recent years have contributed substantially to the availability of cost effective tools that can be used by endemic African countries. These tools are directed towards control of mosquito vector such as reducing breeding sites, insecticides, insecticide treated nets (ITNs) and indoor residual spraying (IRS) (20,21). Other approaches are directed at controlling the parasites in the human host which include efficacious artemisinin based combination therapies (ACTs) (22), Malaria Early Warning Systems (MEWS) to detect and contain outbreaks, intermittent preventive treatment in infant (IPTi) and during pregnancy (IPTp) (23; 24).

### 1.6. Malaria parasite

#### 1.6.1. The life cycle of malaria parasite

All stages in the life cycle of the *Plasmodium* parasite are haploid, apart from the diploid zygote, which immediately after fertilization undergoes meiosis to yield haploid infectious forms (25). The life cycle of *Plasmodium* parasites which affect
human involves two hosts; vertebrate and invertebrate host, have sexual and asexual stages (1) (Figure 1.1).

*Plasmodium falciparum* sporozoites are released from the female mosquito’s salivary glands into the blood stream of the vertebrate host, and within minutes it reach the liver and start to reproduce, mature into hepatic schizonts which subsequently rupture, releasing merozoites. It takes the parasite one to two weeks to mature during the liver stage (pre erythrocytic stage). During the pre erythrocytic stage an infected person shows no signs or symptoms of disease, and malaria can not be detected.

The merozoites which are haploid are released into the blood stream and rapidly infect red blood cells to start the erythrocytic stage. They begin cycles of invading red blood cells, multiplying, and rupturing every 48 hours for falciparum, vivax and ovale malarias and 72 hours for *P. malariae*. It only takes a few seconds for merozoites to invade new blood cells, so merozoites spend much of the time inside them, protected from the immune system. During the blood stage, that infected persons develop the periodic fevers, chills, and other signs of malaria.

After a number of asexual life cycles, some of the merozoites mature into sexual forms (gametocytes), gametocyte production takes approximately 10 days in falciparum malaria compared to only 4 days in vivax malaria. When a female anophelene mosquito, during a blood meal, takes up these sexual forms, they fuse in the midgut to form a zygote (a transient diploid form), which then undergoes meiosis to yield new haploid infectious forms (sporozoites), and completes the life cycle. The parasite is then poised to begin the cycle again with the mosquito’s next blood meal. In vivax and ovale malarias a number of the sporozoites arriving at the liver become dormant hypnozoites, capable of causing a relapse of malaria at a later date. *P. malariae* does not have the hypnozoite stage but may persist in the blood for many years if inadequately treated (26).

1.6.2. Genetics of *Plasmodium falciparum*

The complete genome sequence of *P. falciparum* clone 3D7 was published in 2002 (27). The 23 mega base nuclear genome consists of 14 chromosomes ranging in size from approximately 0.643 to 3.29 Mb, encodes about 5,300 genes with an (A + T) content of 80.6%, and rises to ≈ 90% in introns and intergenic regions. Compared to the genomes of free living eukaryotic microbes, the genome of this intracellular parasite encodes fewer enzymes and transporters, but a large proportion of genes are devoted to immune evasion and host parasite interactions.
Figure 1.1: The life cycle of *Plasmodium falciparum* parasite
Reproduced from www.malariavaccine.org
Many nuclear encoded proteins are targeted to the apicoplast, an organelle involved in fatty acid and isoprenoid metabolism. *Plasmodium falciparum* chromosomes vary considerably in length, with most of the variation occurring in the subtelomeric regions. Field isolates, even those from individuals residing in a single village (28), exhibit extensive size polymorphism that is thought to be due to recombination events between different parasite clones during meiosis in the mosquito (29). About 60% of the predicted proteins appear to be unique to this organism, a proportion much higher than observed in other eukaryotes.

1.6.3. Antigenic determinant genes in malaria parasites

*Plasmodium falciparum* exhibits antigenic polymorphisms. Numerous polymorphic antigens of the asexual stages of *P. falciparum* are now well characterized. Parasite surface proteins of the sporozoite (*CSP*, coding for the circumsporozoite protein) and the merozoite (*MSP-1* and *MSP-2*, coding for the merozoite surface proteins 1 and 2) are known to be extremely polymorphic. Genes coding for these antigenic proteins; have been found to be highly polymorphic and that their multiple allelic forms differ in their ability to escape the host’s immune response (30–32).

1.6.3.1. Merozoite surface proteins 1 (*MSP-1*)

The *MSP-1* gene located on chromosome 9, codes for *MSP-1*, previously referred to as *P195*, *gp185*, *PSA*, or *PMMSA* (34). The *MSP-1* is a glycoprotein (∼195 kilo Dalton) that is processed at the time of schizont rupture to generate several smaller polypeptides on the mature merozoite surface (33). Tanabe *et al.* (34) partitioned *MSP-1* into 17 blocks, based on the degree of amino acid polymorphism. They classified seven blocks (blocks 2, 4, 6, 8, 10, 14, and 16) as highly variable; five blocks (blocks 7, 9, 11, 13, and 15) as semi conserved, and five blocks (blocks 1, 3, 5, 12, and 17, which include the two terminal segments) as conserved. Block 2 region, located within the N-terminal half of *MSP-1*, is highly polymorphic, with different sequence variants identified. All sequence variant on block 2 of *MSP1* belong to one or another of only three main sequence types represented by variants originally identified in the K1, MAD20, and RO33 isolates (35). Variants within the K1-like and MAD20-like types of block 2 differ in tri- or hexapeptide repeats (34,35,36), whereas block 2 of the RO33 type is a nonrepetitive sequence which varies little between isolates (36).
1.6.3.2. Merozoite surface proteins 2 (MSP-2)
The Msp-2 gene located in chromosome 2, codes for MSP-2 (or MSA-2), which is a glycoprotein, a smaller polypeptide of 45 kilo Dalton that is not processed during parasite maturation but, like MSP-1, appears to be anchored in the merozoite membrane by a glycosylphosphatidylinositol moiety (37). Studies of the genes from *P. falciparum* isolates (38,39) revealed that MSP-2 contains highly conserved N and C termini, with 43 and 74 residues, respectively. Bracketed within these conserved regions, is the highly variable repeat region. The MSP-2 of isolate FCQ27/PNG (FC27) contains two copies of a 32 amino acid repeat, whereas the Msp-2 of Indochina 1 (IC1) and 3D7 contain multiple copies of a 4 amino acid sequence with no apparent relationship to the FC27 repeat. Variant sequences are flanking the repeats. The polymorphism at MSP-1 and MSP-2 loci are mainly assessed by the number of repeats (38,40), which can be used to distinguish the different alleles by size after amplification by PCR.

1.7. Chemotherapy of malaria

Recommendations for treatment of malaria vary according to geographic region because it depends on the severity of the infection, the patient’s age, the degree of background immunity, effectiveness of antimalarial drugs, and the cost and availability of such drugs. The antimalarials in common use come from five classes of compounds; the quinolines and arylaminoalcohols, the antifols, the artemisinin (ART) derivatives, the hydroxynaphthaquinones and antibacterial agents (1).

1.7. 1. Quinoline containing drugs

The quinoline containing drugs include the 4-aminoquinolines, chloroquine (CQ) and its relative amodiaquine, the cinchona alkaloids, quinine and quinidine, and the aminoalcohol mefloquine (a 4-quinoline methanol) and halofantrine (a 9-phenathrene methanol). There are also the 8-aminoquinoline primaquine, in addition to lumefantrine, piperaquine, pyronaridine.

Chloroquine is a 4-aminoquinoline which has been used for treatment and prevention of malaria. Chloroquine is characterized by long elimination half life (1-2 months) (5). It is cheap, and was reported as the most widely consumed drug in the world after aspirin and paracetamol (41).
Amodiaquine is a Mannich base 4-aminoquinoline with a mode of action similar to that of CQ. It is more active than CQ against resistant parasites. There are insufficient data on its terminal plasma elimination half life (5).

Quinine, an alkaloid derived from the bark of the cinchona tree (42). The mean elimination half life is around 11 hours in healthy subjects, 16 in uncomplicated malaria and 18 in severe malaria (43). Quinine is combined with an antibiotic such as tetracycline/doxycycline or clindamycin to treat falciparum malaria infections in regions where sensitivity to quinine is reduced (44), and is reserved for cases of severe malaria.

Quinidine is related to quinine and is used intravenously to treat severe malaria (1).

Mefloquine is a 4-methanolquinoline compound, effective against all forms of malaria (5). It has a long elimination half life of around 21 days, which is shortened in malaria to about 14 days, possibly because of interrupted enterohepatic cycling (45). The use of mefloquine is contraindicated in patients with epilepsy, a history of neuropsychiatric disease or in patients recovering from cerebral malaria. It should not be given to treat patients who have already received the drug in the preceding 4 weeks or whose ability to work safely may be impeded by drug side effects. As a treatment it should be combined with 3 days of artesunate (46). A fixed co-formulation of the two medicines is under development.

Primaquine is an 8-aminoquinoline and is effective against intrahepatic forms of all types of malaria parasites. It is used for the treatment of *P. vivax* and *P. ovale* malaria (5). Its elimination half life is 3-6 hours (47). Primaquine has potent gametocytocidal properties against *P. falciparum*. It is contraindicated in pregnancy or in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, as it may cause massive haemolysis (48).

Lumefantrine is a racemic 2, 4, 7, 9-substituted fluorine derivative synthesised in China. It is manufactured as a fixed combination with artemether. Lumefantrine absorption is dependent on co-administration with fat. Each dose should be taken with a fatty meal or with 200 ml milk (49). The terminal elimination half life is around 3 days (5).

Piperaquine is a bisquinoline compound related to CQ. It has been co-formulated with dihydroartemisinin (DHA) as a fixed dose combination that has been registered in a number of Asian countries in recent years. Experience with the drug suggests its effectiveness and safety for the treatment of uncomplicated falciparum malaria (50).
Tafenoquine is a promising new 8-aminoquinoline which is still in phase III trials for the eradication of hypnozoites. It has a much longer half life than primaquine. It is also contraindicated in patients with G6PD deficiency (1).

Pyronaridine which is a Mannich base compound developed in China has been combined with artesunate in a new fixed combination which is in phase II clinical trials. Reported adverse events after treatment with the combination were mild and transient (51).

1.7. 2. Antifolate drugs

The Antifolates (folate biosynthesis inhibitors) include the diaminopyrimidines, such as pyrimethamine and trimethoprim; the biguanides, represented by proguanil (cycloguanil) and chlorproguanil; and the sulfa drugs, including the sulfonamides and the sulfones. A marked synergy between sulfa drug types and pyrimethamine or proguanil was discovered in the 1960s, the most frequently used combinations being sulfadoxine-pyrimethamine (SP) (Fansidar) and pyrimethamine-dapsone (Maloprim) (52). The latest addition to this class is the fixed combination chlorproguanil-dapsone (Lapdap™), it is thought to be more effective than SP against *P. falciparum* in Africa (53).

1.7. 3. Artemisinin derivatives

Artemisinin which is known as qinghaosu in china, was isolated from the leaves of *Artemisia annua* (sweet wormwood plant) in 1972 (54). It has been used in traditional Chinese medicine for centuries as a treatment of fever (55). Artemisinin itself is poorly absorbed, and therefore semisynthetic derivatives have been used; A water soluble compound; artesunate (AS); is a hemisuccinate ester of ART exists as oral, suppository, intramuscular and intravenous forms; and it can be considered a prodrug for DHA. In addition two oil soluble compounds are available; artemether which is the methyl ether of DHA which is available in oral and intramuscular preparations and arteether (Artemotil) which is the ethyl ether of ART, given by intramuscular injection only. Dihydroartemisinin; the main active metabolite of the ART derivatives can be given orally or rectally as a drug in its own right. Artemisinin compounds are active against different species of *plasmodium* that infect humans (56,57,58). They exhibit a rapid action and high efficacy against the blood stages of *Plasmodium*, including the youngest stages (ring forms) (59), and have been shown to reduce the number of gametocytes in the blood (60,61). Artemisinins also decrease the infectivity of gametocytes to mosquitoes (62,63). This property may help reduced
transmission rates in areas of low transmission (64), but it has no benefit in areas of high transmission despite reported reduction of gametocyte rate (65,66). Artemisinin derivatives are characterized by a short half life (67). High rate of recrudescence associated with ART monotherapy, and the attendant risk of the development of drug resistance was reported (68,69). Clinical failure caused by ART resistant *P. falciparum* has not yet been reported. The main concern with the lipid soluble derivatives is related to their toxicity in animals (70), but similar toxicity has not become an issue with humans.

1.7. 4. Hydroxynaphthaquinones

Hydroxynaphthaquinones represented by atovaquone which is used in a fixed combination with proguanil (Malarone™) for malaria prophylaxis and treatment (1). Resistance develops rapidly after atovaquone monotherapy (71).

1.7. 5. Antibacterial drugs

Antibacterial drugs with antimalarial activity are clindamycin, tetracycline, doxycycline which is used for chemoprophylaxis in travelers and azithromycin which is closely related to erythromycin. Antibiotics are weak antimalarials and slow acting and should not be used as monotherapy to treat malaria (5). The tetracyclines in combination with quinine or ART derivatives may be used for the treatment of *falciparum* malaria but they are contraindicated in young children and pregnancy (5). Antimalarials which are easily affordable by many malaria endemic countries are restricted to CQ, SP, quinine, amodiaquine and mefloquine in SE Asia, ART derivatives are increasingly used throughout tropical world (72).

1.7. 6. Criteria for antimalarial treatment policy change

In 2006 World Health Organization (WHO) recommended that antimalarial treatment policy should be changed at treatment failure rates considerably lower than those recommended previously. It has been recommended that a change of first line treatment should be initiated if the total failure proportion exceeds 10% (5). However, it is acknowledged that a decision to change may be influenced by a number of additional factors, including the prevalence and geographical distribution of reported treatment failures, health service provider and/or patient dissatisfaction with the treatment, the political and economical context, and the availability of affordable alternatives to the commonly used treatment (5).
1.8. Resistance to antimalarial drugs

Antimalarial drug resistance is defined as the ability of a parasite strain to survive and/or multiply despite the proper administration and absorption of an antimalarial drug in the dose normally recommended (5). Malaria treatment failure is a failure to clear malarial parasitaemia and/or resolve clinical symptoms despite the administration of an antimalarial. So while drug resistance may lead to treatment failure, not all treatment failures are caused by drug resistance (5).

The emergence of antimalarial drug resistance has increased the global malaria burden and is a major threat to malaria control. Resistant falciparum malaria has been a major contributor to the global resurgence of malaria in the last three decades (73). The clinical consequences of antimalarial drug resistance are well described in terms of increased morbidity and mortality, especially amongst young African children (74,75).

Resistance to antimalarials has been documented for *P. falciparum*, *P. vivax* and, recently, *P. malariae*. In *P. falciparum*, resistance has arisen to all classes of antimalarials except, as yet, to the ART derivatives.

1.8.1. Global distribution of antimalarial drug resistance


Chloroquine has had a reasonable period of effective deployment. Chloroquine resistance has been reported from wherever falciparum malaria is endemic, except Central America and the Caribbean (78). Recent molecular studies favour importation of chloroquine resistance to Africa from East Asia (79,80).

The first reports of possible quinine resistance occurred in Brazil almost 100 years ago. Clinical resistance to quinine is reported sporadically in SE Asia and western Oceania, and resistance in Africa and South America is much less frequent (5). *In vitro* studies suggested quinine resistance in South America (81) and Africa (82).
Figure 1.2: Malaria transmission areas and the distribution of reported resistance or treatment failures with selected antimalarial drugs, September 2004. Reproduced from WHO, 2006 [5]
Resistance to mefloquine is confined to those areas where it has been used widely (Thailand, Cambodia and Vietnam) (83). It was first reported in the late 1980s (84) near the Thai Cambodian border. Migrant gem-miners returning from Cambodia may have been the means of the spread of mefloquine resistance to Bangladesh and India (85). The level of resistance to mefloquine in South America is lower than in SE Asia, there are also case reports of mefloquine resistance from the Amazon Basin (78), but. Low mefloquine sensitivity in Africa has been suggested by in vitro studies (82,86) but clinical mefloquine resistance is rare in Africa.

