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# Factors Influencing Evolution to Antimalarial Drug Resistance *in Plasmodium falciparum* in Sudan and The Gambia

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**Abstract**
Kheir, A. 2011. Factors Influencing Evolution to Antimalarial Drug Resistance in *Plasmodium falciparum* in Sudan and The Gambia. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 661. 54 pp. Uppsala. ISBN 978-91-554-8044-8.

Drug resistance is a major obstacle to management and control of malaria and currently progressing at a rapid rate across Africa. This thesis has examined factors influencing evolution of resistant *P. falciparum* at two sites in Africa, including parasite migration, cross mating and fitness cost of resistance. In Asar village, eastern Sudan, the frequencies of drug sensitive and resistant parasites were monitored throughout the dry season in the absence of anti-malarial drug usage to examine whether persistence of resistant parasites is reduced in the absence of drug pressure. Two cohorts of *P. falciparum* infected patients were treated with chloroquine in the transmission season (Oct-Dec), and followed monthly in the dry season into the next transmission season. A large proportion of the cohort maintained sub-patent asymptomatic *P. falciparum* infections throughout the entire study period. Alleles of the chloroquine resistance transporter (*Pfcrt*) and multi-drug resistance protein (*Pfmdr*1) were examined. Mutant alleles of *Pfcrt* reached fixation following CQ treatment and remained high in the transmission season. However, at the start of the dry season, wild type alleles of both genes started to emerge and increased significantly in frequency as the season progressed. The mutant *Pfcrt* haplotype was invariably CVIET, indicating migration of CQ resistant parasites into an area; otherwise the CVMNK haplotype is normal. In addition, microsatellite haplotypes of dihydrofolate reductase (*dhfr*) gene and dihydropteroate synthase (*dhps*) genes, which control the parasite response to pyrimethamine and sulfadoxine respectively, were characterized. One major *dhfr* haplotype with double *dhfr* mutations and two major mutant *dhps* haplotypes were seen in eastern Sudan. These haplotypes are distinct from those prevailing in other African countries, suggesting the likely local origin of *dhfr* and *dhps* haplotypes conferring drug resistance.

Transmission capacities of different *P. falciparum* clones within a single infection in The Gambia have a high ability to produce gametocytes and infect *Anopheles* mosquitoes even when they exist at levels not detectable by microscopy and PCR. These findings emphasize the crucial role of gametocyte complexity and infectivity in generating the remarkable diversity of *P. falciparum* genotypes seen in infected people. Parasites with different resistant dihydrofolate reductase (*dhfr)* haplotypes have the ability to infect *Anopheles* mosquitoes following drug treatment, and cross-mating between parasites with different *dhfr* haplotypes was detected. Our results showed that the major *dhfr* haplotype in the Gambia is similar to the common one seen in other African countries, suggesting that parasite migration plays a major role in spread of resistance. Indeed, the dominant resistant haplotype seen in infected patients was readily transmitted to infect mosquitoes.

*Keywords:* cross-mating, fitness, microsatellite haplotypes, mosquito infectivity

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*To the memory of my father*
*To my mother*

# List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Kheir A**, Villalta T, Nassir E., Abdel-Muhsin AM., Swedberg G., Babiker HA. Fitness cost of drug resistance in malaria parasites in eastern Sudan. Manuscript

II. Al-Saai S**, Kheir A**, Abdel-Muhsin AM, Al-Ghazali A, Nwakanma D, Swedberg G, Babiker HA. Distinct haplotypes of *dhfr* and *dhps* among *Plasmodium falciparum* isolates in an area of high level of sulfadoxine pyrimethamine (SP) resistance in eastern Sudan.2009 Infect Genet Evol Sep; 9(5):778-83

III. Nwakanma D**, Kheir A**, Sowa M, Dunyo S, Jawara M, Pinder M, Milligan P, Walliker D, Babiker HA. High gametocyte complexity and mosquito infectivity of *Plasmodium falciparum* in The Gambia 2008. Int J Parasitol. 38(2): 219-27.

IV. **Kheir A**, Nwakanma D, Al-Gazali A, Akbarova Y, Al-Saai S, Swedberg G, Babiker HA. Transmission and cross-mating of high level resistance *Plasmodium falciparum* dihydrofolate reductase haplotypes in The Gambia. Am J Trop Med Hyg. 2010 Apr;82(4):535-41

# Contents

# Abbreviations

| | |
|---|---|
| bp, | Base pair |
| CQ, | Chloroquine |
| CQR, | Chloroquine resistance |
| CQS, | Chloroquine sensitive |
| DHF, | 7,8-dihydrofolate |
| DHFR, | Dihydrofolate reductase |
| DHFS, | Dihydrofolate synthase |
| DHN, | 7,8-dihydroneopterin |
| DHNA, | Dihydroneopterin aldolase |
| DHP, | 7,8 dihydropteroate |
| DHPS, | Dihydropteroate synthase |
| DHPT, | 7,8-dihydro-6-hydroxymethylpterin |
| EIR, | Entomological inoculation rate |
| Kb, | Kilo base pair |
| MDR, | Multi-drug resistance |
| MS, | Microsatellite |
| Pfcrt, | P.falciparum chloroquine resistance transporter gene |
| Pfg377, | P. falciparum gametocyte antigen |
| Pfmdr, | P. falciparum multi-drug resistant gene |
| SNPs, | Single nucleotide polymorphisms |
| SP, | Sulphadoxine/pyrimethamine |
| THF, | Tetrahydrofolate |
| ACT | Artemethinine combination therapy |
| LLINs | Long lasting impregnated bed nets |
| IPT | Intermittent preventive treatment |
| IRS | Indoor residual Spraying |
| PCR | Polymerase chain reaction |
| RT | Reverse transcriptase |
| Pfmsp-1 | Merozoite surface protein-1 |
| RFLP | Restriction fragment length polymorphism |

# Introduction

## Global Burden of Malaria

Malaria is a massive global health problem and an important cause of death and illness among children younger than 5 years old and pregnant women. It puts half of the world's populations (3.3 billion) at risk, it occurs in 109 countries (Who 2008c). With an estimated mortality over a million and about 250 million malaria cases registered in 2006. Malaria varies in epidemiology and clinical appearance in different parts of the world. This variability is attributed to many factors including the species of malaria parasites that occur in a specific area, the parasite's vulnerability to commonly available malaria control strategies, environmental conditions and the level of acquired immunity of the exposed human populations (Who 2000). Malaria is correlated with economic and social development, greatly affecting poor communities (Malaney, Spielman et al. 2004). Several decades ago efforts were focused on the goal of eradication of malaria but because of the dramatic acceleration of the disease rates and deaths, efforts are now rather aimed at malaria control. (Gregson and Plowe 2005).

## The malaria parasite

Malaria is caused by members of genus *Plasmodium*, and there are nearly 120 species able to infect birds, reptiles and mammals but only four of them cause the disease in humans. These are *Plasmodium vivax*, *P. malariae, P. ovale* and *P. falciparum,* which is behind most deaths, since it causes the most severe form of malaria (Gregson and Plowe 2005). In addition *P.knowlesi*, which normally infects the macaque monkeys, has been reported to infect humans as well, thus having been proposed the fifth human malaria parasite. (Singh, Kim Sung et al. 2004; Cox-Singh and Singh 2008).

Different malaria parasite species varies in geographical distribution, microscopic morphology, clinical presentation, and susceptibly to anti-malarial drugs (WHO 2000).With the introduction of the first draft of the *P. falciparum* genome sequence in October 2002 malaria research have been moved into a new era as a result of the massive structural and functional information obtained from this data. It has significantly improved the quality of gene

finding by providing different approaches for identification of key functional genes involve in parasite biology and pathogenesis of malaria.

## Life cycle of malaria parasite

All of the clinical symptoms and pathogenic manifestations associated with mammalian malaria infections are caused by the asexual erythrocytic phase of the plasmodium life cycle. Part of the cycle, which occurs in the human host is asexual, although gametocytes are formed in the host (Bruce-Chwatt 1985). The sexual cycle take place in the female *Anopheles* mosquito, thus gametocytes bridges the two hosts, beginning in the human host and ending in the mosquito midgut, **Figure 1**. Natural infections in man are initiated by a bite of an infected female *Anopheles* mosquito, inserting sporozoites in the blood circulation, and within 40 minutes they can be detected in the hepatic cells. Within these cells the parasites divide asexually to form tissue-stage schizonts (exo-erythrocytic schizonts), which ultimately occupy the entire host liver cells. In 7-10 days the infected liver cells rupture and liberate thousands of merozoites, which invade the red blood cells in the general circulation. After several generations of erythrocytic schizogony some ring-stage parasites develop into the sexual forms: female macrogametocytes and male microgametocytes. (Miller, Baruch et al. 2002; Daily 2006).

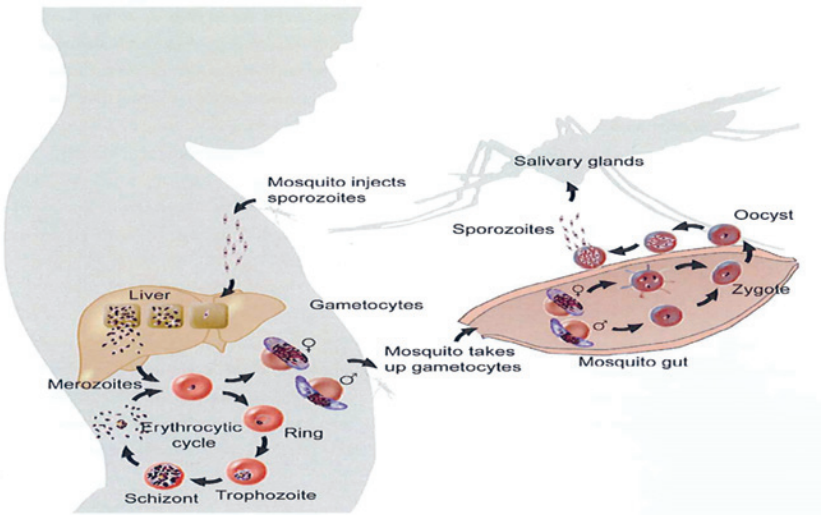


**Figure 1**: The life cycle of *P. falciparum.*
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## Genetic structure of malaria parasite population

The genetic complexity of *P. falciparum,* particularly its ability to cross-mate and generate mutant variants, makes it a successful pathogen. Genetic variants are involved in pathogenicity and in immune responses and have led to the emergence of resistance against virtually all available anti-malarial drugs. Such variants are under strong selective pressure, but analyses of sequence variation in gene segments that are not directly subjected to selection permit the study of inter population diversity of the parasite and its evolutionary origin. (Meyer, May et al. 2002).