Resistance to the antifolates developed more quickly after their introduction. Resistance to SP was first reported in the mid 1960s on the Thai-Cambodian border (77). Currently, high level resistance is observed in a part of SE Asia, southern China, and the Amazon Basin (87,88,89). Lower level of resistance is found on the Pacific coast of South America, and in southern Asia and western Oceania (90). In Africa, SP resistance was reported in 1980s. Resistance to SP exist throughout Africa in various rates (91-97).

Multidrug resistance of \textit{P. falciparum} is the resistance to more than two operational antimalarial compounds of different chemical classes (85). Areas classified as having established multidrug resistance when the drugs of the first two chemical classes 4-aminoquinolines and antifolates, in addition to a third drug are no longer operationally effective. Established multidrug resistance occurs mainly in SE Asia, particularly the border regions of Thailand and in the Amazon basin (5).

In \textit{P. vivax} SP resistance is more wide spread (98). In \textit{P. vivax} resistance to SP (99), to CQ (100,101) and to primaquine (102) has been documented.

\textbf{1.8.2. Mechanisms of resistance to antimalarial drugs}

\textbf{1.8.2.1. Chloroquine}

Chloroquine attacks the dividing erythrocytic stages of the malaria parasite and interferes with parasite hemin (ferriprotoporphyrin IX) detoxification. The site of action of CQ as also of other schizocides such as quinine, mefloquine and halofantrine; is within the digestive vacuole (DV) of the blood stage parasite, CQ is thought to accumulate to high levels in the DV, where it complexes with heme released from hemoglobin digestion and interferes with its polymerization to non toxic hemozoin (103,104), resulting in the accumulation of hemin which is toxic for
the parasite and damages the enzymes as well as the membrane of its digestive vacuole (105). Resistance to CQ is related to diminished uptake of the drug. This is achieved by prevention of access of CQ to the DV. Yet the biochemical mechanism of CQ resistance has remained enigmatic, with different theories. Export of the drug to the cytoplasm by means of a protein was suggested, P glycoprotein homologue 1 (pgh1) in the vacuole membrane, which is specified by *P. falciparum* multidrug resistance (*Pfmdr1*) gene in chromosome 5 (106). Another protein produced within the vacuole, and specified by the gene cg2 on chromosome 7 was postulated to confer CQ resistance (107). Further analysis of the polymorphisms of cg2 suggested that it is not the chloroquine resistance effector. Instead an additional transmembrane protein located in the DV membrane; encoded by *P. falciparum* chloroquine resistance transporter (*Pfcr*) gene which is located in chromosome 7 is postulated to be a CQ transporter (108).

Various hypotheses have been proposed to explain the mechanism of *P. falciparum* resistance to CQ, were reviewed by Jiang and others in 2006 (109). The majority of studies have focused on regulation of the DV physiological changes (pH, ion exchange and CQ concentration) and CQ efflux.

Lower pH in the DV of CQ resistant (CQR) parasites was observed to be coupled with decreased accumulation of CQ (104, 110, 111, 112). A subsequent study confirmed these observations (113). A more acidic pH inside the DV of CQR parasites is believed to directly or indirectly increase the aggregation of heme and reduce its capacity to bind CQ and therefore reduce toxicity to the parasite.

Other studies have suggested that *Pfcr* may affect chloride ions (Cl\(^-\)) transport across the DV (114, 115), thereby influencing the movement of other ions, and eventually affecting heme crystallization, CQ binding kinetic and/or CQ partitioning (116). A plasmodial sodium-hydrogen ions (Na\(^+\)-H\(^+\)) exchanger was suggested in 1997 to be responsible for the reduced CQ import in CQR parasites (117), however in 1998 the steady state accumulation of CQ inside the digestive vacuole was demonstrated to be due to the binding affinity of CQ to heme rather than Na\(^+\)-H\(^+\) exchanger (106), where the affinity of saturable CQ heme binding was greatly reduced in CQR parasites (105, 118, 119), although the number of heme binding sites does not differ in comparison with CQ sensitive parasites (105, 113).
Chloroquine efflux across the DV membrane was also proposed as a cause of decreased CQ accumulation; the hypothesis was supported by many studies which demonstrated an inverse association between CQ efflux and its accumulation (112, 115,118,120,121,122). Chloroquine efflux was supposed to be an energy dependent mechanism, is temporally, indirectly associated with adenosine triphosphate (ATP) production after glucose feeding (112,121). Johnson and colleagues (118) have argued that CQ efflux is not an energy dependent process but due to a process called “charge-leak efflux”, an assumption of this model is that Pfert can transport drugs directly, as predicted by recent bioinformatics analysis studies (123,124). According to this model protonated CQ$^{2+}$ in the digestive vacuole leaks out along its electrochemical gradient through Pfert, a possible anion channel (118). In CQR parasites carrying Pfert mutations that lack the positively charged residue such as lysine at codon 76 to Threonine or isoleucine or asparagines may allow CQ$^{2+}$ to leak outside the DV.

1.8.2.2. Antifolates

*Plasmodium spp.* de novo synthesize the folates they need from the simple precursors guanosine triphosphate (GTP), P-aminobenzoic acid and glutamate (125), Not all of the enzymes involved in folate metabolism have been identified in *Plasmodium spp.* Folate exogenous salvage pathway has been reported (126). Antifolates and sulfad drugs target two enzymes in the folate synthesis pathway. Pyrimethamine and sulfadoxine act synergistically when used in combination. They exert their antimalarial activity by binding and inhibiting dihydrofolate reductase (dhfr) and dihydropteroate synthase (dhps) enzymes, respectively (127). These two enzymes are involved in folate synthesis; disruption of folate synthesis by dhfr and dhps inhibitors leads to decreased levels of fully reduced tetrahydrofolate, a necessary cofactor in important one carbon transfer reactions in the purine, pyrimidine, and amino acid biosynthetic pathways (125). The lower level of tetrahydrofolate results in decreased conversion of glycine to serine, reduced methionine synthesis, and lower thymidylate levels with a subsequent arrest of deoxyribonucleic acid (DNA) replication (128, 129).

Resistance to the antifolate drugs is the result of point mutations in the substrate binding site of the target enzymes (130,131). *In vitro* resistance of *P. falciparum* to pyrimethamine and to chlorcycloguanil is due to specific point mutations in *P. falciparum* dihydrofolate reductase (*Pfdhfr*), which is encoded by a bi-functional gene also encoding thymidylate synthase (106,132-135).
The Pf\text{dhfr} mutations alter the shape of the active cavity where the dhfr inhibitors bind the enzyme, resulting in differential binding affinities for different drugs. The sulfa drugs inhibit dhps by acting as analogues of \textit{p}-aminobenzoic acid, a folate precursor (127). Point mutations in the \textit{P. falciparum} dihydropteroate synthetase (\textit{Pfdhps}) gene encoding dhps have been associated with resistance to sulfa drugs and sulfones. This gene is bi-functional, and also encoding hydroxymethylpterin pyrophosphokinase.

1.8.3. Molecular basis of antimalarial drugs resistance

1.8.3.1. Chloroquine, quinine and mefloquine resistance

Polymorphisms in \textit{Pfcrt} and \textit{Pfmdr1} are the focus of studies on resistance to CQ. In \textit{P. falciparum} CQ resistance is conferred by mutations in the parasite \textit{PfCRT} (108). One mutation, the substitution of threonine for lysine at codon 76 (76T), was perfectly associated with \textit{in vitro} resistance among a set of geographically diverse parasite isolates (136). The absolute specificity of the \textit{pfcrt} 76T to clinical chloroquine resistance has been confirmed by several field studies (79). Specific mutations at codons 72-76 of \textit{Pfcrt} define three major haplotypes; CVMNK, CVIET and SVMNT (137). The CVMNK haplotype is the worldwide identical wild type, CVIET found in most of African/Asian isolates and SVMNT represents the South American/Papua New Guinea (PNG) allele (137). The CVIET and SVMNT represent the two major CQ resistance selective sweeps in SE Asia and South America, respectively. Additional haplotypes were reported in individual samples; SVMIT, RVMNT (138), CVMNN (139), SVIET, CVIKT (140), CVIDT and CVTNT (141). SVMNT haplotype appeared to be associated with decreased efficacy of amodiaquine (115).

Mutations in a second transporter (\textit{Pfmdr1}) have been studied extensively. The aspartic acid to tyrosine point mutation in codon 86 (86Y) has been associated with CQ resistance in some clinical and \textit{in vitro} studies (142-146), but not in others (147-150). Several other \textit{pfmdr1} polymorphisms, notably phenylalanine-184 (184F), cysteine-1034 (1034C), aspartic acid-1042 (1042D), and tyrosine-246 (1246Y), have been implicated as potential contributors to CQ resistance. A parasite transfection experiment showed that polymorphisms in the \textit{pfmdr1} gene modulate susceptibility to chloroquine (151), but their role in determining the \textit{in vivo} resistance has yet to be substantiated (146). Linkage disequilibrium between \textit{pfcrt} 76T and \textit{pfmdr1} 86Y has been noted in Africa (145,152).
Many studies suggested \textit{pfmdr1} as the key modulator of quinine and mefloquine resistance. In a study of \textit{pfmdr1} mutations (86Y, 184F, 1034C, 1042D, 1246Y), chloroquine resistant strains were found to have low susceptibility to quinine (81). Weak association between \textit{pfmdr1} 86Y and decreased sensitivity to quinine was reported (153). Some studies suggested an inverse association between \textit{pfmdr1} 86Y mutation and resistance to mefloquine (143, 153).

\textbf{1.8.3.2. Sulfadoxine-pyrimethamine resistance}

The molecular basis of resistance to SP is the best characterised of all antimalarial resistance. Specific mutations in \textit{P. falciparum} that lead to resistance to both sulfadoxine and pyrimethamine have been identified.

At the present time, point mutations in six codons of \textit{Pfdhfr} gene are known to be associated with pyrimethamine resistance by reducing drug binding capacity in resistant \textit{P. falciparum} strains; include alanine to valine at codon 16 (16V), cysteine to arginine at 50 (50R); asparagine to isoleucine at 51(51I), cysteine to arginine at 59 (59R), serine to asparagine (108N) or to threonine at 108 (108T), and isoleucine to leucine at 164 (164L). A serine to asparagine change at codon 108 is the critical mutation found in all pyrimethamine resistant isolates (154,155). The 108N is the principal mutation causing resistance to pyrimethamine or cycloguanil in Africa and in South America (156). The most frequent additive mutations are 51I and 59R (157). Asparagine-108 confers pyrimethamine resistance \textit{in vitro} with only a moderate loss of susceptibility to cycloguanil and chlorcycloguanil. 108N in addition to 51I and/or 59R mutations results in higher levels of pyrimethamine resistance. On the other hand a 108T coupled with a 16V, confers resistance to cycloguanil and chlorcycloguanil with only a moderate loss of susceptibility to pyrimethamine. Asparagine-108 and 51I and/or 59R confers high level resistance to both pyrimethamine and cycloguanil, with a more modest effect on susceptibility to chlorcycloguanil when combined with 164L (132-136), 164L is absent in Africa and found in areas of high sulfadoxine-pyrimethamine resistance in Southeast Asia and South America. The mutation 50R is found in some areas of South America, and seems to have an effect similar to that of the 59R mutation (131,155). The \textit{Pfdhfr} Triple mutant 108N/51I/59R has been most strongly associated with resistance to SP in Africa (156,157).

Point mutations in five codons of the \textit{Pfdhps} gene are associated with \textit{in vitro} resistance to sulfadoxine and the other sulfas and sulfones include; serine at codon 436 to alanine (436A) or pherylalanine (436F); alanine at 437 to glycine (437G);
lysine at 540 to glutamic acid (540E); alanine at 581 to glycine (581G); alanine at 613 to serine (613S) or threonine (613T) (158-160). Glycine-437 and 540E have been reported to occur together or singly in various parts of the world (106,161-163). Glycine-581 which is rare in Africa, has been observed alone or with 437G in South America (131,164). Among these, 437G, followed by 540E, is most strongly associated with SP treatment failure in Africa (94,131).

Both the Pf\textit{dhfr} and Pf\textit{dhps} mutations occur in a progressive, step wise fashion, with higher levels of \textit{in vitro} resistance occurring in the presence of multiple mutations (131,165). The importance of the mutations in Pf\textit{dhfr} and Pf\textit{dhps} genes in conferring \textit{in vivo} resistance to SP has been debated (166,167). An association between the prevalence of mutations in both Pf\textit{dhfr} and Pf\textit{dhps} genes and SP treatment failure rates has been demonstrated (131,165,168,169). Checchi and others demonstrated a strong association between SP treatment failure and the presence of the Pf\textit{dhps} double mutant 437G/540E together with Pf\textit{dhfr} triple mutant (95).

\textbf{1.8.3.3. Artemisinin resistance}

\textit{Plasmodium falciparum} sarco/endoplasmic reticulum calcium-dependent adenosine triphosphatase 6 (SERCA-Pf\textit{ATPase}6) has recently been identified as target for artemisinins. The S769N mutation in SERCA-Pf\textit{ATPase}6 was associated with decreased \textit{in vitro} susceptibility to artemether (170).

\textbf{1.8.3.4. Atovaquone resistance}

Atovaquone is supposed to target cytochrome b (Cyt B) in the pyrimidine metabolism pathway (171). Resistance to atovaquone is associated with point mutations in the Cyt B gene, which encodes cytochrome b.

\textbf{1.8.4. Molecular markers for antimalarial drugs resistance and outcome of treatment}

Associations between clinical treatment outcomes and resistance conferring mutations have not been straightforward. This is because treatment outcomes are affected by factors other than parasite resistance, including host immune responses (79), micronutrient levels (172) and bioavailability and pharmacokinetics (173).

However, molecular markers are now available that can predict resistance to CQ (146) and to SP (174). In the case of CQ, \textit{pf}\textit{crt} 76T provides a single marker for chloroquine resistance.

A strong association between failure to SP and the presence of Pf\textit{dhfr}/Pf\textit{dhps} quintuple mutant (Pf\textit{dhfr} 108N, 51I, 59R and Pf\textit{dhps} 437G, 540E). A high specificity
of \( Pf\text{dhfr} \) 59R and \( Pf\text{dhps} \) 540E for the presence of \( Pf\text{dhfr}/Pf\text{dhps} \) quintuple mutant has been observed by Kublin and others (174), and thus these alleles can be used as reliable markers for SP failure. This hypothesis has been supported by another study which reported a strong association between clinical failure to SP treatment and the presence of \( Pf\text{dhfr} \) 59R and \( Pf\text{dhps} \) 540E (175).

Markers for resistance to most other antimalarial drugs are lacking because the molecular basis of resistance are not yet understood.

Molecular markers are used as research tools for surveillance for CQ and SP resistance. Molecular markers can provide direct evidence of selection for resistant parasites by antimalarial drug treatment (94,146,169,176) or prophylaxis (177).

1.8.5. Evolution of antimalarial drugs resistance

Many factors contribute to the development and spread of resistance. Gene mutations conferring resistance to antimalarial drugs do occur in nature; independently of drug effect. Single or multiple point mutations in the \( plasmodium \) genome may confer resistance to the antimalarials drugs. A number of factors have been identified as playing a role in development and spread of drug resistance.

Characteristics of the antimalarials are important determinants of resistance. This argument have been discussed by Mackinnon (178), mutants arising in hosts treated with drugs with short half lives or low efficacy will have a fitness only slightly higher than the wild type and therefore will only slowly increase in relative frequency. Under such condition mutant will take far longer to reach a frequency at which they are likely to be transmitted than if hosts were treated with high efficacy drugs or drugs with long half lives. In the later situation the drug persists at sub therapeutic concentrations in the plasma for extended period of time; after the treatment of the primary infection and may exert substantial residual selection on new infections (85) especially in areas with intense malaria transmission. Sub therapeutic drug concentrations eliminate the most susceptible parasites and leave those that may be more fit, to recover and reproduce. Wide spread use of drugs at high intensity serves to increase drug pressure and is a determinant for selection of resistant parasite populations.

The nature of the mechanism of resistance can determine the rate at which resistance may develop. There are three components to this point; First, the probability of establishment of resistance when multiple genes are involved is much reduced compared with that for resistance conferred by single genes, since the chance of
multiple mutations arising de novo in a single parasite is very small compared to that for a single mutation (178). Second the action of the genes involved in resistance; if the genes act epistatically; the spread of resistance will be slowed down compared to genes act independently (179,180). Third when multiple mutations are required for resistance, and they are not at the same locus, recombination, which in malaria occurs every transmission cycle, will act to break down (as well as build up) combinations of resistance alleles (178).

The intensity of transmission determines the rate at which drug resistance may develop and spread. There are conflicting arguments as to whether resistance spreads more rapidly in high transmission or low transmission areas. As endemicity level increases the number of parasite genotypes per infection increases (181), thus promoting the spread of resistance (180,182). On the other hand recombination breakdown which is higher in high transmission areas, would slow the spread of resistance (179,180,182,183). Another factor reduces the rate of spread of resistance in high transmission areas is that the host population are generally more immune, and the drug usage might be lower, thus exposing proportionally fewer parasites to drugs. More potent immune responses increase the efficacy of chemotherapy, where a semi immune patient might be cured by a drug despite the fact that his parasites are partially drug resistant than do non immune hosts (184). Non immune malaria patients generate a non specific immune response that is not as effective as the specific immunity elicited by repeated infections. Thus, the former situation increases manifestation and spread of resistance.

Vector and environmental factors may influence the proliferation of resistant parasites. For example, CQ resistant parasites may be more fit for reproduction in certain anopheline mosquitoes than non resistant strains (85).

The emergence of antimalarial drug resistance is dependent on the occurrence of a spontaneous genetic change in a malaria parasite. However for resistance to spread, the spontaneous occurrence of a mutation in itself is not sufficient.

In the absence of the antimalarial, a parasite with the resistant mutation does not have a survival advantage and there may even be a survival disadvantage, a so called fitness cost to having the mutation (185). Hence antimalarial resistance in malaria parasites spreads because it confers a survival advantage in the presence of the antimalarial to which it is potentially resistant, where the multiplication of the sensitive parasites is inhibited allowing the drug resistant mutants to survive and
multiply and therefore increasing the probability of transmission for resistant than for sensitive parasites.

For resistance to be propagated, recrudescence and subsequent transmission of an infection that generated resistant malaria parasites de novo are essential (69). As resistant infections are more likely to recrudesce, both increased rate of recrudescence and slow initial response to treatment increase the likelihood of generating sufficient gametocytes densities to transmit compared with drug sensitive infections (98).

1.8.6. Origin of antimalarial drug resistance genes

The number of origin of spread of resistance is determined by genotyping of molecular markers; single nucleotide polymorphisms and microsatellites situated very close to drug resistance genes. Microsatellites are useful markers since they are abundant, easily genotyped and have multiple alleles per locus (186). Due to the high recombination rate in *P. falciparum* (187) markers should be positioned as close as possible to the resistance genes.

Two independent origins of CQ resistance was suggested by it's simultaneously appearance in SE Asia and South America (188). Chloroquine resistance appeared in east Africa 17 years after its occurrence in Asia, followed by systematic progression in Africa with little evidence of leapfrogging. (188).

Four origins of CQ resistance alleles were identified by examining point mutations in *Pfcrt*, including a single origin in SE Asia and Africa, two independent origins in South America, and an independent origin in PNG (108). Using microsatellite markers these four independent origins were confirmed (137). Two independent origins of identical coding changes were demonstrated in PNG and South America in 2001 (189). Sequencing analysis which is needed to be confirmed by microsatellite markers suggested two additional origins of resistant *Pfcrt* alleles in Cambodia (141), and in the Philippines (190). Transfer of the *Pfcrt* mutant alleles from SE Asia to Africa (107,188), and the spread of the same alleles to South America (80) and to the Philippines (190) were suggested.

It was assumed that mutations in *Pfdhfr* conferring resistance have multiple origins (177). Three molecular studies using different microsatellite markers to investigate parasites from three different continents demonstrated that this previously suggested theory of pyrimethamine resistance evolution is only partially correct (191,192,193). All three studies suggested multiple origins of low level resistance, and very limited numbers of origins of high level pyrimethamine resistance (with ≥3 mutations). A
study comparing parasites from SE Asia carrying two to four mutations with samples from South Africa, demonstrated that the triple mutant Pf dhfr alleles spreading through Africa have identical or very similar flanking regions to those from SE Asia, indicating that these alleles result from import of parasites from SE Asia (194). Similarly studies on South American and African parasites suggested; very few origins of high level Pf dhps resistance. In South America Pf dhps genes containing a single mutation were found on many different microsatellite backgrounds, suggesting multiple origins. However, only two different origins of the triple mutant allele were demonstrated (191). In Africa, the double mutant Pf dhps allele (A437G/K540E) has a single origin and is found on the same genetic background in two studied countries (192). In SE Asia, microsatellite diversity is reduced around the Pf dhps locus. However, two to three microsatellite haplotypes are found associated with Pf dhps alleles, and recombination and mutation muddy the picture (195).

Duraisingh and others (153) reported limited numbers of origin of Pfmdr alleles containing the mutant Tyr-86 associated with CQ resistance, suggested by significantly lower levels of microsatellite variation in this allele. Triglia and others have investigated the nature and origin of pfmdrl amplicons, they concluded that amplification of the pfmdrl gene in P. falciparum has arisen as multiple independent events, therefore multiple origins have occurred (196). A study analyzed P. falciparum isolates from Thai with high levels of chloroquine and mefloquine resistance has suggested two separate origins of pfmdr alleles in the study area (148).

1.8.7. Assessment of Plasmodium falciparum susceptibility to antimalarial drugs

Antimalarial drug susceptibility is commonly assessed by therapeutic response (in vivo test). Clinical studies are the gold standard for monitoring antimalarial drug efficacy to provide information used by policy makers to establish drug treatment policy in endemic areas of Africa. In vivo test is the assessment of clinical and parasitological outcomes of treatment over a certain period after the start of treatment, to check for the presence and level of the parasite density and taking into account clinical factors.

In vivo response to drugs was originally defined by WHO in terms of parasite clearance; sensitive and three degrees of resistance (RI, RII, RIII) (197). It is difficult to apply this classification in areas with intense transmission where new infections may be mistaken for recrudescences. Therefore, WHO introduced in 1996 a modified protocol based on clinical outcome (adequate clinical response, early treatment
failure, and late treatment failure) targeting a practical assessment of therapeutic responses in areas with intense transmission (198), where parasitemia is common in the absence of clinical signs or symptoms (87,90). Then the protocol has also been adapted for use in areas of low to moderate endemicity, taking into consideration that the objectives of malaria treatment are both parasite clearance and disappearance of symptoms (199). However, in vivo testing is costly, laborious and is not ideal for large scale epidemiologic surveys.

Antimalarial drug susceptibility can also be assessed by in vitro drug sensitivity assays which measure the sensitivity of P. falciparum from the inhibition of growth or schizont maturation (90,200). In vitro test is impractical due to technical limitations and poor correlation with clinical outcomes, largely owing to the role of human host immunity (201). However it is useful for validating molecular markers and for following changes in resistance patterns. Recently, the use of molecular markers has been proposed as an additional tool for the early detection of drug resistance in malaria (79). In vitro test results, in particular, do not necessarily correspond to in vivo outcomes. Evaluation of molecular markers of drug resistance may offer a simple, low cost means of drug efficacy surveillance (168).

1.9. Prevention of resistance by use of combination therapy

Combination therapy (CT) has been advocated as the standard treatment to prevent the spread and development of resistance to antimalarial drugs (202). As a response to increasing levels of resistance to antimalarial medicines, WHO recommends that all countries experiencing resistance to conventional monotherapies, such as chloroquine, amodiaquine or sulfadoxine-pyrimethamine, should use CT, preferably those containing artemisinin derivatives (ACTs – artemisinin based combination therapies) for falciparum malaria (87,203).

Antimalarial CT is the simultaneous use of two or more blood schizontocidal drugs with independent modes of action and thus unrelated biochemical targets in the parasite (5). The concept is based on the potential of two or more simultaneously administered schizontocidal drugs with independent modes of action to improve therapeutic efficacy and also to delay the development of resistance to the individual components of the combination.

The impact of CT on drug resistance is based on the assumption that drug resistance essentially depends on mutation, the probability of a parasite arising that is resistant
simultaneously to two drugs with unrelated modes of action is the product of the per parasite mutation frequencies multiplied by the total number of parasites exposed to drugs (69).

1.10. Artemisinin based combination therapies (ACTs)
Artemisinin derivatives are particularly effective in combinations with other antimalarials because of their very high killing rates, lack of adverse effects and absence of significant resistance (22). The primary disadvantage of artemisinin drugs is that they are characterized by a short half life. Treatment with artemisinin drugs causes reduction of parasite burden below detectable levels without eliminating all parasites; this results in a higher risk of recrudescence (67,204). In addition, a fraction of the parasites exposed to the drug are thought to become dormant and unsusceptible to further dosing until reactivation (205). In order to completely eliminate the parasites and prevent the emergence of resistant *P. falciparum*, combinations with other, longer acting drugs are necessary (206,207). Using ART derivatives with other effective drugs ACTs allow a reduction in the duration of ART treatment. Several ACTs are currently recommended by WHO as the first line antimalarial treatment for *P. falciparum* malaria, these include the combination of artemether with lumefantrine, and the combination of AS with amodiaquine, mefloquine or SP. Amodiaquine plus SP may be considered as an option where ACTs cannot be made available, provided that efficacy of both is high (206). Other ACTs in the pipeline recommended for accelerated development, particularly combination of piperaquine-DHA-trimethoprim (Artecom™), Artecom™-primaquine (CV8™), Lapdap plus AS, Naphthoquine plus DHA and pyronaridine plus AS (203).

1.11. Malaria in Sudan

1.11.1. The burden of Malaria in Sudan
In Sudan, malaria is a leading cause of morbidity and mortality resulting in an estimated 7.5 million cases and 35,000 deaths annually (208), it accounted for 37.2% of all maternal deaths in Sudan at hospital level (209).

Malaria is one of the top 10 most common diseases that cause outpatient attendance and deaths in Sudan (210). Case fatality rate is high ranging between 0.9 to 6.9%. It is increasing every year due to poor case management, low awareness and the emergence of drug resistance.
1.11.2. Malaria epidemiology in Sudan
Malaria is endemic throughout the Sudan. Endemicity varies from hypo endemic in the North to hyper or holo endemic in the South (Figure 1.3) (210). Considering the prevailing epidemiological factors Sudan has been stratified into five malaria strata (Figure 1.4); 80% of the population is living in epidemic-prone areas (unstable malaria transmission) (210).

The disease is caused in >90% of cases by *P. falciparum* though other species are also available. In Khartoum it has been shown that about 15% of cases are due to other types of *Plasmodium* (*P. ovale* =6.2% and *P. vivax* =8.2%) (211). *Anopheles arabiensis* is the primary vector all over the Sudan but *A. gambiae* and *A. funestes* are widely found in many areas mainly in the south (210).

1.11.3. Antimalarial drug resistance in Sudan
In Sudan, CQ had been used as first line treatment for uncomplicated malaria and SP as the second line over a long period of time. Treatment failure of *P. falciparum* to CQ and SP is wide spread in the country. Chloroquine treatment failure has been reported firstly in 1978 in Gezira area, central Sudan (212). In 1983 it was documented in Khartoum (213), in 1989 and 1990 in eastern Sudan (214,215), and in 1992 in Gezira (216). Then the situation was worsening where it reached 32.1% in Khartoum (219); and more than 75% in eastern Sudan (217,218). In 2001 treatment failure to CQ was reported in five sentinel posts (219); and in 2003 in eastern part of the country (220). Treatment failure to SP in Sudan has been documented in varying levels ranging from 8-69.9% that have been reported from Khartoum (221), eastern (222,223,224,225) and southern (226) parts of the country. Four reports have indicated treatment failure to quinine in Sudan (218,227,228,229).

1.11.4. Malaria treatment policy in Sudan
Wide spread resistance of malaria parasites to commonly available antimalarial drugs necessitated the deployment of new antimalarial drug policy. Consistent with WHO recommendations; Sudan has changed its national first and second line antimalarial treatment from CQ and SP to AS plus SP and artemether plus lumefantrine, respectively since June 2004.
Figure 1.3: Malaria endemicity level in Sudan, 2001
Reproduce from the Annual Statistical Report of the Federal Ministry of Health (210)
Figure 1.4: Sudan Major Malaria Strata, 2001 (212)
Reproduce from the Annual Statistical Report of the Federal Ministry of Health (210)
Objectives of the study

General objectives
This study aimed at contributing data to help policy makers in planning malaria control to adopt drug policies that might delay the spread of antimalarial drug resistance.

The main objective of the study was to examine therapeutic efficacies of two antimalarial drug combinations to provide an effective and affordable alternative first line treatment for uncomplicated malaria in Sudan. In addition the study aimed at examining efficacy of the recently adopted national first line antimalarial treatment (AS plus SP) and at providing baseline information on the distribution of SP resistance genotypes alleles in *P. falciparum* in Sudan.

Specific objectives
1. To evaluate the safety and efficacy of CQ plus SP and CQ plus DHA for treatment of uncomplicated falciparum malaria in Gedaref area.
2. To assess the prevalence of key mutations in the *P. falciparum* genes conferring resistance to CQ and SP in the same area.
3. To evaluate the safety and efficacy of the first line antimalarial treatment (AS plus SP) for treatment of uncomplicated falciparum malaria in Khartoum.
4. To assess the frequency of SP resistance marker haplotypes in *P. falciparum* in Khartoum, New Halfa and Kassala.
2. Materials and Methods

2.1. Study sites

2.1.1. Eastern Sudan

In eastern Sudan four sites situated in Sudanese savannah were selected (Figure 2.1). The climate in the selected sites is characterized by well defined wet and dry periods, with a short rainy season from July to September. Malaria is mesoendemic in the four areas, characterized by markedly seasonal, unstable transmission, which peaks in October following the short rainy season.

2.1.1.1. Daraweesh and Kajara Villages

Daraweesh and Kajara, Gedaref State, eastern Sudan. The two villages are about two kilometers (km) from each other, and 16 km from the capital of the State (Gedaref town), they are inhabited by 550 and 2000 individuals, respectively.

2.1.1.2. New Halfa

New Halfa lies 500 km to the east of Khartoum. The area is made up of 250 villages, together inhabited by about 400,000 individuals. New Halfa is located in the middle of an agricultural scheme with a permanent irrigation system.

2.1.1.3. Kassala

Kassala is located in eastern Sudan, about 405 km east of Khartoum, near the border with Eritrea; it is surrounded by rounded hills. The population of Kassala is estimated to be 149,000.

2.1.2. Haj Yousif (Central Sudan)

Haj Yousif, Sharg Alneel locality, Khartoum State (Figure 2.1), situated some 15 km northeast center of Khartoum. Haj Yousif is inhabited by about 500,000 individuals according to census, 2001. Malaria is mesoendemic and characterized by seasonal, unstable transmission. A small peak of transmission occasionally occurs in December and January (230), otherwise general malaria epidemiology in the area is similar to that in eastern Sudan.

2.2. Assessment of efficacy of antimalarial drug combination

A randomized controlled clinical trial of CQ plus DHA versus CQ plus SP was conducted in two neighboring villages, Daraweesh and Kajara, eastern Sudan, in the period between October 2002 and January 2003. In addition a clinical trial was carried out in Haj Yousif, central Sudan (Alban Jadid hospital), from November 2004 to February 2005, to evaluate the recently adopted national first line curative antimalarial for uncomplicated falciparum malaria in Sudan (AS plus SP).
Figure 2.1: Study areas in Sudan
The WHO in vivo protocol for antimalarial drug efficacy (231), with few modifications that suit the study sites, was followed in recruitment of patients and in evaluating treatment outcome. The modifications included the age of patients and parasite density, where patients in all age groups were enrolled because malaria transmission in the study areas is density unstable and all inhabitants are susceptible, and parasite density was not considered as a criterion for recruitment, since the malaria diagnosis is not based on parasite count in these areas, otherwise many cases would have been excluded.

Patients were observed for 28 days using a standardized antimalarial drug efficacy record form.