## Microsatellite loci

Microsatellites (MS) are molecular markers that can provide valuable information on population structure in malaria parasite and are common in the *P. falciparum* genome (Su and Wellems 1996; Su, Ferdig et al. 1999). They can be defined as nucleotide repeats of ranges between 2 and 6bp, one MS can be found in every kilo-base genome-wide. MS are highly polymorphic due to variation of length of these multiple alleles, and more neutral than SNPs among parasite isolates. Fortunately, mutations that are associated with drug resistance in *P. falciparum* provide a unique chance to identify allelic associations because these mutations have occurred within the past 50 years.

These drug resistant genotypes are characterized by reduced diversity around the major resistance alleles (Su and Wootton 2004). In Asia and Africa, microsatellite markers with multiple alleles flanking genes associated with drug resistance such as *dhfr* and *Pfcrt* were investigated and allowed typing of specific haplotypes flanking these regions and enabled identification of drug resistance origin as well as tracing spread of these mutations (Nair, Williams et al. 2003; Roper, Pearce et al. 2003).

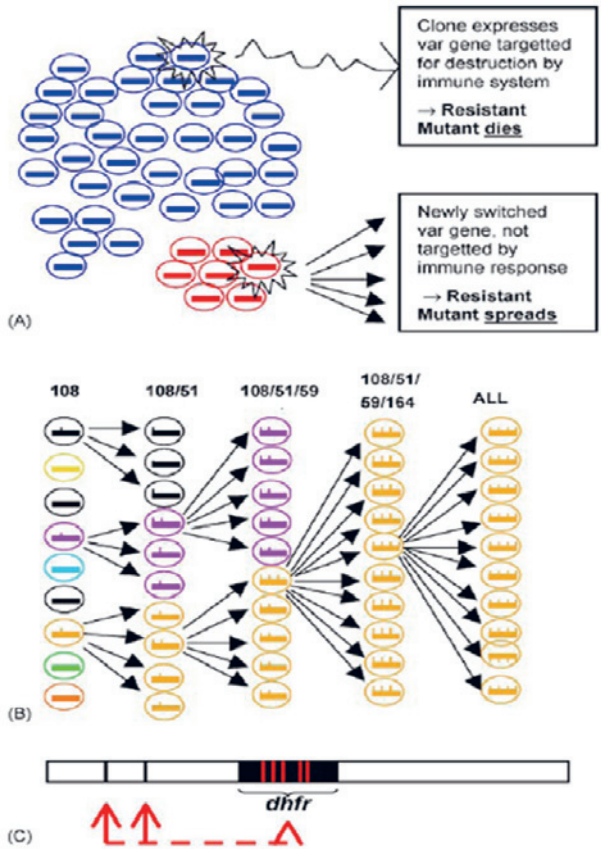



**Figure 2**: Factors describing why alleles with multiple mutations have few origins (A) Only parasites with switched *var* gene are likely to escape immune surveillance and achieve transmission since this gene is a major target for destruction by the immune system of the host. (B) Alleles with multiple mutations have few origins that arises from parasites that have passed through a series of sequential accumulated mutations as bottlenecks (C) Evolution of a resistance gene such as *dhfr* might require additional compensatory mutations around the mutation point to overcome fitness cost thus occurs less frequently. Source: Acta Trop. 2005 (Anderson and Roper 2005).

## *P. falciparum* gametocyte antigen gene (*Pfg377*)

*Pfg377* is a 9360bp intronless gene found on chromosome 12 and encoding a protein of 377 kDa that is expressed only in the gametocyte stage, and may play a role in parasite maturity in mosquito midgut. The protein is highly hydrophilic, and has an essentially non-repetitive structure; *Pfg 377* has four regions of tandem repeats. Studies showed polymorphisms in these regions, and region one is the least polymorphic with no allelic variation detectable and the most polymorphic among them is region 3 which encodes seven

degenerate amino-acid repeats resulting in different alleles that varies by multiples of 21 base-pairs (Alano, Read et al. 1995). Typing of region 3 has provided a good tool for analysis of parasite diversity within a population as well as the presence or absence of multiple clones infections within an isolate and can detect presence of sub-patent gametocytes in infected blood, **Figure 3** (Alano, Read et al. 1995). The repetitive region of this gene is under no selection, thus typing of this gene along with genes under selection such as drug pressure can discriminate the effects of drug influence on population structure (Abdel-Muhsin, Mackinnon et al. 2003).

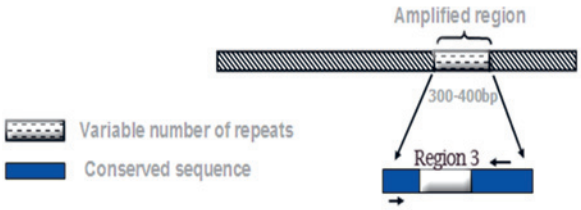



**Figure 3:** Region 3, the most polymorphic part in *pfg377* gene. This region encodes seven degenerate amino-acid repeats resulting in different alleles that vary by multiples of 21 base-pairs. Typing this region is a useful tool for analysis of parasite diversity within a population as well as the presence or absence of multiple clones genotype infections within an isolate.

## Merozoite Surface Proteins -1 gene (*msp1*)

Two antigens located on the surface of the *P.falciparum* infected merozoites, have been considered as potential vaccine candidates. These antigenes are denoted as merozoites surface protein1(*MSP-1*) (McBride, Walliker et al. 1982) and merozoite surface protein 2 (*MSP-2*) (Kemp, Cowman et al. 1990). Both proteins are polymorphic in natural populations of *P.falciparum* (Walliker 1998*)*.The polymorphism is suspected to be a major cause of the parasite surviving their host immune responses.

## Multiplicity of infection

People living in malaria endemic countries are subject to frequent bites of infected mosquitoes; this can lead to accumulation of different parasitic genotypes within infected individuals. Typing of polymorphic single copy genes allows identification of presence of multiple numbers of haploid *P. falciparum* clones within an infection (Conway, Greenwood et al. 1991).

Clonal multiplicity in individuals within communities varies depending on the seasonal and epidemiological situation and on variable exposure patterns, properties of the mosquito vector, and even the time of day of sample collection. (Babiker, Creasey et al. 1991; Meyer, May et al. 2002).

## Malaria Control Strategies

Early attempts to control malaria were to target the mosquito vector(s). In 1955 WHO started malaria eradication programs in many African countries. However, this approach has been hindered by resistance of vectors to insecticides (Curtis 1993). So effective control and treatment of malaria present enormous challenges, by reducing the burden of malaria in an integrated approach like using long lasting impregnated bed nets (LLINs), which has a major impact on reducing both mortality and morbidity (Lengeler 2004), as well as indoor residual spraying (IRS), and intermittent preventive treatment (IPT), that reduces the incidence of maternal anemia and improve infant birth weight (ter Kuile, van Eijk et al. 2007). Ultimately a vaccine development for management and control of malaria in endemic areas would be a major improvement, but for the time being antimalarial drugs are important in control of malaria. (Gregson and Plowe 2005; Reetha, Sharma et al. 2007).

## Combination therapy

In response to the widespread occurrence of chloroquine-resistant parasites in Africa (Bruce-Chwatt 1986), and the more recent signs that resistance to pyrimethamine/sulphadoxine is reducing the efficacy of that drug (Ronn, Msangeni et al. 1996; Trigg, Mbwana et al. 1997), calls have been made for the deployment of new effective antimalarial combinations. Of particular interest are the artemisinins. Artemisinin, a chemical extract from the plant *Artemisia annua* is a novel antimalarial with an encouraging effect against multidrug resistant forms of *Plasmodium falciparum*. Artesunate (ART) is a water-soluble derivative of artemesinin. Drug combinations of ART are currently in their infancy in Africa, a number of safety and efficacy trials have taken place over the last five years (Seidlein, Milligan et al. 2000; Mutabingwa, Maxwell et al. 2001). The introduction of artemisinin based combination therapy (ACT) coartem®, (artemether/lumefantrine, AL) has made a significant impact on the treatment of malaria, by reducing the transmission through reducing gametocyte development which have short elimination half-lives, in various combinations with longer-acting drugs (Watkins and Mosobo 1993; White, Nosten et al. 1999).
The basic premise behind the use of combination therapy is that the chance of a mutant parasite emerging that is simultaneously resistant to two antima-

larial drugs is very low. The rate of spread of resistance could be further reduced if the drugs also had an effect on gametocytes (Targett, Christopher et al. 2001). Studies in Thailand showed that when artemisinin derivatives were introduced as a component of first-line treatment, there was a significant reduction in the incidence of clinical *Plasmodium falciparum* malaria during the next two years. Furthermore, studies in The Gambia (Von Seidlein, Bojang et al. 1998) and Tanzania (Hatz, Abdalla et al. 1998) gave a strong indication that artemisinin derivatives reduce the transmissibility of *Plasmodium falciparum*. More recent studies in The Gambia, which assessed the use of artesunate in combination with pyrimethamine-sulfadoxine, showed markedly lower gametocyte prevalence after the treatment with the combination than after treatment with pyrimethamine-sulfadoxine alone (Doberty, Sadiq et al. 1999; von Seidlein, Milligan et al. 2000). It remains unclear from these studies whether the observed reduction in gametocyte numbers is due to the gametocytocidal effect of artemisinins, to the lower production of gametocytes due to rapid reduction in the total parasite burden or to a combination of both. (Targett, Drakeley et al. 2001).

## Drug resistance

Drug resistance is the ability of a parasite to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than recommended, but within the limits of tolerance of the subject (Who 1986). Parasite survival is achieved by developing mutations in enzymes related to drug targets to encode a protein that enables it to skip an anti-malarial drug; this then is followed by selection of parasites with genetic changes that confer reduced susceptibility (Sharma 2005).

There is a continuous increase in resistance to anti-malarial drugs as a result of indiscriminate use, which in turn drives a strong selective force in favor of the resistant parasites. This facilitates global spread of drug resistance and hence worsening the status of management and control of the disease, escalating morbidity and mortality (Who 2006). Resistance to all known anti-malarial drugs, including the newly introduced artemisinin based combination therapy (Carrara, Zwang et al. 2009), has developed to various degrees in several countries.