2.2.1. Study population

Patients in all age groups were enrolled if they were permanent residents in the study sites, proved to have a thick blood film showing asexual P. falciparum monoinfection, an elevated axillary temperature (≥37.5°C) or a history of fever during the preceding 24 hours, and if an informed consent was signed by adult patients or a parent/guardian for children. However, pregnant women, patients with severe malnutrition, danger signs of severe and complicated malaria defined by WHO guidelines (232), with history of hypersensitivity to any of the studied drugs, concomitant febrile infections, mixed Plasmodium infections or children who cannot swallow tablets were excluded. Criteria for withdrawal were clinical deterioration necessitating hospital referral and patient’s request.

2.2.2. Clinical examinations and diagnosis

At enrolment (Day 0) axillary temperature was measured using a digital electronic thermometer, then a standardized medical history was obtained and physical examination was performed by the study physician. All patients were re-examined on each day of follow up in the clinic, including a clinical assessment for adverse reactions.

Thick and thin blood films were made in duplicate; all slides were stained with 3% Giemsa stain at pH 7.2 and examined by an expert microscopist according to the standard WHO procedure (233). Parasitemia, expressed as the number of asexual parasites / µl blood (P/µl) was determined from thick blood smears. The number of asexual parasites was counted against 200 white blood cells (WBC) or against 500, if the number of parasites was less than 10 per 200 WBC, and then calculated assuming 8000 WBC per µl as mean WBC. The count was stopped (after completing the last
field) if more than 500 parasites have been counted without having reached 200 WBC. A blood film was considered negative when asexual parasites were not detected after the examination of 100 thick film fields. A thin blood smear was also examined to confirm parasite species. If *P. falciparum* gametocytes are seen, a gametocyte count is performed against 1000 WBC. Blood smears taken during patient follow up were examined in the same manner.

### 2.2.3. Samples

Finger prick blood specimens were collected from all enrolled patients on Day 0, on all scheduled follow up days and on any other day if the patient became unwell or developed fever. These were used to prepare thick and thin smears and blood spots on Whatmann 3 mm filter paper, which were dried and kept at room temperature, each sealed in a separate plastic bag until DNA extraction.

### 2.2.4. Treatment

All enrolled patients at Daraweesh and Kajara site (50 patients), were randomly assigned to receive either CQ + DHA (25 patients) or CQ + SP (25 patients) regimens. While those enrolled at Haj Yousif site (120 patients) were treated with AS + SP.

Twenty five mg/kg body weight CQ was administered over 3 days (10 mg/kg on day 0 and day 1, and 5 mg/kg on day 2); SP was administered in a single dose on day 0, based on a pyrimethamine level of 1.25 mg/kg of body weight. Dihydroartemisinin was given at a dose of 2 mg/kg body weight once in the first day, then 1 mg/kg for 6 days, and AS was given as 4 mg/kg/day for three days.

Patients were treated according to the guidelines of the WHO (87,231), all patients were treated orally, and a full treatment dose of each combination was adjusted and co administered simultaneously. All doses were given under direct supervision. After treatment, patients were directly observed for 1 hour, and the full dose was repeated if vomiting occurred. Patients who repeatedly vomited their first dose of study medication were excluded from the study. Patients received paracetamol (10 mg/kg/dose) when needed.

Treatment failure cases were treated with quinine and Artemether + lumefantrine at Daraweesh and Kajara site, and in Haj yousif site, respectively.

### 2.2.5. Clinical and parasitological follow ups

Follow up was scheduled for days 1, 2, 3, 7, 14, 21, and 28. Patients were asked to return to the clinic any time if became unwell or developed fever. During each visit
standardized forms were completed to facilitate the detection of any adverse events, a complete physical examination including axillary temperature was performed, thin and thick blood smears were examined. Blood spots on filter paper were prepared on days 0, 3, 7, 14, 21, and 28 or on any day of recurrent parasitemia after day 3, for molecular analysis.

2.2.6. Treatment outcomes

2.2.6.1. Primary and secondary end points

Primary end points were therapeutic response on the basis of parasitological and clinical cure by day 28, and safety which was based on the incidence of adverse reactions; an adverse reaction was defined as the development of any symptom or sign that did not exist before the initiation of treatment, that was not a classic symptom or sign of malaria, or as sign or symptom worsening during the course of treatment and follow up. Fever clearance time, parasite clearance time, and gametocyte carriage on days 7 and 14 were considered as secondary end points. Fever and parasite clearance time were defined as the time (days) from initiation of treatment to fever and parasite clearance, respectively; fever was judged by temperature of 37.5°C or higher, and by history of fever during the preceding 24 hours. Gametocyte carriage was the proportion of patients with gametocytes, stratified by those with and without gametocytes on day 0.

2.2.6.2. Clinical and parasitological outcomes

Clinical and parasitological responses were classified into four groups:

Early treatment failure (ETF) was defined as the development of one of the following during the first three days of follow up: danger signs or severe malaria on day 1, day 2, or day 3 in the presence of parasitemia; a day 2 parasitemia greater than on day 0 irrespective of axillary temperature or parasitemia on day 3 with axillary temperature \( \geq 37^\circ C \); parasitemia on Day 3 \( \geq 25\% \) of day 0.

Late clinical failure (LCF) was defined as the development of one of the following during the follow up period from day 4 to day 28, without previously meeting any of the criteria of ETF: danger signs or severe malaria in the presence of parasitemia; unscheduled return of the patient because of clinical deterioration in the presence of parasitemia.
Late parasitological failure (LPF), defined as the presence of parasitemia on any of the scheduled days of return (days 7, 14, 21 or 28) and axillary temperature < 37.5ºC without previously meeting any of the criteria of ETF or LCF.

Adequate clinical and parasitological response (ACPR) was defined as the absence of parasitemia on days 7, 14, 21 and 28 without developing any of the criteria of early or late treatment failure.

2.2.7. Molecular genotyping

2.2.7.1. Deoxyribonucleic acid extraction

Parasite DNA was extracted from the blood spots on filter papers using two different methods during different times of this study:

2.2.7.1.1. Qiagen kit

Parasite DNA was extracted using commercial kits (QIAmp blood mini kit, Qiagen, Hilden, Germany) with minor modifications of the manufacturer's instructions.

A small piece of the dried blood spot was cut, after cleaning the scissors and scalpels with sodium hydroxide. The spot was placed into 1.5 ml micro centrifuge tube and incubated for 15 minutes at 85ºC after adding 180 µl of the lysis buffer (ATL). The mixture was incubated with 30 µl of proteinase K stock solution at 56ºC for 75 minutes, and then with 200 µl of the lysis buffer (AL) for 15 minutes at 70ºC, the tube was centrifuged after each incubation to remove drops from inside the lid. The sample was mixed thoroughly with 200 µl ethanol (96-100%) and the mixture was applied to the QIAamp column (in a 2 ml collection tube). QIAamp column was centrifuged two times at 8000 round per minute (rpm) for 1 minute and the QIAamp spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded, the second centrifugation was carried out after adding 500 µl of the washing buffer (AW1). Five hundred µl of the washing buffer (AW2) was added to the QIAamp spin column which was centrifuged again at full speed (14,000 rpm) for 3 minutes and placed in a clean 1.5 micro centrifuge tube, then the column was incubated with 150 µl of the elution buffer (AE) for 3 minutes at room temperature and then centrifuged at 8000 rpm for 1 minute. The filtrate was kept at -20ºC until used.

2.2.7.1.2. Chelex

Extraction of the parasite DNA from the blood spot on filter paper was performed in 96 well plate format, using the saponin/lysis/chelex extraction method developed by
Wooden and others (234) with some modifications described by Pearce and others (235).
Segments of the blood spots were cut using scissors and scalpels which were cleaned after each spot by 95% alcohol and flame. The spots were then transferred to 96 format deep well plates, without using the last column (column 12), 0.8 ml of 0.5% saponin in freshly prepared phosphate buffered saline (PBS) was added to each spot, and the plate was shaken for 10 minutes at 150 rpm and incubated overnight at room temperature. The spot was then washed twice in 0.8 ml of PBS, using a 96 well sucker to remove the supernatant, the sucker was cleaned after each wash with 5 M hydrochloric acid (HCL) followed by water in deep well format plates. The plate was then boiled for 8 minutes, after adding 100 µl of PCR quality water and 50 µl of 20% Chelex suspension in distilled water (pH 9.5) to each spot. The plate was cooled for 10 minutes at room temperature and spinned down at maximum speed for 5 minutes. A part of the supernatant was transferred to a new 96 well plate, leaving chelex in the original deep well plate, both plates were then stored in -20°C for subsequent analysis.

2.2.7.2. Typing of merozoite surface protein 1 and 2 genes
To distinguish recrudescence from new infections molecular genotyping was performed using paired samples from all patients experiencing late clinical or parasitologic failure on follow up days 4–28.
Polymorphic repetitive regions of two *P. falciparum* antigen loci, block 2 of *MSP-1* and block 3 of *MSP-2* were genotyped for allelic variation using nested PCR assays as described previously after minor modifications (236).
Outer PCR assays using primers corresponded to conserved sequences flanking these regions, followed by five separate nested PCR reactions were carried out to amplify the MAD20, K1 and RO33 allelic families of *MSP-1*, the FC27 and the IC1 allelic families of *MSP-2*, outer PCR product and allelic specific oligonucleotide primer pair were used in each nested PCR reaction, the sequences of the primers for both outer and nested reactions are presented in Table 2.1.
All outer and nested PCR reactions, took place in a total volume of 20 µl containing a final concentration of 0.125 µM each primer, 125 µM each of the 4 deoxynucleoside triphosphate (dNTPs); (dATP, dCTP, dGTP and dTTP) and 0.4 units of HotStart Taq polymerase.
To initiate all the outer reactions 1 µl of the extracted DNA was used, and 1 µl of the outer PCR product was used to initiate each of the 5 separate nested reactions.
Genomic DNA from laboratory *P. falciparum* clones 3D7, HB3, and RO33 were used as controls for MAD20, K1 and RO33 *MSP-l* families respectively, and HB3 and 3D7 for FC27 and ICI *MSP-2* families respectively.

All the outer and nested reactions for *MSP-l* and *MSP-2* were performed with an initial denaturation for 2 minutes (min) at 94°C, followed by 30 amplification cycles with annealing at 58°C and extension at 65°C for 2 min for the outer reaction and 1 min for the nested reaction. The last extension step was carried out for 5 min.

Another method was used to perform the outer and nested PCR reactions at the beginning of this study using 26 µl outer PCR amplification mixture contained a final concentration of 0.16 µM of each primer (MWG Biotech, UK), 125 µM each dNTPs (Invitrogen, UK), 1 unit of Taq polymerase (Amersham, UK), 0.1 mg/ml gelatine. A 50 µl nested reaction mixture was prepared as described for the outer reaction with increasing the final concentration of each dNTPs to 175µM.

Two µl of extracted DNA were used as a template in the outer reactions, and 1 µl of the product generated in the first reaction was used in each of the nested reactions.

Outer reactions for *MSP-l* and *MSP-2* were performed with an initial denaturation for 5 min at 94°C, followed by annealing at 58°C for 2 min, and extension at 72°C for 2 min. The last extension was carried out for 10 min. The same cycling conditions were applied to perform the nested reactions after adjusting the annealing temperature to 63°C and the last extension to 5 min.

Thirty five cycles were performed for the outer reactions and 30 cycles for all nested reactions.

**2.2.7.3. Visualization of the amplification product**

Nested PCR products were analyzed by electrophoresis using 1.5% GTG Nusieve agarose gels or metaphorous gels (Cambrex, Germany), stained with ethidium bromide in TBE buffer (100 mM Tris, 100 mM boric acid and 5 mM ethylenediaminetetraacetic acid "EDTA"). A mixture of 8 µl of the amplified DNA and 2 µl of loading buffer were loaded on the gels; paired samples from the same patient were run on adjacent lanes. DNA molecular weight marker; 50 base pair (bp) ladder, XIV (Roche, UK) was run in the first and last lanes. The gel was run in TBE at 60 voltages for about 45 to 60 min. DNA was visualized by ultraviolet transillumination and the gel was photographed.
2.2.7.4. Assessment of treatment outcomes

Treatment outcomes were assessed by comparing genotyping patterns on the day of treatment failure with genotyping patterns on the day of enrolment. Each band assigned a molecular weight within the expected size of \(MSP-1\) and \(MSP-2\) genes, was considered an individual strain.

If all strains present on the day of failure were present on the day of enrolment the treatment failure was classified as true recrudescence. However it is classified as a new infection if none of the strains present on the day of failure were present on the day of enrolment. If the sample on the day of failure contained any of the strains present on the day of enrolment and any strain not present on the day of enrolment the treatment failure was considered as mixed.

2.3. Detection of polymorphisms in sulfadoxine-pyrimethamine and chloroquine resistance marker genes

Studies to detect polymorphisms in the molecular markers of \(P. falciparum\) resistance to \(SP\), \(Pfdhfr\) and \(Pfdhps\) genes and to \(CQ\), \(pfmdrl\) and \(pfcrt\) genes were conducted in different areas.

2.3.1. Parasite deoxyribonucleic acid

Pretreatment parasite isolates used in these studies were obtained from uncomplicated falciparum malaria patients participated in the two clinical trials in Gedaref and Haj Yousif areas, and in other two ongoing malaria treatment studies in New Halfa and Kassala, eastern Sudan. Parasite DNA was extracted as described in section 2.2.7.1. Samples obtained from in New Halfa at Alhara Aloula Health Centre and from in Kassala at Alsawagi Alganoubia Health Centre during the period September to November 2005.

Fifty parasite isolates were obtained from Daraweesh and Kajara site, 108 from Haj Yousif, 79 from New Halfa and 49 from Kassala.

2.3.2. Methods

During the course of this study two different methods were used to detect polymorphisms in the drug resistance marker genes \((Pfdhfr\) and \(Pfdhps\)).

(a) Polymerase chain reaction followed by restriction fragment length polymorphism (RFLP) assay, and (b) PCR followed by enzyme linked immunosorbent assay (ELISA). Polymorphisms in \(Pfcrt\) and \(Pfmdrl\) genes were detected using PCR-RFLP method.
2.3.2.1. Polymerase chain reaction and restriction fragment length polymorphism

Polymorphisms in *Pfdhfr*, *Pfdhps*, *Pfcr1* and *Pfmdr1* were detected using PCR assays; followed by RFLP analysis. DNA samples of known alleles of the above genes were used as positive controls.

Single base changes in the PCR products were detected by RFLP analysis. Restriction digestion was carried out overnight, 5 µl of the PCR product was incubated with mutation specific restriction enzymes in a 15 µl final concentration at the optimum temperature according to supplier protocol (New England Biolab, UK). Digestion products were separated by electrophoresis in an ethidium bromide stained 1.5-3% agarose (Seakem™) gel (Cambrex), 100 bp marker (New England Biolab, UK) were used to size the bands. Mixed alleles (both wild type and mutant alleles present) were considered mutant.

2.3.2.1.1. *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthetase genes

To detect polymorphisms in *Pfdhfr* and *Pfdhps* PCR-RFLP method was carried out as described before (237,238).

A nested PCR method was used to amplify the regions surrounding the polymorphisms in the *Pfdhfr* gene at codons 16, 51, 59, 108 and 164, and those at codons 436, 437, 540, 581, and 613 in the *Pfdhps*. Primer sequences are presented in Table 2.2.

In each outer PCR assay 2 µl DNA were amplified in a 50 µl reaction mixture containing 0.25 µM of each primer (MWG Biotech, Germany), 200 µM of each dNTPs (Invitrogen, UK), 1 unit Taq polymerase (Amersham, UK).

For *Pfdhfr*, amplification was performed for 45 cycles with denaturation at 94°C for 1 min, annealing at 45°C, extension at 72°C; each for 1.5 min. An Initial denaturation was at 94°C for 3 min and a final extension was for 10 min at 72°C. For *Pfdhps* cycling conditions as for *Pfdhfr* but the first five annealing and extension steps were carried out for 2 and 1 min, respectively.

Second round PCR was carried out in volumes of 50 µl, prepared as described for the first round PCR; to amplify 2µl of the amplified DNA. Thirty five cycles were used, cycling conditions were as the first round PCR, but each of the annealing and extension steps were carried out for 1 min.
A 648 bp portion of the \textit{Pfdhfr} gene amplified by using the primers M1 and M5 in the first round PCR was used as a template for two separate second round PCR using F-M4 and M3-F/ primer pairs. Similarly K-K/, K/-J and L-L/ primer pairs were used to carry out three separate second round PCR using as a template 710 bp portion of the \textit{Pfdhps} gene which was amplified in the first round PCR by using R2 and R/ primers. For the \textit{Pfdhfr} gene, the PCR product (522 bp) generated in the amplification with M3-F/ primer pair was used to discriminate between the three alternative forms of codon 108 by digestion with restriction enzymes \textit{AluI}, \textit{BsrI} and \textit{BstNI} to detect serine, asparagine and threonine respectively. In addition the restriction enzyme \textit{NlaIII}, \textit{Tsp509I} and \textit{DraI} were used to discriminate between the polymorphic variants of codon 16 (alanine and valine), codon 51 (asparagine and isoleucine) and codon 164 (isoleucine and leucine), respectively. To examine codon 59 the 329 bp PCR product of the primers M4 and F was digested by \textit{XmnI} restriction enzyme to differentiate between cysteine and arginine variants.

For the \textit{Pfdhps} gene, to distinguish between serine and alanine at codon 436; the 438 bp PCR product given by the primers K and K/ was digested by \textit{MnII}. The PCR product of the primer pair K/-J (436 bp) was digested by \textit{HindIII} to identify the 436-phenylalanine mutation, in the same way PCR product was digested by \textit{MwoI} to distinguish between alanine and glycine at codon 437, and by \textit{FokI} to distinguish between lysine and glutamic acid at codon 540. The 161 bp PCR product produced by the primer pair L-L/ was digested by \textit{BstUI} to discriminate between 581-A and 581-G, the same PCR product was digested by \textit{MwoI} to identify 613-A, and by \textit{AgeI} to differentiate between 613-S and 613-T.

2.3.2.1.2. \textit{Plasmodium falciparum} chloroquine resistance transporter and multidrug resistance genes

Polymerase chain reaction followed by RFLP analysis was used to detect the two alleles of \textit{Pfcrt} gene at codon 76 (146) and of \textit{Pfmdr1} at codon 86 (144,239). Primer sequences are shown in Table 2.3.

A primary round of PCR using flanking primers TCRP1 and TCRP2 and 2 µl DNA to amplify a 537 bp region around the mutation K76T was performed, in the second round, TCRD1 and TCRD2 amplified a 134 bp portion of the \textit{Pfcrt} gene. For both Primary and secondary PCR a reaction volume of 50 µl contained a final concentration of 1 µM of each primer, 200 µM of each dNTPs, 2.5 mM MgCl\textsubscript{2} and 1.5 units of Taq polymerase was used. The first and second reactions conditions
consisted of an initial denaturation step of 5 min at 94°C followed by amplification cycles each consisted of denaturation for 30 seconds at 94°C, and annealing for 30 seconds at 56°C and 48°C for the first and second reaction respectively, extension for 1 min at 60°C and final extension at 60°C for 3 min. Forty five cycles were performed for the first reaction and 30 cycles for the second reaction.

Restriction enzyme Apo1 which indicate the wild type at codon 76 (76K) was used to digest the 134 bp PCR product.

A 330 bp DNA fragment of the Pfmdrl gene was amplified under the following conditions: 3 µl of DNA template, 0.5 µM of the primers Pfmdr1–1 and Pfmdr1–2, 200 µM dNTP and one unit of HotStart Taq polymerase in a 50 µl reaction. Denaturation at 95°C was performed for 16 min, followed by 45 cycles of denaturation at 94°C for 45 seconds, annealing at 49°C for 1 min, and extension at 72°C for another min, with final extension at 72°C for 10 min.

To identify the mutant type tyrosine (86Y) the amplification product was digested by AflIII. Apo1 enzyme was used to confirmed mixed alleles.

2.3.2.2. Polymerase chain reaction and enzyme linked immunosorbent assay

Single nucleotide polymorphisms in the Pf dhfr and Pf dhps genes were detected using PCR and ELISA as described by Alifrangis and others in 2003 (240).

2.3.2.2.1. Amplification of Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthetase genes

Fragments of Pf dhfr (594 bp) and Pf dhps (711 bp) which contain the coding sequence where known sites of sequence polymorphism are found, namely codons 50, 51, 59, 108, 164 of the Pf dhfr gene, and codons 436, 437, 540, 581, 613 of the Pf dhps gene, were amplified in a 96 well plate format by independent nested PCR (236,241). PCR primers are indicated in Table 2.4, M9 and R/ primers for Pf dhfr and Pf dhps nested PCR were biotinylated at the 5’ end by the supplier (MWG, Biotech, Riskov, Denmark).

The 20 µl of each of Pf dhfr, Pf dhps outer PCR mixture contained 0.25 µM final concentration of either set of the primers M1/M7 or N1/N2 of Pf dhfr and Pf dhps respectively, 0.3 mM of each dNTPs and 1 unit HotStart Taq polymerase (Qiagen, Albertslund, Denmark), 1 µl of the template DNA was added to the outer reaction mixture. A standardized control panel of P. falciparum isolates which represent almost all known SNPs of Pf dhfr and Pf dhps were added in column 12. For nested PCR 1 µl of each the Pf dhfr and Pf dhps outer PCR product was added to 25 µl PCR
reaction mixture which was the same as the outer PCR mixture, with decreasing the concentration of each dNTP to 0.2 mM and using primer set M3b/M9 (Pfdhfr) and R2/R/ (Pfdhps).

Each of Pfdhfr and Pfdhps outer PCR was carried out with an initial incubation for 2 min at 94°C, followed by a total of 40 cycles with denaturation at 94°C for 1 min, annealing at 52°C for 2 min and elongation at 65°C for 1 min and final extension for 10 min at 65°C. Nested PCR cycling conditions for Pfdhfr were 94°C for 2 min followed by 5 cycles (94°C/1 min, 44°C/2 min and 65°C/1 min) followed by 35 cycles (94°C/1 min, 44°C/1 min and 65°C/1 min). However Pfdhps nested PCR was performed with starting incubation in 94°C for 3 min and 40 cycles (94°C/1 min, 51°C/2 min and 65°C/1 min) and extension at 65°C for 10 min.

2.3.2.2.2. Sequence specific oligonucleotide probes - enzyme linked immunosorbent assay

The ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with streptavidin in PBS (1 µg/ml), covered, and left overnight at 4°C.

The nested PCR products were diluted 1:10 in water in a 96 well PCR plate, denatured at 95°C for 5 min, and immediately cooled to 4°C until use.

To enable simultaneous probing with sequence specific oligonucleotide probes (SSOPs) targeting the full panel of Pfdhfr and Pfdhps SNP haplotypes, replicate of the coated ELISA plates were washed three times in washing buffer (PBS containing 0.05% Tween 20). The plates were then incubated for one hour at room temperature; with 100 µl of cold washing buffer and 2 µl of the diluted PCR products in each well and then washed three times in washing buffer.

The 3´end digoxigenin conjugated SSOPs (MWG Biotech, Denmark), (Table 2.5) were diluted in tetramethyl ammonium chloride (TMAC; Sigma Aldrich, Germany) solution (3 M TMAC, 50 mM Tris, pH 8.0, 0.1% sodium dodecyl sulfate, 2 mM EDTA, pH 8.0) to 20 nM, 4 nM final concentration of 50/51 CN/C1 and each of the other used SSOPs respectively, the mixtures were heated to 53°C, and 100 µl was added to each well.

The plates were then incubated in a hybridization oven (AH Diagnostics, Aarhus, Denmark) at 53°C on a shaking device for one hour, and washed three times in washing buffer. This was followed by two rounds of a stringent washing and incubation for 10 minutes per round in TMAC solution at the temperatures indicated in Table 2.5. The plates were then washed three times in washing buffer to remove
TMAC, and peroxidase-conjugated anti-digoxigenin antibody in dilution buffer (1:1,000) (Roche Diagnostics, Mannheim, Germany) was added to each well and incubated for one hour at room temperature. After washing the plates three times in washing buffer, 100 µl an o-phenylenediamine solution of 1.5 mg/ml of 1,2-phenyldiamine dihydrochloride (Dako, Glostrup, Denmark) dissolved in water containing 0.015% H₂O₂ was added to each well. The reaction was stopped after 30 minutes by adding 1.25 M H₂SO₄ and the optical density (OD) was measured in an ELISA reader at 492 nm.

2.3.2.2.3. Scoring of enzyme linked immunosorbent assay results

To score the ELISA results, a simple analysis test for the positive and negative control samples was performed to set a threshold for positivity for each SNP test. For each SNP analysis parasite samples were categories into single genotype when only one SNP was present at OD value above the threshold of positivity. Mixed with a dominant SNP genotype single genotype was considered when the OD value of the weakly reacting SSOP was less than half the OD value of the strongly reacting SSOP. The infections was categorized as mixed with no dominant genotype, if the OD value of the weakly reacting SSOP was higher than half the OD value of the strongly reacting SSOP.

For samples that contained infections categorized as single or mixed with a dominant SNP type, at all analyzed codons, results were combined to construct haplotypes.

2.4. Statistical Analysis

Statistical analysis was performed using Statistical package for Social Science (SPSS) software version 11. Qualitative data were compared using Pearson’s chi-square or Fisher’s exact test. Mean values of normally distributed continuous data were compared using Student’s t test and one way analysis of variance. Data not conforming to a normal distribution were compared by the Mann-Whitney U test or the Kruskal-Wallis one-way analysis of variance on ranks. Dependence of quantitative values was controlled by correlation. A P value < 0.05 was considered significant.

2.5. Ethical considerations

The protocols of the studies were reviewed and approved by the Ethics committee, Ministry of Health, Khartoum, Sudan or by the Ethical Committee, Faculty of Medicine, University of Khartoum. Informed consent was obtained before enrolment from each patient or legal guardians of minors. The recruited subjects were informed
that they were free to withdraw their consent at any time during the study. Treatment was provided free of charge. A medical team was available in the study sites on daily basis during the study period, and the patients were under close medical supervision.
Table 2.1: Polymerase chain reaction primer sequences used for genotyping of merozoite surface protein -1 and 2 (MSP-I and MSP-2)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP-I</td>
<td>M1 – OF</td>
<td>5’-CTAGAAGCTTTAGAAGATGCAAGTATGTTT-3’</td>
</tr>
<tr>
<td></td>
<td>M1 – OR</td>
<td>5’-CTTAAATAGTATTCTAATTTCAAGTTTGGATCA-3’</td>
</tr>
<tr>
<td></td>
<td>M1 – KF</td>
<td>5’-AAATGAAGAGGAAATTACTACAAAAAGGTGC-3’</td>
</tr>
<tr>
<td></td>
<td>M1 - KR</td>
<td>5’-GCTTGCAUGCTGGAGGGGCTTGACCAGA-3’</td>
</tr>
<tr>
<td></td>
<td>M1 – MF</td>
<td>5’-AAATGAAGGCAAGTGGAAACAGCTGTAC-3’</td>
</tr>
<tr>
<td></td>
<td>M1 – MR</td>
<td>5’-ATCTGAAGGATTTGTACGGTCTGATTAC-3’</td>
</tr>
<tr>
<td></td>
<td>M1 – RF</td>
<td>5’-TAAGGATGGAGCAATCTCAAGTTTGTG-3’</td>
</tr>
<tr>
<td></td>
<td>M1 - RR</td>
<td>5’-CATCTGAAGGATTTGCAACCTGGAGATC-3’</td>
</tr>
<tr>
<td>MSP-2</td>
<td>M2 – OF</td>
<td>5’-ATGAAGGTAAATTAAAAACATTGTCTATTATA-3’</td>
</tr>
<tr>
<td></td>
<td>M2 – OR</td>
<td>5’-ATTGTTACCATCGTACATTCTTT-3’</td>
</tr>
<tr>
<td></td>
<td>M2 – FCF</td>
<td>5’-AATACGAGGTAGTGGCARATGCTCCA-3’</td>
</tr>
<tr>
<td></td>
<td>M2 – FCR</td>
<td>5’-TTTATTGTTGATGGCAGAATTGGAAC-3’</td>
</tr>
<tr>
<td></td>
<td>M2 – ICF</td>
<td>5’-AGAAGTATGGAGAAAGTAAKCCCTACT-3’</td>
</tr>
<tr>
<td></td>
<td>M2 - ICR</td>
<td>5’-GATTGTAATTCGGGAGATTCTACT-3’</td>
</tr>
</tbody>
</table>
Table 2.2: Sequences of the primers used for the detection of polymorphisms in the *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) genes using polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pfdhfr</em></td>
<td>M1 5’-TTTATGATGGAACAAGTCTGC-3’</td>
</tr>
<tr>
<td></td>
<td>M5 5’-AGTATATACATCGCTAACAGA-3’</td>
</tr>
<tr>
<td></td>
<td>M3 5’-TTTATGATGGAACAAGTCTGCAAGT-3’</td>
</tr>
<tr>
<td></td>
<td>F/ 5’-AAATTCTTGATAACAAACGGAACCTttTA-3’</td>
</tr>
<tr>
<td></td>
<td>F 5’-GAAATGTAATTCCTAGATATGGAATATT-3’</td>
</tr>
<tr>
<td></td>
<td>M4 5’-TTAATTTCCCAAGTAAAACTATATAGAGCTTC-3’</td>
</tr>
<tr>
<td><em>Pfdhps</em></td>
<td>R2 5’-AACCTAAACGTGCTGTTCAA-3’</td>
</tr>
<tr>
<td></td>
<td>R/ 5’-AATTGTGATTTGTCCACAA-3’</td>
</tr>
<tr>
<td></td>
<td>K 5’-TGCTAGTGTTATAGGATATAGGATGAGCATC-3’</td>
</tr>
<tr>
<td></td>
<td>K/ 5’-CTATAACGAGGTATTGCATTTAATGCAAGAA-3’</td>
</tr>
<tr>
<td></td>
<td>J 5’-TGCTAGTGTTATAGGATATAGGATGAGCATC-3’</td>
</tr>
<tr>
<td></td>
<td>L 5’-ATAGGATACACTATTTGATATTGGACCAGGATTCG-3’</td>
</tr>
<tr>
<td></td>
<td>L/ 5’-TATTACAACATTTTGATATTGCAGCAACCCG-3’</td>
</tr>
</tbody>
</table>
Table 2.3: Primer sequences used for the amplification of *Plasmodium falciparum* chloroquine resistance transporter (*Pfcrt*) and multidrug resistance (*Pfmdr1*) genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRP1</td>
<td>5’-CCG TTA ATA ATA AAT ACA CGC AG-3’</td>
</tr>
<tr>
<td>TCRP2</td>
<td>5’-CGG ATG TTA CAA AAC TAT AGT TAC C-3’</td>
</tr>
<tr>
<td>TCRD1</td>
<td>5’-TGT GCT CAT GTG TTT AAA CTT-3’</td>
</tr>
<tr>
<td>TCRD2</td>
<td>5’-CAA AAC TAT AGT TAC CAA TTT TG-3’</td>
</tr>
<tr>
<td>pfmdr1–1</td>
<td>5’-AGA TGG TAA CCT CAG TAT CA-3’</td>
</tr>
<tr>
<td>pfmdr1–2</td>
<td>5’-TTA CAT CCA TAC AAT AAC TTG-3’</td>
</tr>
</tbody>
</table>
Table 2.4: Primer sequences used for the detection of polymorphisms in *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) genes using polymerase chain reaction and enzyme linked immunosorbent assay

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pfdhfr</em></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>5’-TTTATGATGGAACAAAGTCTGC-3’</td>
</tr>
<tr>
<td>M7</td>
<td>5’-CTAGTATAGTAGCTGCTAACA-3’</td>
</tr>
<tr>
<td>M3b</td>
<td>5’-TGATGGAACAAAGTCTGCAGCTG-3’</td>
</tr>
<tr>
<td>M9</td>
<td>5’-CTGGAAAAAATACATCACCATCATTGCATG-3’</td>
</tr>
<tr>
<td><em>Pfdhps</em></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>5’-GATTCTTTTTTCAGATGGAGG-3’</td>
</tr>
<tr>
<td>N2</td>
<td>5’-TTCCTCATGTAATTCACTCTGA-3’</td>
</tr>
<tr>
<td>R2</td>
<td>5’-AACCTAAAACGTGCTGTCAAA-3’</td>
</tr>
<tr>
<td>R</td>
<td>5’-AATTGTGTGATTGTCACACAA-3’</td>
</tr>
</tbody>
</table>
Table 2.5: Sequences of the specific sequence oligonucleotide probes (SSOP) used for the *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) polymerase chain reaction and enzyme linked immunosorbent assay