## Epidemiology of drug resistance

Malaria epidemiology varies in the tropical zones, as the prevalence of parasite and the disease varies significantly from one area to another. One limiting factor to differences in magnitude of malaria is the abundance of the mosquito vector. The first reports (Bruce-Chwatt 1985), of *P.falciparum* chloroquine (CQ) resistance came from southeastern Asia and south America in the late 1950s, the earliest report of antimalarial resistance was that of *P. falciparum* to quinine reported from Brazil in 1910 (Spencer 1985). The resistant parasite then has spread out to all known malaria-endemic area except the countries located north of the Panama Canal and Haiti Island. Increasing CQ resistance has driven those countries in which resistance has developed to switch their first line treatment from CQ to SP, which is inexpensive, relatively safe, and has simple dosing. By the late 1980s, resistance to SP became prevalent on the Thai-Cambodian and Thai-Myanmar (Thai-Burmese) borders which later became a multi-drug resistance (MDR) area. Only in the 1980s severe resistance started to emerge in east Africa and spread across the continent. As a consequence most African countries have switched their first-line drug to SP but unfortunately the efficacy of this drug in Africa is progressively deteriorating (Bjorkman and Phillips-Howard 1990). In west or central Africa, and in Madagascar, around 40% of *P.falciparum* isolates remained sensitive to CQ. It is important to note that SP efficacy life span was relatively short compared to CQ because *P. falciparum* has developed resistance to SP within only five years of introducing the drug while CQ have been in use for several decades before the emergence of CQ resistance in Asia. Difference in the rate of development of resistance towards SP and CQ could be attributed to their mode of action and the target molecules involved. CQ resistance could involve multiple genes whereas the target molecules for SP are fairly well established (Wongsrichanalai, Pickard et al. 2002; Berry, Vessiere et al. 2004; Talisuna, Bloland et al. 2004; Sharma 2005).

## Mechanisms of drug resistance

Many factors contribute to development and spread of resistance, including the interaction of drug pattern, characteristics of drug itself, human host, parasite, vector and environment factors.

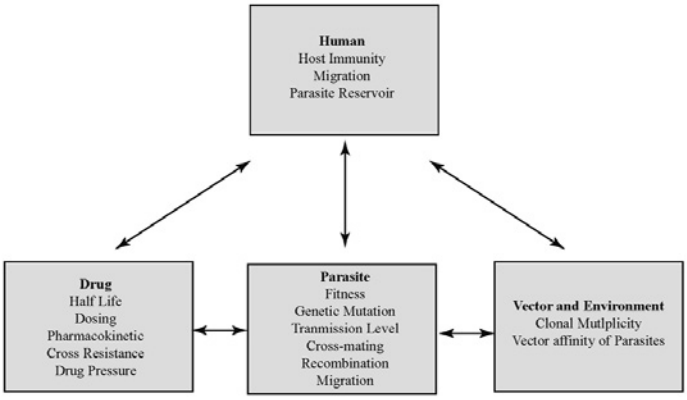



**Figure 4:** Factors and characteristics affecting spread of malaria drug resistance

## Fitness of drug-resistant mutants

Anti-pathogenic drugs target metabolic and structural proteins, which are essential for growth and multiplication. Therefore, genetic changes (mutations) in genes whose products are associated with these proteins/processes can encode resistance but often have deleterious effects (Abdel-Muhsin, Mackinnon et al. 2003). Consequently, development of drug resistance may incur a fitness cost in the absence of drug selection. This has been demonstrated in many pathogens, including parasites (Binks, Baum et al. 2001), bacteria (Maiga, Djimde et al. 2007) and viruses (McCollum, Poe et al. 2006).

For example, in HIV-1 virus, it has been shown that mutations in a protease gene causing resistance to a protease inhibitor can markedly reduce the virus replication capacity (McCollum, Poe et al. 2006). Drug-resistant mutant forms of HIV, have been found to infect patients approximately 20% less than might be expected (Certain, Briceno et al. 2008). Fitness costs may manifest as reduced within-host growth and/or duration of infection and/or increased clearance of resistant pathogens. Ultimately the cost is paid by reduced transmission between hosts (Binks, Baum et al. 2001; Su, Ferdig et al. 1999; Mehlotra, Mattera et al. 2008). Fitness cost is often measured via controlled *in vitro* laboratory experiments in culture or animal models in absence of drug. Despite their limited fitness, resistant pathogens do not always revert to wild-type when serially passaged in drug-free conditions. However, additional compensatory mutations can be acquired and ameliorate the fitness cost associated with antimicrobial resistance (Mehlotra, Mattera et al. 2008). With regard to malaria parasites, the question of whether drug-resistant mutants can be competitively suppressed by the sensitive forms, in the absence of the drug, was first considered decades ago (Rosario 1976). However, unambiguous experimental evidence of competitive superiority of sensitive parasites is limited due to the laborious nature of

laboratory competition analysis and ethical issues associated with adequate study design in the field. Here we review accumulating epidemiological and laboratory evidence consistent with fitness deficit in drug resistant malaria parasites and discuss its implications for spread and management of drug resistance.

An early opportunity to monitor the effect of discontinued therapy on the survival of drug resistant *P. falciparum* parasite arose after the withdrawal of pyrimethamine-based medicated salt as a feasible effective control measure in 1950s. Following cessation of the programme in Tanzania and withdrawal of the drug, the prevalence of pyrimethamine sensitive *P. falciparum* parasites revived significantly (Clyde 1967). However, the resistant parasite remained in the area for several years after withdrawal of the drug, demonstrating that some resistant parasites had persisted in the absence of the drug. In this case a competitive advantage of pyrimethamine sensitive parasite was noticeable; nonetheless the persistence of the resistant clones can be explained by development of stabilizing compensatory mutation. Broad epidemiological observations suggest that drug resistant forms of *P. falciparum*, once developed, can persist despite intermittent use of therapy. However, their prevalence parallels the magnitude and pattern of drug usage. Large scale molecular surveys in Mali, demonstrated that pyrimethamine resistant genotypes concurrent with higher SP usage, were high in urban areas where the drug is readily accessible and low in rural areas with limited drug dispersal (Plowe, Djimde et al. 1995; Plowe, Djimde et al. 1996). These results illustrate the possible selective disadvantage of drug resistance in an environment of reduced drug pressure or, more simply, that selection is less intense in rural areas so that resistance is spreading more slowly. Similar findings have been reported from southern Mozambique, in an area neighbouring KwaZulu-Natal, South Africa. Longitudinal surveys have revealed a clear parallel between prevalence of *dhfr* and *dhps* mutations and deployment of SP. The frequency of *dhps* double mutations in the area peaked in 2001 (0.22) but declined to baseline levels (0.07) by 2004. Similarly, parasites with both *dhfr* triple and *dhps* double mutations had increased in 2001 (0.18) but decreased by 2004 (0.05). The peaking of SP resistance mutations in 2001 coincided with a SP-resistant malaria epidemic in neighbouring KwaZulu-Natal, while the reduction in mutant genotypes corresponded with replacement of SP with artemether-lumefantrine in KwaZulu-Natal (Raman, Sharp et al. 2008).

Additional associations between community usage of SP and prevalence of resistant genotypes came from the outcomes of an effective vector control trial. The use of insecticide treated bednets (ITNs) in villages in Tanzania has been associated with significant decrease in *P. falciparum* resistant genotypes, presumably because ITNs reduces transmission, disease incidence, drug usage and selective advantages of resistant parasites. The prevalence of mutant *dhfr* and *dhps* genotypes in children less than five years old

living in the above villages reduced dramatically during 1998 to 2000 following ITNs use. Conversely, the prevalence of *dhfr* wild type has increased significantly (Alifrangis, Lemnge et al. 2003). This matches prediction of drug resistance models which suggest that lowering transmission can reduce the spread of drug resistance (Hastings 1997; Mackinnon and Hastings 1998). Although the interaction can be complex with minimal selection at intermediate levels of transmission depending on the underlying genetics of resistance (Hastings 2003).

Further evidence for lower fitness of pyrimethamine resistant parasite, in the face of lower drug pressure, has been inferred from the observation of an inverse relation between age and prevalence of resistant *dhfr* genotypes (Mockenhaupt, May et al. 1999). Since infections in older children are generally asymptomatic, it could be speculated that the survival advantage of mutant genotype populations is limited in the absence of therapy. In infants and younger children with higher incidence of malaria resistant genotype can have a selective advantage and may consequently be more prevalent in this age group. On the other hand the nature of frequent asymptomatic parasite carriage among older semi-immune children and adults can present an unfavorable environment for the resistant parasite. Among asymptomatic parasite carriages the selective disadvantage of resistant parasite can be amplified by continuous asexual parasite multiplication over time. Such a pattern has been seen among asymptomatic parasite carriers, during the dry season, in areas with seasonal transmission. (Abdel-Muhsin, Mackinnon et al. 2003).

# Genes involved in resistance to Chloroquine and Sulfadoxine - Pyrimethamine

Here mainly four genes relevant to the study are discussed, the protective chloroquine transporter genes *Pfcrt* and *Pfmdr1*, which are associated with resistance to chloroquine and genes encoding dihydrofolate reductase *dhfr* and dihydropteroate synthase *dhps* that are associated with resistance to Pyrimethamine-Sulfadoxine (SP).

## *P. falciparum* Chloroquine transporter gene (*Pfcrt*)

The gene is located on chromosome 7 and encodes *P. falciparum* chloroquine resistance transporter protein, a trans-membrane protein located in the membrane of the digestive vacuole. (Fidock, Nomura et al. 2000; Tinto, Ouedraogo et al. 2003; Berry, Vessiere et al. 2004). Transfection studies have also demonstrated a link between *P. falciparum* resistance to CQ and mutations in the *Pfcrt* gene, especially the substitution of threonine to lysine

at position K76T was found to be an important mutation associated with CQR. However, other mutations in the *Pfcrt* gene at positions 72 to 76, appears to play a crucial role in CQR (Foote, Kyle et al. 1990; Reed, Saliba et al. 2000; Babiker.HA 2002). Thus CQR appears as a progressive accumulation of mutations in the *Pfcrt* gene where K76T mutation seems to have central role leading to CQ clinical failure and is now used as a marker for CQR surveillance (Djimde, Doumbo et al. 2001; Sidhu, Verdier-Pinard et al. 2002; Lakshmanan, Bray et al. 2005).