<table>
<thead>
<tr>
<th>SSOP (temperature)</th>
<th>SSOP sequence</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pfdhfr</em> 50/51CN 58°C</td>
<td>TGG AAA <strong>TGT</strong> AAT TCC CTA</td>
<td>3D7, FCR3, K1, HB3</td>
</tr>
<tr>
<td>50/51CI 58°C</td>
<td>TGG AAA <strong>TGT</strong> ATT TCC CTA</td>
<td>DD2, 7G8</td>
</tr>
<tr>
<td>59C 60°C</td>
<td>AAT ATT <strong>TTT</strong> GTG CAG TTA</td>
<td>3D7, FCR3, 7G8, HB3</td>
</tr>
<tr>
<td>59R 60°C</td>
<td>AAT ATT <strong>TTC</strong> GTG CAG TTA</td>
<td>DD2, K1</td>
</tr>
<tr>
<td>108S 62°C</td>
<td>A AGA ACA <strong>AGC</strong> TGG GAA AG</td>
<td>3D7</td>
</tr>
<tr>
<td>108N 62°C</td>
<td>A AGA ACA <strong>AAC</strong> TGG GAA AG</td>
<td>DD2, K1, HB3, 7G8</td>
</tr>
<tr>
<td>108T 62°C</td>
<td>A AGA ACA <strong>ACC</strong> TGG GAA AG</td>
<td>FCR3</td>
</tr>
<tr>
<td>164I 62°C</td>
<td>GT TTT ATT <strong>ATA</strong> GGA GGT T</td>
<td>3D7, FCR3, DD2, 7G8, K1, HB3</td>
</tr>
<tr>
<td>164L 62°C</td>
<td>GT TTT ATT <strong>TTA</strong> GGA GGT T</td>
<td>Patient's isolates (TN518, TN542)</td>
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<tr>
<td><em>Pfdhps</em> 436/437AA 60°C</td>
<td>GAA TCC <strong>GCT</strong> GCT CCT TTT</td>
<td>Patient's isolate (AA J.A)</td>
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<td>GAA TCC <strong>GCT</strong> GGT CCT TTT</td>
<td>Patient's isolate (TN518)</td>
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<td>FCR3, HB3</td>
</tr>
<tr>
<td>436/437SG 60°C</td>
<td>GAA TCC <strong>TCT</strong> GGT CCT TTT</td>
<td>3D7, 7G8, K1</td>
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<tr>
<td>436/437FG 60°C</td>
<td>GAA TCC <strong>TTT</strong> GGT CCT TTT</td>
<td>DD2</td>
</tr>
<tr>
<td>540K 60°C</td>
<td>ACA ATG GAT <strong>AAA</strong> CTA ACA</td>
<td>3D7, FCR3, DD2, K1, 7G8, Hb3</td>
</tr>
<tr>
<td>540E 60°C</td>
<td>ACA ATG GAT <strong>GAA</strong> CTA ACA</td>
<td>Patient's isolates (TN518, TN542)</td>
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<td>A GGA TTT <strong>GCG</strong> AAC AAA CA</td>
<td>3D7, FCR3, DD2, 7G8, HB3</td>
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<tr>
<td>581G 62°C</td>
<td>A GGA TTT <strong>GGG</strong> AAC AAA CA</td>
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<tr>
<td>613S 62°C</td>
<td>GA TTT ATT <strong>TCC</strong> CAT TGC</td>
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3. Results

3.1. Efficacy of chloroquine plus sulfadoxine-pyrimethamine and chloroquine plus dihydroartemisinin

3.1.1. Treatment outcomes

A total of 50 patients were included in the study, these were most of the patients who had developed uncomplicated *P. falciparum* malaria during the malaria season in Daraweesh and Kajara villages. Baseline characteristics of the patients are shown in Table 3.1.

Twenty five patients were treated with CQ+DHA, and 25 were treated with CQ+SP. Forty six patients completed the 28 days follow up, and had a treatment outcome that could be evaluated, four patients withdrew from the study after completing the treatment doses (three patients refused to continue and one patient had left the village).

Table 3.1: Baseline characteristics of uncomplicated falciparum malaria patients treated with chloroquine plus dihydroartemisinin (CQ+DHA) and with chloroquine plus sulfadoxine-pyrimethamine (CQ+SP) in Daraweesh and Kajara villages, eastern Sudan

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CQ+DHA</th>
<th>CQ+SP</th>
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</thead>
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<tr>
<td>Median ± 25th; 75th age in years</td>
<td>12 ± 8; 20.5</td>
<td>18 ± 9; 22</td>
</tr>
<tr>
<td>Children: adults (%)</td>
<td>56:44</td>
<td>44:56</td>
</tr>
<tr>
<td>Male: female (%)</td>
<td>56:44</td>
<td>56:44</td>
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<tr>
<td>History of previous antimalarial (%)</td>
<td>92</td>
<td>88</td>
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<tr>
<td>Mean ± SE body weight (kg)</td>
<td>33.6 ± 3.01 (45)</td>
<td>34.64 ± 2.86</td>
</tr>
<tr>
<td>Mean ± SE axillary temperature (°C)</td>
<td>37.87 ± 0.18 (3.1)</td>
<td>37.41 ± 0.17</td>
</tr>
<tr>
<td>Median ± 25th; 75th parasitemia (/µl)</td>
<td>8760 ± 1380; 16000</td>
<td>14800 ± 2880; 20000</td>
</tr>
<tr>
<td>Proportion with gametocytes (%)</td>
<td>28</td>
<td>12.5</td>
</tr>
</tbody>
</table>

3.1.1.1. Primary outcomes

All patients tolerated the two regimens well, no adverse reactions were reported. In approximately one third of the patients treatment failed with no significant difference in cure rates between the two treatment groups; (CQ + DHA, 68.2% (15/22); CQ +
SP, 62.5% (15/24), P= 0.46. Early treatment failure (ETF) was not observed, LPF occurred slightly more frequently than LCF in both groups (Table 3.2). Of the 16 patients experiencing treatment failure, recrudescence was observed in 15 patients (93.8%); and treatment failure was due to reinfection in one patient.

Table 3.2: Treatment outcome of the two studied antimalarial drug combinations in Daraweesh and Kajara villages, eastern Sudan

<table>
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<tr>
<th>Antimalarial drug combination</th>
<th>Treatment outcome</th>
<th>Total treatment failure</th>
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<tr>
<td></td>
<td>Percentage (Number)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACPR</td>
<td>LCF</td>
</tr>
<tr>
<td>Crude PCR adjustment</td>
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<tr>
<td>CQ plus DHA</td>
<td>68.2</td>
<td>13.6</td>
</tr>
<tr>
<td>(15)</td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>CQ plus SP</td>
<td>62.5</td>
<td>12.5</td>
</tr>
<tr>
<td>(15)</td>
<td>(3)</td>
<td>(6)</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>6</td>
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</table>

- CQ = Chloroquine
- DHA = Dihydroartemisinin
- SP = Sulphadoxine-pyrimethamine
- ACPR = Adequate clinical and parasitological response
- LCF = Late clinical failure
- LPF = Late parasitological failure

3.1.1.2. Secondary outcomes

Fever clearance time (median ± 25th; 75th) was 1 day ± 1; 1.5 in both groups; range was 2 and 6 in CQ+DHA and CQ+SP regimen, respectively.

Asexual parasitemia was cleared more rapidly in patients receiving CQ+DHA compared to CQ+SP (median parasite clearance time ± 25th; 75th) were 1 ± 1; 2 and 2 ± 1; 2, respectively. On day 1 of treatment prevalence of asexual parasitemia, was reduced to a larger extent in the CQ+DHA group (72%) compared to CQ+SP (28%) group. By day 3, 1/23 (4.3%) and 2/24 (8.3%) patients were parasitemic among the CQ+DHA and CQ+SP group, respectively (Figure 3.1). Among patients treated with CQ+DHA; day 0 median parasite density was 8,760 P/µl (range = 25920), it was 200
P/µl (range = 10200) and 360 (range = 680) on day 1 and 2, respectively. Among those treated with CQ+SP; median parasite density was 14,800 P/µl blood (range = 103440), 2040 P/µl blood (range = 17320) and 760 P/µl (range = 240) on day 0, day 1 and day 2.

![Diagram](image)

**Figure 3.1:** Proportion of parasitemic patients following treatment with chloroquine plus dihydroartemisinin (CQ+DHA) and with chloroquine plus sulfadoxine-pyrimethamine (CQ+SP) in Daraweesh and Kajara villages, eastern Sudan

In CQ+DHA regimen; at baseline the proportion of patients with gametocytes was 28% (7 of 25). Of these, 5 (71.4%) and 3 (42.9%) were still gametocytemic on day 7 and 14, respectively, no gametocytes were detected on day 28. While in CQ+SP regimen; the proportion at baseline was 12% (3 of 25). All of these were still gametocytemic on day 7 and 14, on day 28 two of them were gametocytemic (Figure 3.2). Among the patients who had no gametocytes at baseline, in CQ+DHA regimen; two patients were gametocytemic on day 7. While in CQ+SP regimen; 9 and 2 patients were gametocytemic on day 7 and 14, respectively.
Figure 3.2: Proportion of gametocytes carriers among patients treated with chloroquine plus dihydroartemisinin (CQ+DHA) and with chloroquine plus sulfadoxine-pyrimethamine (CQ+SP) in Daraweesh and Kajara villages, eastern Sudan

3.1.2. Polymorphisms in \textit{Plasmodium falciparum} dihydrofolate reductase, dihydropteroate synthetase, chloroquine resistance transporter and multidrug resistance genes in Daraweesh and Kajara Villages

No mutations were detected in \textit{Pfdhfr} at codons 16, 59, or 164 (Table 3.3). For the other two codons, 51 and 108, the percentage of mutations was 84% (42/50) each, and there were no mixed wild/mutant infections (Figure 3.3). There was strong linkage between the two SNPs (P $\leq$ 0.001), where all isolates had mutation at codon 51 also had mutation at codon 108, and vise versa.

Similarly, SNPs in five codons of \textit{Pfdhps} were investigated. Codons 436, 437, and 613 all contain wild type (Table 3.3). High frequency of mixed wild/ mutant alleles for \textit{Pfdhps} K540E was seen in 80% (39/49) of the examined parasites. Mutant \textit{Pfdhps}
581G allele occurred in 20% of the examined isolates. No association between \textit{pf}\textit{dhps} 540E and 581G (P = 0.14) was observed.

As shown in Table 3.3, the frequency of the pure mutant allele \textit{Pfcrt} 76T was 66% (33/50). However, considering mixed infections with both wild type and mutant alleles as mutant this frequency was 90%. The frequency of the mutant \textit{Pfmdr1} 86Y allele was 86% (43/50); including one isolate of mixed \textit{Pfmdr1} 86N/Y alleles.

Among the 45 isolates with the \textit{Pfcrt} 76T mutation, 42 (93%) also carried the mutant \textit{Pfmdr1} 86Y allele. In contrast, only one (20%) of the five parasites with the wild type \textit{Pfcrt} 76K allele exhibited the \textit{Pfmdr1} 86Y mutation. Likewise, 42 of the 43 (97.7%) isolates with \textit{Pfmdr1} 86Y also had the mutant \textit{Pfcrt} 76T allele, whereas only 3 (42.9%) of the 7 with \textit{pfmdr1} wild type isolates exhibited \textit{Pfcrt} 76T (Table 3.4). Whether or not the mixed infections were excluded from the analysis, the association between the two loci was significant (P = 0.001).

As shown in Table 3.4, all 42 isolates carrying both, \textit{Pfcrt} 76T and \textit{Pfmdr1} 86Y also exhibited the \textit{Pfdhfr} 51I and 108N mutations (P < 0.001). The frequency of the mutant \textit{Pfdhps} 540E allele was significantly associated with \textit{Pfcrt} 76T and \textit{Pfmdr1} 86Y; P = 0.001, P = 0.009, respectively. In contrast, the \textit{Pfdhps} 581G variant occurred in these isolates in 21.4% (9/42) only. The four isolates with \textit{Pfcrt} and \textit{Pfmdr1} wild type alleles also exhibited wild type codons for \textit{Pfdhfr} and \textit{Pfdhps}. The reverse, however, was not true since three of the eight isolates with \textit{Pfdhfr} wild type alleles contained \textit{Pfcrt} 76T, and one \textit{Pfmdr1} 86Y (Table 3.4).

There was no association between the treatment outcome and the prevalence of the different mutations for any of tested drug resistance genes/codons in both treatment arms (P = 0.5-1).
Table 3.3: Frequency of wild, mutant and mixed alleles of *Plasmodium falciparum*; chloroquine transporter (*Pfcrt*), multidrug resistance (*Pfmdr1*), dihydrofolate reductase (*Pfdhfr*) and dihydprteroate synthetase (*Pfdhps*) genes, in 50 parasite isolates obtained from Daraweesh and Kajara, eastern Sudan

<table>
<thead>
<tr>
<th>Genotype</th>
<th><em>Pfcrt</em></th>
<th><em>Pfmdr1</em></th>
<th><em>Pfdhfr</em> gene</th>
<th><em>Pfdhps</em> gene</th>
</tr>
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<tr>
<td></td>
<td>Codon</td>
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<td>A16V</td>
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<td>Mutant</td>
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<td>0.14</td>
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<td>0.84</td>
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<td>Mixed</td>
<td>0.24</td>
<td>0.02</td>
<td>0</td>
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Table 3.4: Polymorphism profile of *Plasmodium falciparum*; chloroquine transporter (*Pfcrt*), multidrug resistance (*Pfmdr1*), dihydrofolate reductase (*Pfdhfr*) and dihydrpteroate synthetase (*Pfdhps*) genes of the 50 parasite isolates obtained from Daraweesh and Kajara, eastern Sudan

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<th><em>Pfmdr1</em>-86</th>
<th><em>Pfdhfr</em>-51</th>
<th><em>Pfdhfr</em>-108</th>
<th><em>Pfdhps</em>-540</th>
<th><em>Pfdhps</em>-581</th>
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</table>

\[\text{Mutan} = \text{Mutan} \]

Mixed = presence of wild and mutant allele
3.2. Efficacy of artesunate plus sulfadoxine-pyrimethamine

3.2.1. Treatment outcomes

To evaluate efficacy of AS plus SP in Haj Yousif area, Khartoum State, a total of 118 patients with uncomplicated falciparum malaria were enrolled in the study. Baseline characteristics are summarized in Table 3.5. A total of 108 patients were monitored for 28 days, 10 patients (8.5%) were dropped from the analysis, because of either: loss to follow-up (4), travel from the study area (3), self medication with other antimalarial during the follow up period (1), unwillingness to participate in the study (1), or death (1).

Table 3.5: Baseline characteristics of uncomplicated falciparum malaria patients treated with artesunate plus sulfadoxine-pyrimethamine in Haj Yousif area, Khartoum State

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median ± 25th; 75th</th>
</tr>
</thead>
<tbody>
<tr>
<td>age in years</td>
<td>18 ± 11; 28</td>
</tr>
<tr>
<td>Children: adults (%)</td>
<td>32.4: 67.6</td>
</tr>
<tr>
<td>Male: female (%)</td>
<td>59.3: 40.7</td>
</tr>
<tr>
<td>History of previous antimalarial (%)</td>
<td>87</td>
</tr>
<tr>
<td>Mean ± SE body weight (kg)</td>
<td>46.8 ± 1.9</td>
</tr>
<tr>
<td>Median ± 25th; 75th axillary temperature (ºC)</td>
<td>38.5 ± 37.9; 39.1</td>
</tr>
<tr>
<td>Median ± 25th; 75th parasitemia (/µl)</td>
<td>10920 ± 4650; 20780</td>
</tr>
<tr>
<td>Proportion with gametocytes (%)</td>
<td>6.5</td>
</tr>
</tbody>
</table>

3.2.1.1. Primary outcomes

Before PCR correction, of the 108 patients who completed follow up to day 28, 97 patients (89.8%) had an ACPR. Overall treatment failure by day 28 was 10.2% (11). All treatment failures, except one, were LTF that occurred between days 14 and 28 (Table 3.6).

PCR corrected results using paired samples of day 0 and the day of recrudescence showed genetic heterogeneity (new infections) in two out of the 10 cases. In addition to heterogeneity, no heterogeneity was detected in the other 8 pairs, which suggested the presence of recrudescence together with new parasite populations. The
polymerase chain reaction corrected cure rate was 91.7%. No adverse events that could have been related to antimalarial medication were reported.

Table 3.6. Crude and genotyping adjusted clinical and parasitological response of uncomplicated falciparum malaria to artesunate plus sulfadoxine-pyrimethamine in Haj Yousif area

<table>
<thead>
<tr>
<th>Treatment outcome</th>
<th>Number (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude</td>
<td>PCR adjusted</td>
</tr>
<tr>
<td>Early treatment failure</td>
<td>1 (0.9)</td>
<td>01 (0.9)</td>
</tr>
<tr>
<td>Late clinical failure</td>
<td>7 (6.5)</td>
<td>05 (4.6)</td>
</tr>
<tr>
<td>Late parasitological failure</td>
<td>3 (2.8)</td>
<td>03 (2.8)</td>
</tr>
<tr>
<td>Total failures</td>
<td>11 (10.2)</td>
<td>9 (8.3)</td>
</tr>
<tr>
<td>Adequate clinical and parasitological response (ACPR)</td>
<td>97 (89.8)</td>
<td>99 (91.7)</td>
</tr>
<tr>
<td>Total</td>
<td>108 (100)</td>
<td>108 (100)</td>
</tr>
</tbody>
</table>

3.2.1.2. Secondary outcomes

Fever clearance time (median ± 25\textsuperscript{th}; 75\textsuperscript{th}) was 1 day ± 1; 1. All evaluated patients (107) except the patient classified as ETF were afebrile by day 3. Whereas 27 (25.2%) and one patient (4.7%) had fever on day 1 and 2, respectively (Figure 3.3).

![Figure 3.3](image.png)

Figure 3.3. Proportion of febrile malaria patients during the 28 days following artesunate plus sulfadoxine-pyrimethamine treatment in Haj Yousif
Parasite clearance time (median ± 25th; 75th) was 1±1; 1 day, 11.2% (12 of 107) of patients were parasitemic by day 1, and only 3.7% (4 of 107) by day 2. All the evaluated patients were aparasitaemic by day 3 (Figure 3.4). The day 0 median parasite density was 10920 P/µl (109040), it decreased to 480 P/µl (8800) by day 1 and to 180 P/µl by day 2 (1960).

![Graph showing parasitemia](image)

**Figure 3.4: Proportion of patients with asexual *Plasmodium falciparum* parasitemia during 28 days following artesunate plus sulfadoxine-pyrimethamine treatment in Haj Yousif**

The overall gametocyte prevalence decreased gradually during the follow up period: At baseline, the proportion of patients with gametocytes was 6.5% (7 of 107). Of these, 3 (42.9%) and 2 (28.6%) were still gametocytemic on day 7 and 14, respectively. No gametocytes were detected on day 21 or 28 (Figure 3.5). Among the patients who had no gametocytes at baseline, only one patient was gametocytemic on day 7.
Figure 3.5: Proportion of patients with *Plasmodium falciparum* gametocytemia during the 28 days following artesunate plus sulfadoxine-pyrimethamine treatment in Haj Yousif

3.2.2. Distribution of *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthetase haplotypes among the study population in Haj Yousif

Single nucleotide polymorphisms in *Pfdhfr* and *Pfdhps* genes were determined in 86 and 91 isolates, respectively.

Mutations in *Pfdhfr* were detected at positions 51 (51I) and 108 (108N) only and in *Pfdhps* at positions 436/437 (436A/437G), 540 (540E) and 581 (581G). No polymorphisms were found at *Pfdhfr* positions 59 and 164 or at *Pfdhps* position 613 (Table 3.7). In table 3.7, the *Pfdhfr* and *Pfdhps* is shown as constructed haplotypes: The double mutant *Pfdhfr* haplotype (CICNI) was the most frequent, existed in 96.5% (83) of the infections. While the single *Pfdhfr* mutant haplotype (CNCNI) was found in 2.3% (2) of the infections, and only one infection had the wild type *Pfdhfr* haplotype (CNCSI). In contrast, *Pfdhps* mutant haplotypes were detected at a low
frequency (9.9%), only two infections (2.2%) had more than one Pf\textit{dhs} mutation, containing the combination of 437G/540E. \textit{Plasmodium falciparum} \textit{dhps} single mutant haplotypes was detected in 7.7% (7) of the infections.

Table 3.7: Distribution of \textit{Plasmodium falciparum} dihydrofolate reductase (\textit{Pfdhfr}) and dihydropteroate synthetase (\textit{Pfdhps}) haplotypes in Haj Yousif

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>\textit{Pfdhfr} 50/51/59/108/164 (N = 86)</th>
<th>\textit{Pfdhps} 436/437/540/581/613 (N = 91)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI-CN-I</td>
<td>91.9% (79)</td>
<td>SAKAA 90.1 (82)</td>
</tr>
<tr>
<td>MC-CN-I</td>
<td>4.7% (4)</td>
<td>SAEAA 3.3 (3)</td>
</tr>
<tr>
<td>CN-CN-I</td>
<td>2.3% (2)</td>
<td>SGKAA 2.2 (2)</td>
</tr>
<tr>
<td>CN-CS-I</td>
<td>1.2% (1)</td>
<td>MKAA 2.2 (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SGEAA 1.1 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SGEGA 1.1 (1)</td>
</tr>
</tbody>
</table>

- Underline = Mutation

Combination of the dominant double mutant \textit{Pfdhfr} haplotype (CI-CN-I) and the mutant \textit{Pfdhps} haplotype (SGEAA) 437G/540E was detected in two isolates (2.6%), shown in Table 3.8.

Table 3.8: Combinations of \textit{Plasmodium falciparum} dihydrofolate reductase and dihydropteroate synthetase haplotypes among the studied population in Haj Yousif area (N = 76)

<table>
<thead>
<tr>
<th>Combination</th>
<th>Frequency (Number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI-CN-I + SAKAA</td>
<td>82.9% (63)</td>
</tr>
<tr>
<td>MC-CN-I + SAKAA</td>
<td>5.3% (4)</td>
</tr>
<tr>
<td>CI-CN-I + SAEAA</td>
<td>3.9% (3)</td>
</tr>
<tr>
<td>CI-CN-I + MKAA</td>
<td>2.6% (2)</td>
</tr>
<tr>
<td>CI-CN-I + SGEAA</td>
<td>1.3% (1)</td>
</tr>
<tr>
<td>CI-CN-I + SGEGA</td>
<td>1.3% (1)</td>
</tr>
<tr>
<td>CN-CN-I + SAKAA</td>
<td>1.3% (1)</td>
</tr>
<tr>
<td>CN-CS-I + SAKAA</td>
<td>1.3% (1)</td>
</tr>
</tbody>
</table>
3.2.3. *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) haplotypes in relation to in vivo response to artesunate plus sulfadoxine-pyrimethamine

Allelic haplotypes within *Pfdhfr* and *Pfdhps* genes were examined for their association with in vivo response to SP plus AS. Based on the WHO 28 day classification system; results are summarized in Table 3.9. No association was observed between *Pfdhfr* mutant haplotypes and treatment failure (P = 0.27), where the predominant *Pfdhfr* haplotype (CI\(\text{CN} I\)) was detected in 92.2% of the ACPR and in 85.7% of treatment failure infections. Similarly no association was found between *Pfdhps* mutant haplotypes and treatment failure. Furthermore, no association was observed between the combinations of *Pfdhfr/Pfdhps* haplotypes and treatment failure (P = 0.14)

Table 3.9: *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) allelic haplotypes in relation to in vivo response to artesunate plus sulfadoxine-pyrimethamine among patients from Haj Yousif

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Number (%)</th>
<th>Adequate clinical and parasitological response</th>
<th>Treatment failure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pfdhfr</strong> 50/51/59/108/164</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CI(\text{CN} I)</td>
<td>71 (92.2)</td>
<td>6 (85.7)</td>
<td></td>
</tr>
<tr>
<td>M(\text{CN} I)</td>
<td>4 (5.2)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>CNC(\text{NI})</td>
<td>1 (1.3)</td>
<td>1 (14.3)</td>
<td></td>
</tr>
<tr>
<td>CNCSI</td>
<td>1 (1.3)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Total (N)</td>
<td>77</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

| **Pfdhps** 436/437/540/581/613 |             |                                                |                   |
| SGEAA | 1 (1.2) | 0 (0)                                  |
| SGFGA | 0 (0) | 1 (14.3)                                |
| SGKAA | 2 (2.4) | 0 (0)                                  |
| MKAA | 2 (2.4) | 0 (0)                                  |
| SAEAA | 3 (3.6) | 0 (0)                                  |
| SAKAA | 75 (90.4) | 6 (85.7)                                |
| Total (N) | 83 | 7                                      |
3.3. Distribution of *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) allelic haplotypes in New Halfa and Kassala

To assess the distribution of SP resistance marker alleles in *P. falciparum* in New Halfa and Kassala, single nucleotide polymorphisms in *Pfdhfr* and in *Pfdhps* genes associated with SP resistance, were detected in *P. falciparum* isolates from New Halfa (79) and from Kassala (49), and allele frequencies within the two genes were determined in pretreatment samples with positive ELISA results. Hundred twenty eight and 119 *P. falciparum* were examined for *Pfdhfr* and *Pfdhps*, respectively. Mixed genotypes and pure mutants were combined in all analysis.

Single nucleotide polymorphisms in *Pfdhfr* gene were detected at codons 50; (50R), 51; (N51I), 108; (S108N) and 59; (C59R). The overall frequency of *Pfdhfr* mutant haplotypes was high and comparable in both sites (82.3% in New Halfa and 91.8% in Kassala) (Table 3.10).

Single nucleotide polymorphisms in *Pfdhps* gene were identified in 79 isolates from New Halfa and 40 from Kassala, mutations were detected at codons S436A, A437G and K540E. Polymorphism at codon A581G was identified only in Kassala isolates. (Table 3.10).

In contrast the overall frequency of *Pfdhps* mutant haplotypes was significantly lower in New Halfa (39.2%) compared to Kassala (90%) (P = 0.000) (Table 3.10).

No polymorphisms at *Pfdhfr* position 164 or *Pfdhps* position 613 were detected. Within the *Pfdhfr* gene, the double mutant *Pfdhfr* haplotype (CICNI) was the predominant haplotype in both sites with a frequency of 57% and 83.7% in New Halfa and Kassala, respectively. *Plasmodium falciparum* dhfr triple mutant haplotype (CIRNI) was rare in both sites; identified in 2 isolates each in New Halfa (2.5%) and Kassala (4.1%), (Table 3.10; Figure 3.6). Single mutant haplotypes (CNCNI or CICSI) at positions 108 and 51 were identified in 7 (8.9%) isolates in New Halfa.

On the other hand within *Pfdhps* gene, the most frequent mutant haplotype in both sites are the ones harboring mutations at codons 437 and 540 (SGEAA) with a frequency of 17.7% in New Halfa and 62.5% in Kassala. Single mutant haplotype (Table 3.10) was found to be most frequent in New Halfa (8.9%) than in Kassala (2.5%).

Isolates harbouring quintuple mutations at *Pfdhfr* positions 51, 59, 108 and at *Pfdhps* positions 437 and 540 were found in 2.9% (2) and 5.9% (2) of the isolates from New Halfa and Kassala, respectively (Figure 3.6).
Table 3.10: Allelic haplotypes distribution within *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) genes in *P. falciparum* isolates from New Halfa and Kassala

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency (N)</th>
<th>New Halfa</th>
<th>Kassala</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pfdhfr 50/51/59/108/164</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CICNI</td>
<td>57% (45)</td>
<td>83.7% (41)</td>
<td></td>
</tr>
<tr>
<td>MCNI</td>
<td>3.8% (3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CJCMI</td>
<td>1.3% (1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CIRNI</td>
<td>2.5% (2)</td>
<td>4.1% (2)</td>
<td></td>
</tr>
<tr>
<td>CIRSI</td>
<td>1.3% (1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CNRNI</td>
<td>-</td>
<td>2% (1)</td>
<td></td>
</tr>
<tr>
<td>CICSI</td>
<td>6.3% (5)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MCSI</td>
<td>7.6% (6)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CICNI</td>
<td>2.5% (2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CNCMI</td>
<td>-</td>
<td>2% (1)</td>
<td></td>
</tr>
<tr>
<td>CNCSI</td>
<td>17.7% (14)</td>
<td>8.2% (4)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100% (79)</td>
<td>100% (49)</td>
<td></td>
</tr>
<tr>
<td><strong>Pfdhps 436/437/540/581/613</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGEAA</td>
<td>17.7% (14)</td>
<td>62.5% (25)</td>
<td></td>
</tr>
<tr>
<td>SGMAA</td>
<td>2.5% (2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AGEAA</td>
<td>-</td>
<td>2.5 (1)</td>
<td></td>
</tr>
<tr>
<td>SGEAA</td>
<td>-</td>
<td>10% (4)</td>
<td></td>
</tr>
<tr>
<td>MEGA</td>
<td>-</td>
<td>5% (2)</td>
<td></td>
</tr>
<tr>
<td>MMAA</td>
<td>2.5% (2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MEAA</td>
<td>1.3% (1)</td>
<td>7.5% (3)</td>
<td></td>
</tr>
<tr>
<td>MKAA</td>
<td>3.8% (3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SAEAA</td>
<td>1.3% (1)</td>
<td>2.5% (1)</td>
<td></td>
</tr>
<tr>
<td>AAMAA</td>
<td>1.3% (1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ΔAEAA</td>
<td>1.3% (1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ΔAKAA</td>
<td>7.6% (6)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>SAKAA</td>
<td>60.8% (48)</td>
<td>10% (4)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100% (79)</td>
<td>100% (40)</td>
<td></td>
</tr>
</tbody>
</table>

- **M** = mixed genotype
- **MEGA:** M = AG/SG
- **MMAA:** M = SG/ΔA or SG/SA
- **MEAA:** M = AG/SG or AG/SA or SG/SA
- **MKAA:** M = ΔA/SA
Figure 3.6: Distribution of haplotypes of *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) genes in New Halfa and Kassala in eastern Sudan
4.1. Discussion

In Daraweesh and Kajara site; frequency of treatment failure was comparable in the two studied groups with 31.8% and 37.5% in CQ+DHA and CQ+S/P group, respectively. Although these findings are unsatisfactory in term of clinical or parasitological efficacy, the rate of treatment failure with the two studied combinations is lower than the previously reported levels with CQ monotherapy in the study area. Considering 28 days follow up period, previous studies on CQ efficacy in eastern Sudan reported 42% CQ resistance in 1989, 48% in 1990, 77% in 2004, and 76% in 2006 (214,215,217,218). The observed treatment failure among CQ+DHA group suggests that addition of CQ to DHA has no advantage over DHA monotherapy, where recrudescence in monotherapy with artemisinin and its derivatives was reported (68,69). On the other hand these findings indicated SP treatment failure in the study area; the reported failure to CQ+SP is consistent with a recent study reported 31.7% failure to SP alone and 36.6% failure to CQ+SP in the same area (224).

The observed faster clearance of asexual parasitaemia, and significant decrease in its prevalence by day 1 in CQ+DHA group compared to CQ+SP, and the lower post treatment gametocyte carriage in CQ+DHA group, in spite of the higher pretreatment levels compared to CQ+SP could be attributed to the efficacy of DHA against the blood stages (59) and its gametocytocidal effect (60,61). Another explanation of the low gametocyte carriage in CQ+DHA compared to CQ+SP regimen, might be the suggested inherent property of SP to increase gametocyte carriage (241,242).