## *P. falciparum* Multi Drug Resistance gene (*Pfmdr1*)

The *Pfmdr1* gene, located on chromosome 5 and encoding a P-glycoprotein homologue1 (Pgh1), has generated interest in resistance to chloroquine and other antimalarials. The aspartic acid to tyrosine point mutation in codon 86 has been associated with chloroquine resistance in some clinical and in vitro studies (Sudan), while several other polymorphisms notably Phe184,Cys 1034, Asp1042 and Tyr 1246 have been implicated to varying degrees in chloroquine resistance , and although evidence for association of *Pfmdr1* with chloroquine is still un clear, polymorphism in *Pfmdr1* may be associated with resistance to chloroquine.

## *P.falciparum dhfr/dhps*

Sulfa drugs are an important group of antimalarial compounds and have generally been used in combination such as Sulfadoxine and Pyrimethamine (**Fansidar™**). The existence of a *de novo* folate biosynthetic pathway in *Plasmodium* has been inferred by the ability of sulfa drugs to inhibit the growth of parasites (Sherman 1979). The major enzyme targets of malaria chemotherapeutic in the folate biosynthetic pathway are (*DHFR)* dihydrofolate reductase and (*DHPS)* dihydropteroate synthase. Pyrimethamine is a potent inhibitor of *DHFR,* which plays three main roles in the folate pathway in *P. falciparum*. It controls *de novo* folate synthesis by catalyzing the synthesis of tetrahydrofolate (THF). It also mediates the salvage of exogenous folate derivatives, 7,8-dihydrofolate (DHF) and the fully oxidized folate, by reducing them to THF. *DHFR* enzyme is also capable of recycling DHF from 2'-deoxythymidine-5'-monophosphate (dTMP) synthesis, by reducing it to THF so that it can re-enter the folate pool **(Figure 5).**

Four point mutations in the *dhfr* gene can alter *P. falciparum* drug response because of changes in the binding active site cavity of *dhfr* enzyme (Cowman, Morry et al. 1988). A change resulting in (S108N) is considered to be the major determinant of pyrimethamine resistance. Additionally, other successive mutations (N51I), (C59R), (I164L) (Peterson, Walliker et al. 1988) were found to confer higher levels of drug resistance than does the mutation (S108N) alone (Plowe *et al.,* 1997; Peterson, Walliker et al. 1988)

However, mutations at codon 164 are not common in Africa but wide spread in Asia (Nzila 2006). It has been suggested that, these mutations occurs in a cumulative (Peterson, Walliker et al. 1988) stepwise fashion in the field (Plowe, Cortese et al. 1997). Sulfadoxine inhibits dihdropteroate synthase DHPS the enzyme is involved in folate synthesis, mediating the synthesis of dihydropteroate. The inhibition of *dhps* activity decreases the synthesis of dihydropteroate, which leads to reduced DHF synthesis, as the amount of DHF, the substrate of *dhfr*, is decreased, the activity of *dhfr* inhibitor increases. In this way, concomitant blockage of DHF synthesis and the inhibition of *dhfr* results in the synergistic action and Interruption of the DNA replication process. Five point mutations have been implicated in resistance by decreasing the binding affinity of enzyme, at codons **S**436**A/F**, **A**437**G** ,**K**540**E**, **A**581**G**, **A**613**T/S**. The triple mutant (437**G**,540**E**,581**G**), (Brooks, Wang et al. 1994; Triglia and Cowman 1994) *dhps* alleles is common seen in parasite populations that already carry the triple mutant *dhfr* allele and has been linked to increased resistance to SP (Plowe, Cortese et al. 1997; Nzila, Mberu et al. 2000; Happi, Gbotosho et al. 2005; Kublin, Dzinjalamala et al. 2002). In vivo combination of mutationsin *Pfdhfr/dhps* has been associated with SP treatment failure (Wongsrichanalai, Pickard et al. 2002).

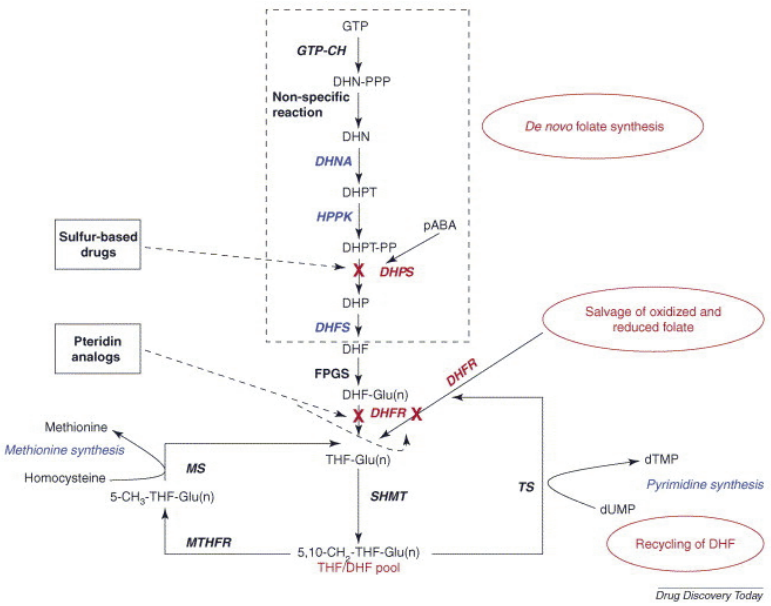



**Figure 5:** Folate biochemistry in *P. falciparum*: Note the role of *dhfr* in the pathway where it controls denovo folate synthesis by blocking the synthesis of THF, mediates the salvage of exogenous folate DHF and the fully oxidized folate, by reducing them to THF as well as DHF recycling from 2'deoxythymidine5'-monophosphate (dTMP) synthesis, by reducing it to THF enabling it to re-enter the folate pool. Source: Drug Discov Today (Nzila 2006).

## Origin and evolution of drug resistance

Two processes are necessary for evolution of drug resistance. First, a resistant genotype is generated by mutation; second, the spread of this mutation within and between parasite populations takes place. (Anderson and Roper 2005). Mutation rates have been measured in *Plasmodium* genes and were found to be in the order of $10^{-9}$ (Paget-McNicol and Saul 2001), and studies have suggested that numbers of parasites per infected host can reach $10^{10–12}$ (White, Nosten et al. 1999), hence within a single host it is probable to see around 10–1000 origins of point mutations conferring resistance. However, data from different studies suggest that mutations are rarely established within parasite populations and spread of resistant parasites could have evolved from few origins rather than evolving repeatedly. (Anderson and Roper 2005).

Recent molecular surveys have shown that resistance to commonly used anti-malarial drugs has rather few independent genotypes. This suggests that *de novo* mutations, that confer resistance to these drugs, do not occur frequently. However, limited number of drug resistant genotypes is spreading across different parasite populations and then increase in frequency to hinder management and control measures. The spread of limited drug resistant genotype(s) can lead to removal of genetic variation from the chromosomal regions surrounding the selected site, such as flanking neutral polymorphisms which are carried along with a mutation site resulting in what can be described as hitchhiking. The spread of the selected allele also results in increased linkage disequilibrium (LD) with flanking markers and skews the allele frequency spectra at loci nearby on the chromosome. The size of genomic regions affected is influenced by the strength of selection, as well as the rates of recombination and mutation. These characteristic patterns of variation are tools for identifying regions of the genome that are under selection (Pearce, Malisa et al. 2005). Many factors have been suggested to contribute to the idea that resistant mutations have raised from few independent origins: these include the followings:

1. Only 1% of the parasite lineages are committed to become gametocytes and transmission stage and hence resistance alleles must be found within this small proportion to be passed into the second generation.
2. Only mutations occurring in parasites expressing recently switched *var* gene are likely to escape immune surveillance and achieve transmission because this gene is a major target for destruction by the immune system of the host.
3. It is suggested that mutations occur spontaneously, starting with a parasite with a single mutation spreading through a population, followed by parasites among them which have accumulated additional

mutation and again spreading among the population and so on. This process results in sequential bottlenecks in the population of resistant alleles.

4. Fitness is another factor playing a major role in limiting the spread of resistant parasite due to the fact that parasites with resistant allele are less fit for survival compared to wild parasites, the probability of their transmission become dramatically less unless they acquire compensatory mutation that enables them to restore their fitness after the mutation event or having an advantage of selective pressure such as drug pressure.

## Malaria in Eastern Sudan

Eastern Sudan is an area with a poor savannah where malaria is considered to be hypoendemic and transmission is restricted to the period of October to December following the main rain season. From January to June, the area become dry and rarely any cases of malaria would be reported. *P. falciparum* is the predominant parasite species accounting for more than 90% of malaria cases, while *P. vivax* occurs at a frequency of about 5%. *Anopheles arabiensis* is the main mosquito vector. The mean annual entomological inoculation rate (EIR) has been estimated to be less than one infective bite per person during the transmission period (Hamad, Nugud Ael et al. 2002).

A large proportion of individuals who contract malaria in the transmission season, retain long lasting *P. falciparum* infection at an asymptomatic sub-patent level. Such infections are capable of producing gametocytes and hence are capable of infecting mosquitoes that appear following the annual rains. *P. falciparum* parasites that cause clinical malaria during the transmission season were found to carry multiple clone of infection per patient and hence characterized by great diversity, suggesting that these parasites belong to a larger parasite reservoir existing prior to the start of the transmission season (Babiker, Abdel-Muhsin et al. 1998; Abdel-Wahab, Abdel-Muhsin et al. 2002).

## Evolution of drug resistance in Eastern Sudan

Studies in villages in eastern Sudan have clearly demonstrated the presence of *P. falciparum* clones with a wide range of response to chloroquine and pyrimethamine along with clinical failure to the two drugs. Chloroquine resistance (CQR) first emerged in this area in the mid 1980s (Bayoumi, Babiker et al. 1989), however subsequent surveys demonstrated that the rate of parasitological failure to CQ remained stable over a period of seven years (1986 - 1993). Nevertheless, the CQR level varied between years in associa-

tion with the amount of annual rain. Resistance to SP is well established in Asar and other neighboring villages in the region and during the course of this study chloroquine (CQ) and SP were in use in the village (Babiker, Satti et al. 1995; Ali, Mackinnon et al. 2006; IE, Elbashir et al. 2006).However recently CQ has been replaced by combination therapy, artesunate plus sulfadoxine/pyrimethamine (AS+SP) in Sudan (Malik, Mohamed et al. 2006). Detailed molecular surveys revealed significant temporal fluctuations in the frequency of resistant *P. falciparum* genotypes, increasing during the dry season but waning at the start of the next transmission season (Abdel-Muhsin, Mackinnon et al. 2004). When *P.falciparum* in Sudan is compared to other African countries, parasite population in Sudan appears to be less diverse which could be influenced by relatively concise transmission period. (Hill and Babiker 1995; Conway, Roper et al. 1999).