No association was observed between the detected mutations in Pf\textit{dhfr}, Pf\textit{dhps}, Pf\textit{crt} and Pf\textit{mdr1} and treatment failure to the studied combinations. However, such results are expected because the sample size in each treatment arm was small and the frequency of relevant mutations was high.

In this study, associations between mutations that confer resistance to CQ and SP were explored. These results revealed a strong association between Pf\textit{crt} 76T (chromosome 7) and Pf\textit{mdr1} 86Y (chromosome 5) as reported before in different sites (152,243,244,245). Inter and intragenic association of Pf\textit{dhfr} and Pf\textit{dhps} mutant codons was not seen among the parasites examined in this study, although it was indirectly proved in the studies where SP resistance was found to be associated with mutations in both genes (131,165,174). There were strong associations between the
mutations Pfcrt 76T, Pfmdr1 86Y, Pf dhfr 51I, Pf dhfr 108N and Pf dhps 540E, which convey the parasite resistance against both CQ and SP. The frequencies of the CQ resistance mutations Pfcrt 76T (90%) and Pfmdr1 86Y (86%) in the area have only slightly increased over the last 2 years from 81 to 87 and 79%, respectively (245,246).

Plasmodium falciparum dihydrofolate reductase 51I and 108N alleles occurred in more than 80% of the examined isolates. Although the frequency of Pf dhfr 108N was comparable with that of the Pfcrt 76T, longitudinal data proved that the rise in the Pf dhfr 108N allele had happened recently, unlike that of Pfmdr1 86Y and Pfcrt 76T (Figure 4.1).

An unexpected finding was the association of at least five mutations (Pfcrt 76T, Pfmdr1 86Y, Pf dhfr 51I and 108N and Pf dhps 540E) scattered on four different chromosomes and incriminated in resistance to two different antimalarials, CQ and SP. Association between the mutations Pfcrt 76T, Pfmdr1 86Y and Pf dhfr 108N has been reported before from West Africa (243,247). So far, the reason for the observed associations between separate mutations conferring resistance to unrelated drugs is obscure. Remarkably in this regard, selection for high grade pyrimethamine resistance has been reported to be accompanied by an increased number of mutations in P. falciparum (248). In this study the observed association between the CQ and SP resistance mutations could be explained within the context of drug pressure. Where CQ and SP had been the first and second line antimalarial treatment for long time in Sudan, and both drugs are affordable and available in the local markets.

Sudan has changed its national first and second line antimalarial treatment from CQ and SP to AS plus SP and artemether plus lumefantrine (Co-artem). However recent reports have indicated SP treatment failure in different parts of Sudan (221,222,223,225,226,249). Although some efficacy studies of AS + SP in the country are available (223,250,251,252,253), the debatable efficacy of SP necessitate additional studies for continuous monitoring the efficacy of the first line antimalarial regimen and the spread of SP resistance markers.

The present study examined efficacy of AS + SP in a mesoendemic suburban malaria area of Khartoum, 92.6% of the infections were cured by day 28, while 7.4% showed a LTF response. The results were confirmed using PCR genotyping. PCR results showed that the LTFs were a combination of old and new infections in all recurring
samples. This finding has major epidemiological consequences as it reflects the inability of SP to protect AS and prevent new infections (a role supposed to be played by the partner drug in ACT) after therapy (87). The microepidemiology of resistance of the partner drug has to be considered in ACT. A comparable cure rate (91.2%) was reported in southern (Nuba Mountain) Sudan (251), cure rates to AS+SP ranging between 99-100% have been reported from different parts of Sudan (223,250,252,253). In the present study all patients became afebrile and aparasitemic after three days and gametocytemia was not detected in any infection by the end of the follow up period. The rapid elimination of parasites and clearance of fever showed the clinical benefit of AS, as artemisinin and its derivatives have been reported as potent, short acting antimalarials with rapid clinical recovery and parasite/gametocyte clearance times (59,60,61). The gametocytocidal ability of AS compensates the suggested inherent property of SP to increase gametocyte carriage (242).

All Adverse events reported previously after treatment with ART derivatives and ACTs were mild and none had led to discontinuation of treatment (254,255). In spite the documented failure to treatment with the combination of AS with SP in Haj Yousif, the combination appeared to be safe and well tolerated. In addition it combined the benefits of rapid parasite clearance, symptoms relief and low gametocyte carriage associated with AS, with the longer term efficacy of SP. Nevertheless AS and SP combination is relatively cheap and might reduce the rate at which SP resistance develops.

*Plasmodium falciparum* dihydrofolate reductase and *Pfdhps* allelic haplotypes and their distribution in relation to treatment outcome were investigated in this study. Consistent with previous studies conducted in the same area (221,256) and other two sites in eastern Sudan (249,Osman ME *et al*; unpublished data); the most frequent *Pfdhfr* haplotype in the study area was *Pfdhfr* double mutant (CICNI), however its rate (91.9%) is much higher than in 2002 (79%) and in 2005 (80%) in same area (221,256). This might be attributed to the use SP monotherapy, and of cotrimoxazole (trimethoprim/sulfamethoxazole) in the study area for common bacterial infections, both drugs are affordable and readily available in the local market. This haplotype was found to be predominant in southern Sudan where the majority of parasites in the area harboured wild genotype (220,257).
In the present study no significant association was observed between treatment failure and the frequency of *Pfdhfr* mutant haplotypes (*P* = 0.27), where the predominant *Pfdhfr* haplotype CICNI harbouring mutations at codons 51 and 108 is homogenously distributed between ACP responders (97.4%) and treatment failure groups (85.7%). These findings suggest that (CICNI) haplotype is insufficient to confer SP clinical failure, but since this *Pfdhfr* haplotype is supposed to be prerequisite for increased resistance to pyrimethamine (135,258), and taking into account that *Pfdhfr* mutations are acquired in a stepwise fashion, (131,165), further mutation in *Pfdhfr*, namely at codon 59 might lead to increased clinical failure to SP. Our findings support the hypothesis that resistance associated mutations in *Pfdhfr* gene are useful in monitoring progress of resistance to SP but not the current situation of clinical failure to SP (238,259).

The current study confirms the previously reported poor predictive value of resistance related *Pfdhps* mutations for SP treatment failure, as 90.4% and 85.7% of the ACPR and treatment failure infections, respectively carrying *Pfdhps* wild type haplotype (SAKAA). The *Pfdhps* double mutant (437G/540E) was shown to increase the likelihood of treatment failure to SP when coexisted with the *Pfdhfr* triple mutant variant (157,174,175).

However in the current study, the actual SP failure was most likely underestimated, because some SP resistant parasites might be cleared by the action of AS which was used in addition to SP, or by host immune response (79,260), where existence of certain degree of immunity was reported in areas with moderate transmission level (261).

Since it is now a priority to provide baseline information on the distribution of SP resistance marker alleles in *P. falciparum* in Sudan, the frequency of *Pfdhfr* and *Pfdhps* mutations, associated with decreased sensitivity to SP was investigated in two sites in eastern Sudan; New Halfa and Kassala. Mutations at the key positions in *Pfdhfr* (C51I, C59R and S108N) and *Pfdhps* (S436A, A437G, K540E and A580G) identified in this study, were found to occur commonly in different parts of Sudan (220,221,249,257,258).

The overall high frequency of *Pfdhfr* mutant alleles and the predominance of double mutant haplotype CICNI harbouring mutations at codons 51 and 108, found in both study sites (Table 3.10) are in consistence with the only one previous study which has
estimated the frequency of *Pf dhfr* and *Pf dhps* mutant haplotypes in eastern Sudan (249).

The triple mutant *Pf dhfr* (CIRNI) at positions 51/59/108 detected in this study in New Halfa and Kassala was not identified before in eastern Sudan, a recent study in Gedaref area has found no mutation in codon 59 of *Pf dhfr* in all examined isolates (249). The very low frequency of *Pf dhfr* triple mutant haplotype (CIRNI), which has been seen in two study sites (2.5% in New Halfa and 4.1% in Kassala) (Figure 3.6), is in agreement with a previous study conducted in Bahr El Gazal Province, southern Sudan (257). Detection of this triple mutant haplotype in this part of the country is disturbing, since evidence of correlation between the combination of triple mutation in *Pf dhfr* at codons 51, 59 and 108 and SP treatment failure has been reported (157,262). Similarly the *Pf dhps* haplotype carrying double mutant allele (437G/540E) which dominated in both study sites and frequent at a significantly lower rate in New Halfa (17.7%) compared to Kassala (62.5%) should be considered because it was thought to have a role in strengthening the association between the *Pf dhfr* triple mutant allele and SP resistance (157).

*Plasmodium falciparum* with quintuple mutations at *Pf dhfr* positions 51, 59, 108 and at *Pf dhps* positions 437 and 540 was found at a low rate in New Halfa (2.9%) and in Kassala (5.9%). A strong association between this quintuple mutant variant and SP treatment failure has been indicated by Kublin and others in 2002 (174). According to the presence of *P. falciparum* quintuple mutant at *Pf dhfr* and *Pf dhps* key positions and *Pf dhfr* triple mutant haplotype in only a small fraction of the investigated isolates from both sites, SP is expected to be effective in New Half and Kassala at the moment.

This study is the first to show the identification of the quintuple mutations in eastern Sudan. This reflects a need for continuous surveillance in the region for *Pf dhfr* and *Pf dhps* haplotypes, as these haplotypes have been shown to be associated with SP clinical failures.

The existence of these new mutant genotypes in eastern Sudan might be triggered by the extensive use of SP in the study areas. Sulfadoxine-pyrimethamine is available in the local markets and affordable which makes self medication with SP much easier. The observed difference in the frequency of *Pf dhps* double mutant allele (437G/540E) between New Halfa and Kassala might not reflect a difference in SP sensitivity between the two areas, since the impact of this double mutant allele on SP resistance
depends on its coexistence with *Pfdhfr* triple mutant allele (51I/59R/108N), which exists in both sites at a comparable low rates.

In Sudan the frequency of SNPs in *Pfdhfr* and *Pfdhps* genes, has been evaluated in central part; Khartoum State (221), in southern Sudan; Eastern Upper Nile and Bahr El Gazal (220,257) and in eastern part, Gedaref State (249). Results obtained from the above studies which represent different regions in Sudan; together with the current results are summarized in Table 4.1. The frequency of *Pfdhfr* mutant haplotypes is comparable in central and eastern Sudan where it represents more than 80% in each of the 4 sites (Khartoum, Gedaref, New Halfa and Kassala). In southern parts, the mutant *Pfdhfr* haplotypes existed at lower rate (40% in Bahr El Gazal and 18% in Eastern Upper Nile). The double mutant *Pfdhfr* genotype at codons 51 and 108 was the most frequent *Pfdhfr* variant in all the investigated regions in Sudan, with much higher rates in central and eastern Sudan (>60%) compared to southern sites (18% and 28%).

Triple mutant *Pfdhfr* haplotype which was detected in this study in New Halfa (2.5%) and Kassala (4.1%) eastern Sudan, was identified before in one site in southern Sudan (Bahr El Gazal; 3%).

The *Pfdhps* (SGEAA), the major mutant haplotype in New Halfa (24%) and Kassala (87.5%), was identified before in southern Sudan in Bahr El Gazal at 9% frequency, and this study detected a lower frequency in Khartoum (1.1%). This haplotype was not found previously either in central or eastern Sudan. No mutant alleles within *Pfdhps* gene were reported in Upper Nile (southern Sudan), where 100% SP cure rate was reported in 2003 (220).

In summary this comparison shows variation in *Pfdhfr* and *Pfdhps* allele frequencies by geographic location, which might be due to difference in magnitude and duration of SP and antifolates use in different regions.
Figure 4.1: Longitudinal data show the relative and stepwise rise in the frequency of mutant alleles, *Pfcrt* 76T, *Pfmdr1* 86Y and *Pfdhfr* 108N, in parasites collected from the same area over 10 years (1993–2003), data until 2001 was obtained with permission from Abdel-Muhsin and Babiker (246)
Table 4.1: *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) allelic haplotypes frequency in different regions in Sudan

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Southern Sudan</th>
<th>Central Sudan</th>
<th>Eastern Sudan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eastern Upper</td>
<td>Bahr El Gazal</td>
<td>Khartoum</td>
</tr>
<tr>
<td><em>Pfdhfr</em> 51/59/108/164</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
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<td>3</td>
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<td>0</td>
<td>0</td>
</tr>
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<td>5.4</td>
</tr>
<tr>
<td>ICNI</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NCNI</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>MCNI</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wild genotype</td>
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<td>60</td>
<td>16.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><em>Pfdhps</em> 436/437/540/581/613</th>
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<th></th>
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<tbody>
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<td>1.1</td>
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<td>SGEAA</td>
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<td>0</td>
<td>1.1</td>
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<td>AGEAA</td>
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<td>SAEAA</td>
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<td>0</td>
<td>3.3</td>
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<tr>
<td>SAEAA</td>
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<tr>
<td>SAKGA</td>
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</tr>
<tr>
<td>AAKAA</td>
<td>0</td>
<td>0</td>
<td>44</td>
</tr>
<tr>
<td>MEGA</td>
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<tr>
<td>MEGA</td>
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<td>MEAA</td>
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<tr>
<td>MKAA</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Wild genotype</td>
<td>100</td>
<td>47</td>
<td>92</td>
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</table>

- **M** = mixed genotype  
- **MMAA**: M = SG/AA or SG/SA  
- **MKAA**: M = AA/SA

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4.2. Conclusions:

- High failure rate to both antimalarial drug combinations containing CQ and SP (CQ plus DHA and CQ plus SP) used in Gedaref State. This is probably attributed to the high frequency of mutations in resistance genes for CQ and SP in eastern Sudan, and supported the withdrawal of both drugs.

- Strong association between \textit{Pfcrt} 76T, \textit{Pfmdr1} 86Y, \textit{Pfdhfr} 51I, \textit{Pfdhfr} 108N and \textit{Pfdhps} 540E, which are located on four different chromosomes, and conferring resistance against two structurally and functionally unrelated drugs, CQ and SP was demonstrated. This association is probably due to common use of the two drugs and co-selection of the above resistant alleles.

- The 7.4% failure to AS plus SP and the high frequency of SP resistance marker alleles reported in Khartoum area is alarming, since recently WHO recommended that a change of first line treatment should be initiated if the total failure rate exceeds 10%.

- This study is the first to identify the quintuple mutations in \textit{Pfdhfr}/\textit{Pfdhps} genes (CIRNI/SGEAA) in Sudan, and to identify the triple mutant \textit{Pfdhfr} (CIRNI) and the double mutant \textit{Pfdhps} haplotype (SGEAA) in eastern Sudan. The existence of these new mutant genotypes is disturbing.
4.3. Recommendations:

● Elaborate studies monitoring the efficacy of AS plus SP in different parts of the Sudan are needed.

● Different antimalarial drug combinations should be assessed to provide future effective and affordable alternatives for the treatment of uncomplicated malaria in Sudan.

● Spatial, longitudinal monitoring of SP resistance markers is needed in Sudan to provide data to guide future policies for treatment of malaria in both countries.
References


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Appendix I

Case Report Form
Antimalarial Drug Combinations

Serial number: ……

Study Site:
Health facility's name: ………….. Town/Village: ……………….. State: ………….. …………..

Patient:
Identity number: …. Full Name: ………….. Age (Years): …. Sex: (M/F): ……..
Weight (kg): …. Name of guardian: …………….. Home Address: …………………..

Antimalarial drugs:
Combination: …………………….. Expiry date: ……………….. Total dose (mg): ……..
Previous intake:
(Y/N/Unknown): ………….. Drug: ………….. Date: ………….. Total dose (mg): …

Complaints

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<th>2</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
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<td>Nausea</td>
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<td>Vomiting</td>
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</table>
Serial number: ……

Study Site:

Health facility's name: …………… Town/Village:……………… State: ……………

Patient:

Identity number: …… Full Name: ………………………………………

Antimalarial drugs:

Combination: ……………………………

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</table>

Overall assessment:

- Early treatment failure (ETF) ☐
- Late clinical failure (LCF) ☐
- Late parasitological failure (LPF) ☐
- Adequate clinical and parasitological response (ACPR) ☐
- Withdrawn ☐
- Loss to follow up ☐

Day of assessment: …… Reason for withdrawn: ……………
Comments: …………………………………………………………………