## Malaria in The Gambia

Malaria in the Gambia is seasonal and hyper-endemic, occurring mainly in the dry season from July to November with a peak in September (Greenwood and Pickering 1993). The entomological inoculation rate (EIR) is more than thirty infected bites/person/year (Lindsay, Wilkins et al. 1991). The Medical Research Council (MRC) maintains an ongoing Demographic area 170 km from Atlantic coast. Resistance to SP is well established in Farafenni and other neighboring villages in the region and during the course of this study Chloroquine (CQ) and SP were in use in the village.However recently CQ has been replaced by combination therapy, artesunate plus sulfadoxine/pyrimethamine (AS+SP) in the Gambia.

# Aim of the Study

1. To characterize *Pfmdr1* and *Pfcrt* genes associated with chloroquine resistance among parasites that persist during the lengthy dry season in eastern Sudan.
2. To examine the origin of *dhfr* and *dhps* genes associated with Sulphadoxine- Pyrimethamine (SP) resistance in a low transmission area in eastern Sudan.
3. To examine the ability of different *P. falciparum* clones within a single infection to produce gametocytes and infect *Anopheles* mosquitoe*s* in The Gambia
4. To examine transmission capacity of different *dhfr* resistance haplotypes in The Gambia; and compare *dhfr* haplotypes detected there with those seen in other African countries.

# Materials and Methods

## Study Sites

Studies in this thesis were conducted in two African countries, eastern Sudan and The Gambia.

### (i) Eastern Sudan

The study was performed in Asar village, Gedaref state, eastern Sudan, where the transmission period for malaria is short (6-8 weeks) and distinctly seasonal, following the annual rains in July to November, and Peaks in October. By January the number of malaria cases decreases substantially and entomological surveys find no evidence of transmission during the long dry season.

**Longitudinal Surveys:**

In eastern Sudan I have examined *P.falciparum* isolates collected in two longitudinal surveys and a third cross sectional survey.

*(a) Cohort1: Longitudinal survey Oct 1993 to Dec 1994*

The first longitudinal survey was conducted Oct1993 to Dec1994, a cohort of 83 patients were followed for 15 month and here we reported 25 patients who showed persistent parasitemia during the whole period (Paper 1).

*(b) Cohort 2: Longitudinal survey Oct 2001 to Dec 2002*

In the second longitudinal survey samples were collected Oct 2001 to Dec 2002, 113 patients were monitored for 15 month and here we are reporting data from 38 patients (Paper I).

*(c) Cross sectional survey Oct 2003*

Finger –prick blood samples were obtained from patients attending the clinic in Asar Village, cross sectional survey were performed during the dry seasons 2003 for 239 patients. (Paper II).

### (ii) The Gambia

The study took place at the Medical Research Council (MRC) field station in Farafenni, The Gambia (13° 28´N, 16° 34´ W). Malaria in the Gambia is seasonal and hyper-endemic; occurring mainly during the rainy season from July to November with a peak in September.The MRC maintains an ongoing Demographic Surveillance System (DSS) covering Farafenni town and 40 surrounding villages. Five study villages within 40 km radius of Farafenni town were selected from the DSS, based on similarity of malaria transmission indices such as EIR, sporozoites rates and distance from mosquito breeding sites.

## Asymptomatic Parasite carriers

Malaria screening clinics were held in the villages of Yallal, Alkali,Kunda, Jarjari, Daru and Dai Mandinka to identify gametocyte carriers during the dry season (April/May) of 2003. Prior to this consultations and meetings with village heads and traditional rules were held to obtain village-wide consent. Blood samples was collected from each consenting inhabitant for thick films for malaria diagnosis and packed cell volume (PCV) estimation. Thick film slides were stained and read over 200 high power fields (HPF) within 2 h of consent Inclusion criteria to participate in the study were presence of at least one gametocyte in 200 HPF; PCV 24%; age between five and 45 years; absence of fever (temperature 37°C) or history of fever in the last 2-3 days. A total of 950 villages were screened in April/May 2003 in Farafenni area.

## Symptomatic drug- treated patients

In the transmission season (August 2003), gametocyte production and transmission was examined as part of an efficacy trial of three combinations of antimalarials : Amodiaquine(AQ) plus Artesunate (AS), AQ plus Sulfadoxine and Pyrimethamine (SP) and Chloroquine (CQ) plus SP. Briefly, children aged 0.5-10 years presenting with symptoms of acute malaria infection, including axillary temperature 37.5°C, *P. falciparum* parasitemia ranging between 2000 and 200,000parasites/µl and a PCV 20% were enrolled in the study after obtaining informed consent from their parents or guardians. Study participants were randomised into three groups to receive one of the above combination therapies. Approximately 3 ml of venous blood was collected from gametocyte-positive individuals and processed for mosquito infection experiment as described below (Targett, Drakeley et al. 2001).

From the same blood samples, 30µl was spotted on filter paper for DNA isolation, and approximately 500µl was processed for RNA extraction.

## Mosquito Infectivity

The mosquito infectivity assay was carried out (Targett, Drakeley et al. 2001). Briefly, venous blood in citrate-phosphate dextrose was centrifuged and the plasma was removed. After being washed in RPMI, red blood cells (RBCs) were resuspended to a PCV of 33% in pooled AB serum from European donors with no history of malaria. The suspension was then fed to 3-5 day old female laboratory reared *Anopheles gambiae* mosquitoes, via an artificial membrane attached to a water-jacketed glass feeder maintained at 37°C. Approximately 50 mosquitoes were fed on each blood sample for 30 min, following which unfed mosquitoes were removed and the fully engorged ones kept on sugar solution until dissection 7 days later. Surviving mosquitoes were dissected and their midguts examined for oocysts under dissecting microscope. Infected midguts were transferred into oocyst lysis buffer, incubated for 1 h at 55°C and then stored at –20°C for DNA isolation and PCR (Ranford-Cartwright, Balfe et al. 1991).

## Genotyping

### Blood Sampling and Storage

Blood samples were collected from asymptomatic adults and children, 2-3 ml of venous blood samples was collected from each participant for RNA isolation and mosquitoes infection experiments, at the same time three to four drops of blood from each individual spotted onto a filter paper (Whatman 3M), left to dry and stored in sealed plastics bags.

### DNA extraction

DNA was extracted from blood on filter paper using the Chelex method for analysis of *Pfcrt, Pfmdr1*, *Pfdhfr* and *Pfdhps* for paper I and II (Plowe, Djimde et al. 1995). Qiagen Micro Isolation Kit (Qiagen, UK), was a second method used for extracting DNA from oocysts from mosquito (mid gut) on filter paper (Paper III and IV).

### RNA isolation

In paper III RNA was isolated from infected blood using ABI Prism TM 610 Nucleic acid Prep_station to detect the presence of mRNA from *Pfg 377*,

which will only be produced by the gametocyte. The extract was digested with DNAase for 15 min at 37 C to remove co-extracted DNA.

## RT-PCR

Reverse transcriptase was used to detect the presence of mRNA *Pfg377* alleles in patients and mosquitoes fed on their blood samples, using RT-PCR and detection indicates presence of gametocytes. PCR products for *Pfg377* were seperated by electrophoresis using high resolution Metaphor™ agarose gels, and sized using ID image Analysis soft ware (Eastman Kodak,UK).

## PCR-RFLP

Two round of PCR (outer and nested) were used to amplify *Pfcrt* by primers flanking the polymorphic residue K76T and *Pf mdr-1* gene Y86N and were then digested using restriction enzyme Apo1 that can differentiate wild and mutant alleles. Typing haplotypes of SP drug resistance genes, *Pfdhfr* and *Pfdhps*, are described on paper II and IV

PCR products and restriction products were analysed on 1.5-2% agarose gels with ethidium bromide and were visualized by UV Transilluminator in a Bio Rad Gel Doc system PCR products were excised and DNA was extracted using Gene clean Kit (Roche,UK).

*Msp-1* was genotyped from children and infected mosquitoes using PCR for the three different alleles K1, Mad20 and RO33 on day zero and day 7, post treatment and in mosquito's oocysts.

## Cloning

PCR products were cloned after purifying the DNA by carrying out a 5-min ligation reaction, using TOPO TA cloning Kit (Invitrogene, UK). Single colonies were checked for inserts after DNA extraction and inserts were sequenced.

## Microsatellite Analysis

Fragment analysis was carried out to determine variation of microsatellites flanking the drug resistance genes, three loci each located 0.3, 4.4 and 5.3kb from position 108 of the *dhfr* gene and 0.8, 4.3 and 7.7kb from the 3`end of the *dhps* gene.

Fluorescent PCR products were analysed in ABI 310 sequencer and alleles were visualized and sized on gene scan (Applied BioSystem). Multiple clones infection in the study area with more than one allele of microsatellites was scored compared to the predominant allele per locus as described on paper II.

### Sequencing

Nested PCR products covering the *Pfcrt* 72-76 haplotypes and relevant parts of the *Pfdhfr*, *Pfdhps* and *Pfg377* were cleaned using EXO SAP-IT R (USB), and sequenced using the cycle sequencing reaction with ABI Big Dye terminator version 3.1 before separation of reaction products on an ABI 3730 DNA analyzer.

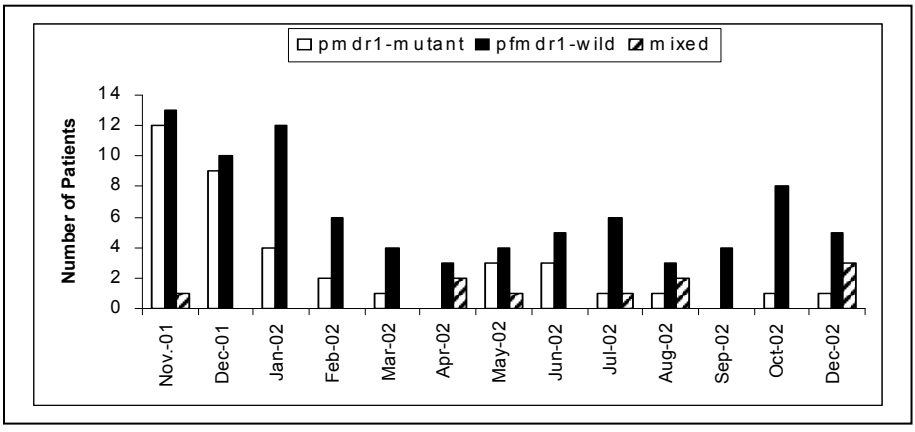
## Ethics

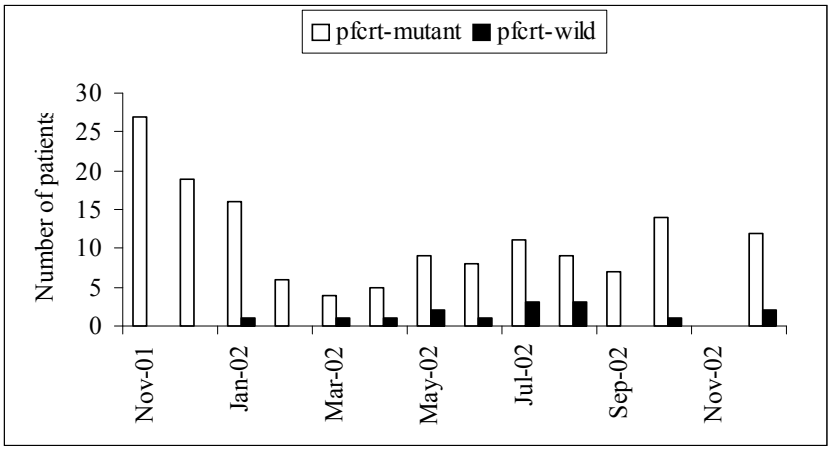
Ethical clearances were obtained from health ministry in Sudan and by the Medical Research Council Scientific Coordinating Committee MRC/Gambian government; Informed consent was obtained from all participants or guardian of the children involved in the studies

# Results

## Paper I

The aim of the study was to estimate the fitness of drug resistant malaria parasites in the absence of drug pressure in the dry season in a low transmission area in eastern Sudan. *Pfcrt* K76T and *Pfmdr1* N86Y were analysed by PCR-RFLP and sequencing for all the cohort study starting from the first transmission wet season, during the whole dry season until the second transmission season. In both cohorts 1993-1994 and 2001-2002 and following treatment mutant alleles of *Pfcrt* showed higher frequency, which is a reflection of selection. At the start of the dry season wild alleles started to emerge in the two cohorts. Mutant *Pfmdr1* allele gave a similar pattern in the two tested cohorts, they existed at a high frequency in the dry season and the new infection that appeared at the second transmission season was encountered by a wild type. So in areas of seasonal transmission and fluctuations between wet-dry seasons, success of resistant parasites is limited, especially when the drug that selected for the resistance is removed.






The fitness cost of *Pfcrt* K76T and *Pfmdr-1* mutation in the absence of drug led to a decline in frequency due to selective disadvantage among asymptomatic parasite carriers in the dry season. A high degree of fluctuation between different parasite clones was seen in the absence of mosquito transmission suggesting fluctuation by competitive interaction within the human host. Follow up have shown similar results, all patients maintained persistent parasitemia through out the dry season when there is no drug pressure.

## Paper II

The aim of the study was to examine mutations within *Pfdhfr* and *Pfdhps* associated with SP resistance and the neighboring microsatellites among isolates in Asar village in eastern Sudan and to see if resistance alleles have developed locally or migrated from another region.

In Asar village in eastern Sudan, findings revealed that *Plasmodium falciparum* parasites have some distinct genetic features and the mutant *Pfdhfr* and *Pfdhps* alleles were responsible for drug resistance of SP and has shed light on the origin and pattern of spread of some anti-malarial drugs. Recent surveys revealed spread of a high level pyrimethamine resistant lineage of *Plasmodium falciparum* of Asian origin across Africa. In contrast our data have shown 84 out of 94 examined isolates carried double mutations at the *dhfr* locus (N51I and S108N), but the C59R and I164L mutations were not seen. Similarly the majority of the isolates carried double mutations in *dhps* (437G and 540 E), in 35 out of 81 isolates. Analysis of neighboring microsatellites revealed one major *dhfr* haplotype with mutations (51I,108N) and one *dhps* haplotype with mutations (436S,437G,540E). These haplotypes differ from the ones thought to drive resistance to SP across Africa. The resistant haplotypes of *dhfr* and *dhps* in Asar share some microsatellites with

the wild genotypes suggesting that they were generated locally. Among isolates successfully examined 40% shared identical haplotypes of the 2 loci comprising a dominant resistant lineage and this lineage plays an important role in clinical failure to SP in this area.

Table 1: *dhfr* haplotypes defined by mutations in positions amino acids 51, 59 and 108, and neighboring microsatellite loci located 0.3, 4.4 and 5.3 kb downstream *dhfr* gene among 94 *P. falciparum* isolates in Asar village eastern Sudan.

| Haplotype | *dhfr* genotype | Microsatellite loci | | | Prevalence |
|---|---|---|---|---|---|
| | | 0.3 kb | 4.4 kb | 5.3 kb | |
| 1 | 51N, 59C, 108S | 84 | 175 | 199 | 3 (3%) |
| 2 | 51N, 59C, 108S | 84 | 175 | 206 | 1 (1%) |
| 3 | 51N, 59C, 108S | 84 | 175 | 213 | 1 (1%) |
| 4 | 51N, 59C, 108S | 84 | 175 | 219 | 1 (1%) |
| 5 | 51N, 59C, 108S | 84 | 172 | 195 | 1 (1% |
| 6 | 51N, 59C, 108S | 84 | 172 | 213 | 1 (1%) |
| 7 | 51N, 59C, 108S | 84 | 170 | 212 | 1 (1%) |
| 8 | 51N, 59C, 108S | 84 | 163 | 218 | 1 (1%) |
| 9 | 51I, 59C, 108N | 107 | 184 | 210 | 1 (1%) |
| 10 | 51I, 59C, 108N | 84 | 155 | 192 | 1 (1%) |
| 11 | 51I, 59C, 108N | 84 | 172 | 192 | 1 (1%) |
| 12 | 51I, 59C, 108N | 84 | 175 | 191 | 69 (73%) |
| 13 | 51I, 59C, 108N | 84 | 175 | 194 | 1 (1%) |
| 14 | 51I, 59C, 108N | 84 | 175 | 312 | 2 (2%) |
| 15 | 51I, 59C, 108N | 84 | 175 | 349 | 1 (1%) |
| 16 | 51I, 59C, 108N | 84 | 175 | 379 | 1 (1%) |
| 17 | 51I, 59C, 108N | 84 | 177 | 189 | 1 (1%) |
| 18 | 51I, 59C, 108N | 84 | 179 | 192 | 1 (1%) |
| 19 | 51I, 59C, 108N | 84 | 180 | 192 | 4 (4%) |
| 20 | 51I, 59C, 108N | 84 | 191 | 199 | 1 (1%) |

Table 2: *dhps* haplotypes defined by mutations in positions amino acids 436, 437, 540 and 581, and neighboring microsatellite loci located 0.8, 4.3 and 7.7kb downstream *dhps* gene among 81 *P. falciparum* isolates in Asar village eastern Sudan.

| Haplotype | dhps genotype | Microsatellite loci | | | Prevalence |
|---|---|---|---|---|---|
| | | 0.8 kb | 4.3 kb | 7.7 kb | |
| 1 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 113 | 108 | 116 | 1 (1%) |
| 2 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 115 | 104 | 114 | 1 (1%) |
| 3 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 115 | 108 | 100 | 1 (1%) |
| 4 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 116 | 104 | 94 | 1 (1% |
| 5 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 120 | 100 | 94 | 1 (1%) |
| 6 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 120 | 104 | 94 | 1 (1%) |
| 7 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 120 | 106 | 123 | 1 (1%) |
| 8 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 120 | 106 | 118 | 1 (1%) |
| 9 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 120 | 108 | 94 | 2 (2%) |
| 10 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 124 | 77 | 94 | 1 (1%) |
| 11 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 124 | 101 | 96 | 1 (1%) |
| 12 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 124 | 106 | 116 | 1 (1%) |
| 13 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 124 | 106 | 94 | 1 (1%) |
| 14 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 124 | 108 | 114 | 1 (1%) |
| 15 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 124 | 108 | 106 | 1 (1%) |
| 16 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 124 | 124 | 114 | 1 (1%) |
| 17 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 126 | 101 | 161 | 1 (1%) |
| 18 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$A | 130 | 104 | 114 | 1 (1%) |
| 19 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$A | 118 | 112 | 94 | 2 (2%) |
| 20 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$A | 120 | 104 | 94 | 1 (1%) |
| 21 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$A | 120 | 106 | 94 | 1 (1%) |
| 22 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$A | 120 | 110 | 94 | 1 (1%) |
| 23 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$A | 120 | 112 | 94 | 35 (43%) |
| 24 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$A | 120 | 112 | 96 | 3 (4%) |
| 25 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$A | 120 | 112 | 189 | 1 (1%) |
| 27 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$A | 120 | 120 | 96 | 1 (1%) |
| 28 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$G | 120 | 104 | 94 | 1 (1%) |
| 29 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$G | 120 | 112 | 94 | 9 (11%) |
| 30 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$G | 120 | 112 | 96 | 1 (1%) |
| 31 | $_{436}$S,$_{437}$G, $_{540}$E, $_{581}$G | 128 | 106 | 128 | 1 (1%) |
| 32 | $_{436}$F,$_{437}$G, $_{540}$E, $_{581}$A | 120 | 112 | 94 | 2 (2%) |
| 33 | $_{436}$S,$_{437}$G, $_{540}$K, $_{581}$A | 128 | 106 | 128 | 1 (1%) |
| 34 | $_{436}$S,$_{437}$A, $_{540}$K, $_{581}$G | 120 | 106 | 94 | 1 (1%) |
| 35 | $_{436}$F,$_{437}$A, $_{540}$E, $_{581}$A | 120 | 112 | 94 | 1 (1%) |

Previous studies in eastern Sudan found *P.falciparum* have some distinct genetic characteristics and this can be due to geographical isolation and the pattern of low seasonal transmission compared to high transmission areas of sub-Saharan African. Asar village showed double mutant *dhfr* allele and the triple mutant of *dhps* allele, which has arisen from the high prevalence background of the double mutant, and this contributes to the SP failure.

# Paper III

The aim of the study was to examine the genetic complexity of *P.falciparum* gametocytes and their infectivity to mosquitoes in the Gambia.

We have seen high degree of complexity and clonal multiplicity of *P.falciparum* gametocyte among asymptomatic infections that persist at sub-microscopic level in the dry season; some of these infections were capable of infecting mosquitoes even when they existed at sub-patent levels in the dry season. The *Pfg 377* gene in asexual forms was detected only in 60 % of the examined individuals. However, using RT-PCR which detects RNA showed higher sensitivity. Some parasite genotypes were found in infected mosquitoes which had been undetectable in the blood on which the mosquitoes were fed. The results showed the ability of different *P.falciparum* clones to transmit from infected individuals to mosquitoes and cross-mate and produce new strains for the next generation.

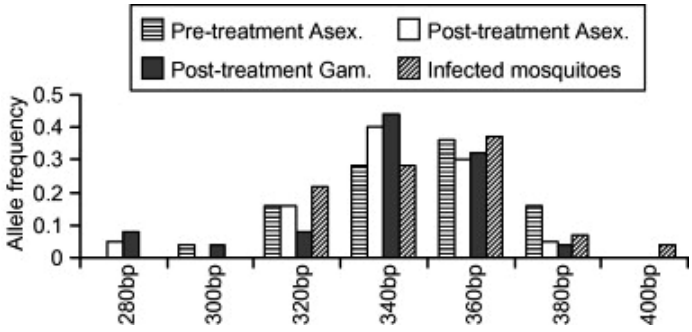
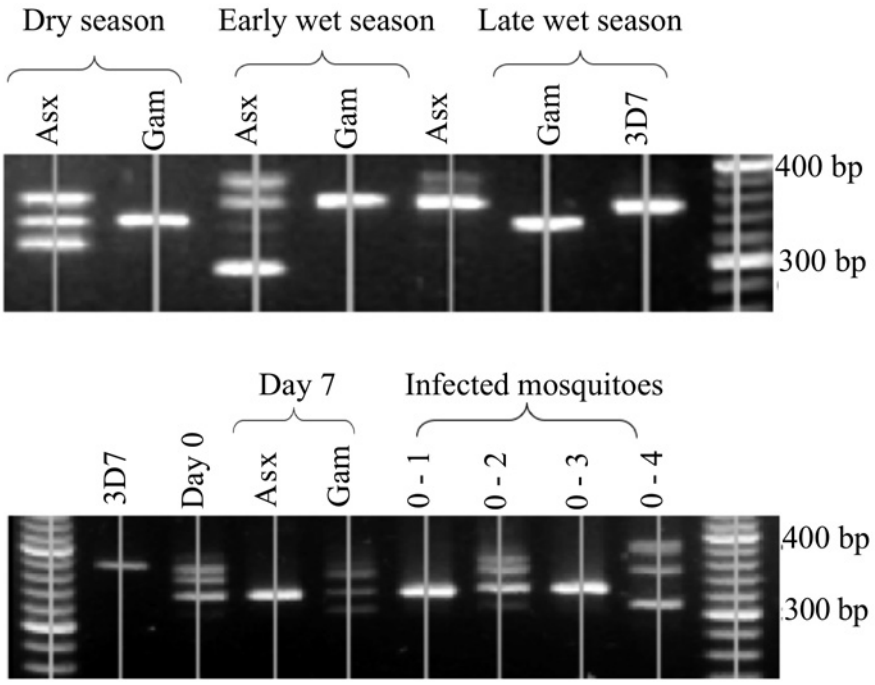



*Fig 2.Pfg377* alleles detected in *Plasmodium falciparum* asexual form, gametocytes and infected mosquitoes in a cohort of 22 drug-treated patients.Asex (Asexual forms), Gam (Gametocytes).



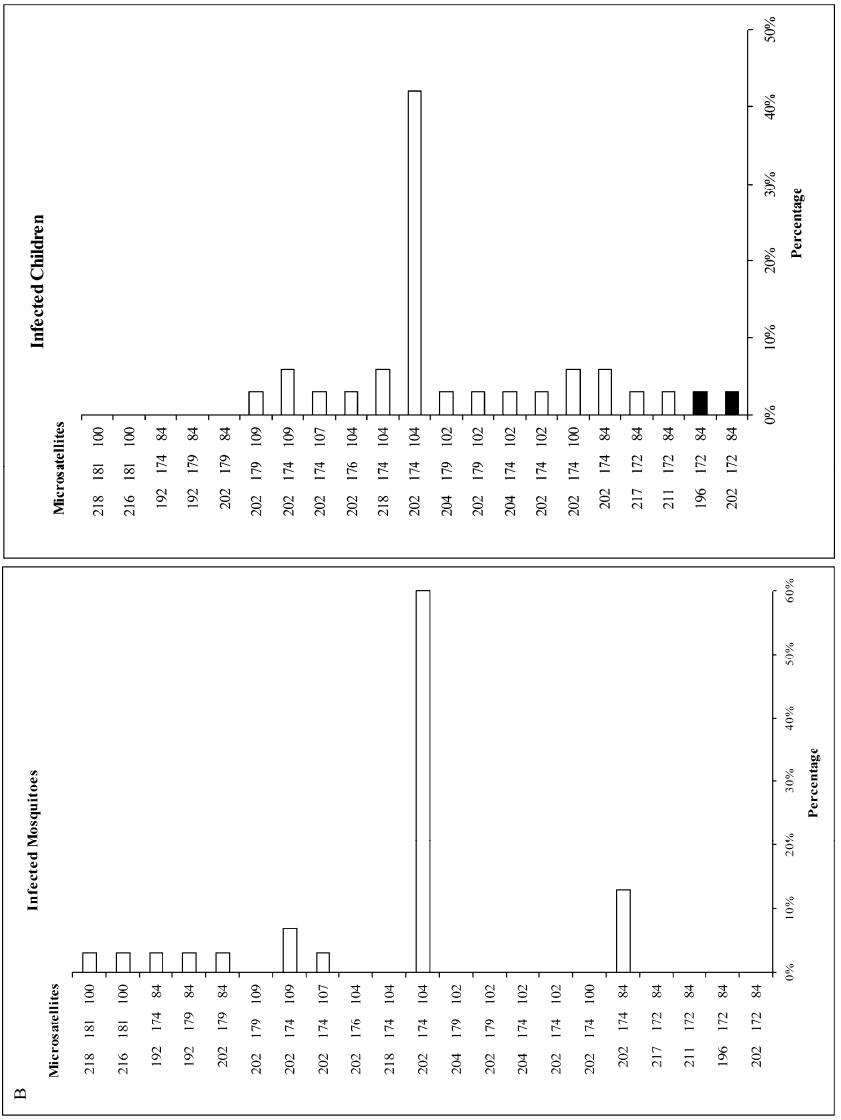

In the asymptomatic chronic infections, the parasite multiplicity is high and it correlates with a high degree of parasite diversity even with asexual parasites at sub-patent levels. Patients can harbour high gametocytes level in the dry season and early transmission season and this will increase in the late transmission season and become capable to infect the mosquito.

## Paper IV

The study was conducted to examine if the dominant triple mutant *dhfr* haplotype in Africa has a higher transmission capacity compared to others, and if it is transmitted as an independent lineage or recombines with others.

We characterised resistant *dhfr* haplotypes among *P. falciparum* infected children, in Farafenni area in The Gambia, and examined their infectivity to *Anopheles* mosquitoes. Twenty microsatellites haplotypes carrying triple *dhfr* mutations were detected among infected children, however a single haplotype existed as a predominant lineage. This haplotype transmitted to mosquitoes more readily following drug usage compared to others. Cross-mating between *P. falciparum* carrying different triple mutant *dhfr* haplotypes was detected; this can result in generation of variable haplotypes that carry triple mutations. The major haplotype seen in The Gambia shares microsatellites with a wide spread haplotype which originated from Asia and is now seen in other African countries (Kenya) where similar analysis was

carried out. These data suggested that gene flow and migration is a primary source of spread of pyrimethamine resistance in Africa. As we have seen in The Gambia, multiple triple mutant *dhfr* haplotypes do exist at limited frequency in many countries and cross-mating between *P. falciparum* clones with different triple mutant *dhfr* haplotypes can result in generation of multiple *dhfr* lineages.




Mosquitoes were infected with one oocyst and harboured more than one *msp-1* allele, this indicated cross mating, However, identical *dhfr* haplotypes were detected in children and in the mosquitoes that fed on blood samples (before treatment).

# Discussion

This thesis has examined factors influencing evolution of resistant *P.falciparum* parasite, including parasite migration, cross mating and recombination and fitness cost of resistance, in two sites with markedly different levels of transmission intensity. In eastern Sudan an area of low and seasonal transmission and The Gambia, where transmission is all year round. In eastern Sudan, I examined fluctuation in frequency of drug resistant *P.falciparum* parasite in absence of drug pressure in the dry season. The frequency of mutant alleles of two genes controlling the response of *P.falciparum* to chloroquine (*Pfcrt* and *Pfmdr1*), reached fixation following CQ treatment and remained high in the transmission season, a reflection of selection. However, at the start of the dry season, wild type alleles of both genes started to emerge and increased significantly in frequency as the season progressed. These findings suggest that drug resistant mutant forms are likely to be less fit than the wild type strains in the absence of selection. However, compensatory mutations may occur which can restore the fitness of mutants to that of sensitive forms. A large body of field data has demonstrated a trend of reduction in prevalence of drug resistant parasites following withdrawal of therapy at the community level (Babiker 2009). The present study however, is one of few examined within host persistence of drug resistant parasites in absence of drug therapy during the dry and transmission-free months in Asar village. Previous cross-sectional surveys in this village showed significant seasonal fluctuation in prevalence of drug resistant *P. falciparum* parasites. This fluctuation reflect the pattern of antimalarial drug usage, and positive selection of resistant parasites in the transmission season followed by selective disadvantage in absence of therapy in the dry season (Abdel-Muhsin, Mackinnon et al. 2004; Babiker, Satti et al. 2005; Babiker, Hastings et al. 2009). Resistance to an antimalarial is defined as the ability of *Plasmodium* parasites to survive despite an adequate blood concentration of anti malarial medicines. However, some wild types parasites have inherent ability to overcome the therapeutic level; the drug may suppress these parasites without clearing them. Following withdrawal of the drug these parasites can then grow faster than mutant parasites (Walliker, Hunt et al. 2005). Previous surveys in Asar village has demonstrated disappearance of *Anopheles* mosquitoes during the dry season and therefore, it is unlikely that the wild type parasites resulted from re-infection (Hamad, Nugud Ael et al. 2002).

This data complements laboratory studies which demonstrated survival disadvantage of resistant parasites in the absence of drugs. Controlled laboratory studies on *P. chabaudi* revealed superior growth and transmission of sensitive strains compared to resistant ones, in the absence of drug therapy (Walliker, Hunt et al. 2005; Nassir, Abdel-Muhsin et al. 2005). In *P. falciparum* within-host competition has been inferred from the frequent occurrence and complex dynamics of multiple clones infections. Competition could eliminate genotypes with survival disadvantages, including resistance mutations in absence of drug pressure (Wargo, Huijben et al. 2007).Thus, over several months in the dry season, the cumulative fitness cost during successive rounds of mitotic propagation in the absence of therapy can lead to steady decline in resistant parasites (Ord, Alexander et al. 2007; Babiker, Hastings et al. 2009).

The source of drug resistant *P. falciparum* parasites has been linked to human migration and gene flow between parasite populations (Nair, Williams et al. 2003; Roper, Pearce et al. 2004). Wide population genetic surveys of parasite across many African countries have demonstrated low level differentiation between them (Anderson, Haubold et al. 2000). This suggests frequent gene flow between them. However, earlier studies suggested that the parasite population in eastern Sudan is geographically isolated from that of other sub-Saharan populations with its distinct genetic characteristics (Abdel-Muhsin, Mackinnon et al. 2003; Binks, Baum et al. 2001). This has prompted the analysis of haplotypes of *dhfr* and *dhps* genes, involved in resistance to SP haplotypes, and allowed comparison with those seen in other African countries. One major *dhfr* haplotype with double *dhfr* mutations and 2 major mutant *dhps* haplotypes were seen in eastern Sudan. These haplotypes are distinct from those prevailing in other African countries, suggesting the likely local origin of these haplotypes (Al-Saai, Kheir et al. 2009).

The predominant resistant haplotypes of *dhfr* and *dhps* in Asar, which exist at prevalence of 73% and 43%, respectively (Table 1 and Table 2), are distinct compared to common haplotypes thought to be the sources of resistance to SP in other African countries (Roper, Pearce et al. 2003; McCollum, Basco et al. 2008; McCollum, Poe et al. 2006; Mehlotra, Mattera et al. 2008) and (Certain, Briceno et al. 2008) *P. falciparum* parasites in eastern Sudan were previously found have some unique genetic characteristics (Babiker, Lines et al. 1997; Binks, Baum et al. 2001). This has been attributed to geographical isolation and pattern of low seasonal transmission compared to high transmission areas of sub-Saharan African where gene flow occurs readily between different parasite populations (Anderson et al., 2000). Newly introduced drug sensitive genotypes into the small *P.falciparum* population of eastern Sudan can probably be weeded out by genetic drift, as a result of seasonal fluctuation in parasite prevalence, nonetheless high-level resistant genotypes can be subjected to directional selection by im-

mense drug pressure that follows the transmission season (Abdel-Muhsin, Mackinnon et al. 2003; Babiker, Creasey et al. 1991), therefore, the distinct predominant resistant *dhfr* and *dhps* haplotypes in Asar suggest that, the drug resistance genotypes in this area may have evolved locally. This is supported by the fact that the resistant haplotypes of both *dhfr* and *dhps* share some flanking microsatellites with the wild-type genotype. Studies from African countries (Maiga, Djimde et al. 2007), have reported that majority of *P.falciparum* isolates with high level of resistance, the triple mutation (51I, 59R, 108N) compromise a single haplotypes, although limited number of different triple mutant *dhfr* haplotypes were seen at low prevalence in some African countries (McCollum, Poe et al. 2006). Therefore, migration can be the driving force for the evolution of pyrimethamine resistance in Africa. However, in the case of eastern Sudan resistance to SP has evolved locally.

In the Gambia, in Farafenni area of high transmission, I have examined the transmission capacity of different *P. falciparum* clones within an infection. At the same time, I tested the transmission capacity of high-level triple mutant pyrimethmine resistant haplotype that has been seen in different endemic sites across Africa. In addition, I examined if the high-level pyrimethmine resistance parasite recombines with other haplotypes or if it transmits independently. We have examined here the genetic complexity of *P. falciparum* gametocytes and their infectivity to mosquitoes in the Gambia. A high degree of complexity and clonal multiplicity of *P. falciparum* gametocytes have been seen among asymptomatic infections that persist, mostly at sub-microscopic level, in the dry season. Some of these infections were capable of infecting mosquitoes, even when they existed at sub-patent levels in the dry season as was the case in one instance in this study. Similar *Pfg377* alleles in asexual forms and gametocytes were seen in 60% of the examined individuals, suggesting that most clones within an infection were simultaneously producing gametocytes. However, in some infections more gametocyte genotypes were detected compared with asexual forms. This could be explained by the higher sensitivity of RT-PCR which detects RNA that can be expressed in multiple copies. An unexpected result was that some parasite genotypes were found in infected mosquitoes but had been undetectable in the blood on which they had fed. These results demonstrate the remarkable ability of different *P. falciparum* clones to co-transmit to mosquitoes and to cross-mate and produce new strains for the next generation, an observation that explains the paramount success of the parasite in nature. Parasites multiplicity in infected people correlated with parasite diversity in *Anopheles* mosquitoes and showed that multiple clones within an infection can be equally infectious to mosquitoes when they feed and this leads to higher transmissibility of multiple *P.falciparum* clones within an infection, even when they exist at low levels (Schneider, Bousema et al. 2006). So, studies confirmed that *P.falciparum* persist as sub-patent level.

To examine transmission success of high level drug resistant *P. falciparum* lineages *dhfr* haplotypes were examined in infected children and *Anopheles* mosquitoes that fed on them. Our study attempted to examine whether the dominant triple-mutant *dhfr* haplotype in Africa, which originated in Asia, has a relative intrinsic higher transmission capacity compared with others and if this haplotype is transmitted as an independent lineage or recombines with others. Twenty microsatellites haplotypes carrying triple *dhfr* mutations were detected among infected children. However, one haplotype existed as a predominant lineage, which is identical to one that originated in Asia and is currently spreading across Africa (Roper, Pearce et al. 2004). This haplotype was also the most frequently observed in the mosquitoes after drug treatment of the children. In a limited number of examined mosquitoes, cross-mating between *P. falciparum* carrying different triple-mutant *dhfr* haplotypes was seen, which indicated recombination between haplotypes that carry triple mutations. These findings suggest that while parasite migration is a major force for dissemination of high level drug resistant parasite lineages local mating and recombination can generate novel drug resistance genotypes. Previous studies in The Gambia have shown a high rate of transmission following SP treatment of uncomplicated malaria, and a prevalence of the triple mutant (IRN) for *dhfr* and 437 (*dhps*) (Sutherland, Ord et al. 2005; Hallett, Dunyo et al. 2006; Nwakanma, Kheir et al. 2008) Therefore treated children may be highly infectious with mutant *dhfr* and *dhps* alleles following treatment. The major triple mutant *dhfr* haplotypes resistant, detected in the present study, shared some flanking microsatellites with other minor haplotypes with triple mutations. This suggests that high level resistance triple mutant *dhfr* may have been in the area for some time and variant of these lineages may have been generated by recombination.

# Conclusions

This thesis studied transmission stages (gametocytes) of the malaria parasite *P. falciparum* in natural infection and examined influence of some genetic factors that enhance spread of drug resistant genotypes in two sites with distinct pattern of malaria transmission.

## Eastern Sudan

In an area of low and seasonal transmission I examined two questions (i) what is the impact of the lengthy and anti-malarial drugs-free period on fitness of drug resistant genotypes, and (ii) was the high-level resistant pyrimethamine genotype thought to drive SP failure across any countries in

Africa, is responsible for the high rate of SP failure in eastern Sudan. The major findings showed the following:

1. During the dry season of this area when drug pressure ceases the drug resistant parasites (resistance to CQ) have shown a sign of lower fitness. The prevalence of alleles associated with CQ resistance started to decline at the start of the dry season, and continued to do so through out the dry season when there is no drug pressure. These findings confirmed that success of resistant parasites is limited, especially when the drug that develops the resistance is removed.

2. This study did not identify the triple mutant *dhfr* haplotype, thought to drive SP failure across Africa, in this region despite high rate of clinical failure of SP. Instead a dominant double mutant *dhfr* haplotype with distinct flanking microsatellites was seen in the region. Microsatellites haplotypes neighbouring the *dhfr* and *dhps* genes were different compared to other microsatellites haplotypes in African countries with triple mutant allele and this contribute to SP failure.

## The Gambia

1. Asymptomatic sub-microscopy infections that prevail in the dry season produce infectious gametocytes capable of infecting Anopheles mosquitoes.

2. The genetic complexity of *P. falciparum* parasites in mosquitoes was sometimes greater than that detectable in infected people on which the mosquitoes had fed. This suggested that gametocytes of clones existing in the blood below PCR detection limits at the time of the feed were at least as infectious to the mosquitoes as the more abundant clones.

3. The major *dhfr* haplotypes in The Gambia is similar to the common one seen in other African countries, emphasising that parasite migration plays a major role in spread of resistance in the region.

4. The dominant resistant haplotype in infected children transmitted readily to infected mosquitoes, in addition minority resistant alleles are also capable of infecting mosquitoes.

5. Cross-mating between parasites with different triple mutanat *dhfr* haplotypes occurs frequently in infected mosquitoes, suggesting that locally generated drug resistance *dhfr* haplotypes is also contributing to evolution of pyrimethmine resistance.

# Future Perspectives

- It is not clear how the dominant *P. falciparum* parasites with double *dhfr* mutations can cause high rate of SP failure in eastern Sudan. The role of *dhps* and possible other genes on the folate pathway need to be studied in some depth.

- Future surveillance to monitor emergence and of triple mutant *dhfr* in eastern Sudan, and if it can evolve from the existing double mutations. Can the process of stepwise mutation generate the triple mutation?

- The decline in drug resistant parasites in the dry season can further be investigated by monitoring the density of mutant and wild drug resistance genes in the dry season use real-time PCR. Such approach will provide desirable data on the extent of fitness cost in natural infection, and selective disadvantages in absence of drug pressure.

- Direct feeding of *Anopheles* mosquitoes on asymptomatic parasite carriers, during the dry season, will provide information on ability of drug resistant parasites to infect mosquitoes.

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