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Dynamics of malaria parasite resistance markers in two areas of different transmission intensity

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Le doyen :
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" Je ne suis pas d'accord avec vos idées mais je me battrai pour que vous puissiez les exprimer "

Voltaire

Au peuple rwandais…

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Abbreviations

ACT	Artemisinin-based combination therapy
AQ	Amodiaquine
bp	Base pairs
CQ	Chloroquine
CQR	Chloroquine resistance
CTXZ	cotrimoxazole
DDT	Dichlorodiphenyltrichloroethane
DHF	dihydrofolate
DHFR	Dihydrofolate reductase
DHFR-TS	Dihydrofolate Reductase-Thymidylate Synthase
DHP	dihydropteroate
DHPPP	2-amino-4-hydroxy-6-hydroxymethyl-7,8 dihydropteridine pyrophosphate
DHPS	Dihydropteroate synthetase
DNA	Desoxyribonucleic acid
EANMAT	East African Network for Monitoring Anti-malarial Treatment
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
FP	ferriprotoporphyrin IX
G6PD	Glucose-6-phosphate dehydrogenase
glurp	Glutamate-rich protein
GDP	Gross domestic product
HIV/AIDS	Human immunodeficiency virus/Acquired immunodeficiency syndrome
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITN	Insecticide treated bednet
LLIN	Long lasting insecticidal net
MIM	Multilateral initiative on malaria
MMV	Medicines for malaria venture
MVI	Malaria vaccine initiative
NACT	Non-artemisinin-based combination therapy
***p*ABA**	Para-aminobenzoic acid

PCR	Polymerase chain reaction
***Pf*ATPase6**	*P. falciparum* ATPase6
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter
pfdhfr	*Plasmodium falciparum* dihydrofolate reductase
pfdhps	*Plasmodium falciparum* dihydropteroate synthase
pfmdr	*Plasmodium falciparum* multidrug resistance gene
Pfmsp2	*Plasmodium falciparum* merozoite surface protein 2
Pgh1	P-glycoprotein homolog 1
PNG	Papua New Guinea
PNGIMR	PNG Institute of Medical Research
PQ	Primaquine
Q	Quinine
RBC	Red blood cell
RBM	Roll Back Malaria
RDT	Rapid diagnostic test
RFLP	Restriction fragment length polymorphism
RNA	ribonucleic acid
SAO	South Asian Ovalocytosis
SERCA	Sarcoendoplasmic reticulum Ca^{2+}-ATPase
SNP	Single nucleotide polymorphism
SP	Sulphadoxine-pyrimethamine
SPR	Sulphadoxine-pyrimethamine resistance
THF	tetrahydrofolate
UNDP	United Nations Development Program
UNICEF	United Nations Children's Fund
UTL	Useful therapeutic life
WHO	World Health Organization

Summary

Every year, 1.5-2.7 million people worldwide die from malaria, while 300-400 million become infected. The groups suffering most are young children and pregnant women, and people who survive a severe malaria attack often suffer permanent development deficits and neurological abnormalities. Malaria's economic impact is huge, reducing economic growth rate by an estimated 1.3% per year in most endemic areas.

In the absence of an effective vaccine, access to safe and effective treatment still remains the mainstay in the control of the disease. However, the efficacy of this control strategy is hampered by the emergence and spread of drug resistant malaria which may lead to excess of mortality. One of the greatest challenges for health authorities of malaria endemic countries is thus to decide on when and how antimalarial drug policy should be changed, so that most of the patients will fully recover from the disease and will be cleared from parasites.

The molecular mechanisms for parasite resistance have been elucidated for the most used antimalarials; sulfadoxine-pyrimethamine (SP) and more or less for chloroquine (CQ). Resistance to CQ has been associated with multiple mutations in *Pfcrt* and *Pfmdr* genes, and resistance to SP with accumulation of multiple mutations in *Pfdhfr* and *Pfdhps* genes. These mutations are now used as molecular markers for drug resistance. The factors influencing the appearance and the spread of resistance to drugs within communities are far from being clear. Drug use has been thought to be one of the key factors leading to drug resistance, but other parameters, such as pharmacokinetics, pharmacogenetics, level of immunity, treatment compliance, dosing, etc., play also a role in clearing symptoms and parasites.

More information about the process determining the emergence and spread of resistance is needed; lessons from the history of the antimalarials and the modes by which resistance arose and disseminated may help prolong the life span of the next generation of chemotherapies. A new approach is to investigate the evolutionary history of drug resistance mutations in historical samples using molecular markers that are closely linked to resistance alleles.

In the present study, we investigated the dynamics of malaria parasite resistance molecular markers in two areas of different transmission intensity with two different drug pressures using a new DNA-microarray based technique that allows analysing all known malaria drug resistance molecular markers simultaneously.

The first goal was to evaluate the impact of a low drug pressure limited in time on the development of resistance against SP in Idete, Tanzania, an area of very high transmission intensity. We used samples which were collected during a vaccine (SPf66) efficacy trial in

children under 5 years in 1994. At that time, the first line antimalarial treatment was chloroquine in Tanzania, but in order to maximize the immune response to the vaccine, every individual was treated with 3 doses of SP to clear the parasite before the vaccination. We found that the use of these 3 doses of SP in children led to an increase in double and triple mutation frequency in *Pfdhfr* and *Pfdhps* genes, which have been associated with increasing levels of resistance to SP.

We also investigated longitudinally the dynamics of treatment effectiveness and those of molecular markers under a sustained drug pressure in Wosera, Papua New Guinea (PNG), an area of moderate transmission intensity. The samples from Wosera were collected during health facility-based morbidity surveillance over a period of 12 years, between 1991 and 2002. In PNG, the 4-aminoquinoline drugs amodiaquine (AQ) and chloroquine (CQ) have been first-line treatment against uncomplicated malaria until the late 1990s.

We found that the clinical treatment effectiveness was diminishing during this period as a consequence of increasing mutation frequency in *Pfcrt* and *Pfmdr* genes which are related to 4-aminoquinolines resistance, particularly to CQ. We found also that SP resistance was developing in Wosera in the absence of high SP use.

To summarize, both studies showed that resistance to SP is developing fast even under very low drug pressure, probably due to cross resistance with other antifolates, as the widely used bactericide, cotrimoxazole. Drug pressure has been shown to be an important factor in the development of antimalarial resistance, even if other factors are likely to play an important role. The molecular markers could be used as an early warning tool, even if their usefulness in predicting treatment response is still limited. Careful baseline evaluation of the molecular resistance background could help when drug policy has to be changed.

The DNA-microarray based technique used in this study to analyze molecular markers proved to be a very useful tool to analyze a lot of samples on a large scale in epidemiological studies.

Keywords: Malaria, *Plasmodium falciparum*, resistance, antimalarial, molecular markers, Tanzania, Papua New Guinea, dynamics, chloroquine, sulfadoxine-pyrimethamine

Résumé

L’incidence du paludisme dans le monde est estimée à 300-500 millions de cas cliniques par an. Chaque année 1.5 à 2.7 millions de personnes meurent de la maladie, surtout les enfants en bas âge et les femmes enceintes. Les patients qui survivent à une attaque sévère du paludisme souffrent souvent de déficits permanents de développement mental et d’anomalies neurologiques. Cette maladie représente également un grand obstacle au développement économique et social des régions dans lesquelles elle sévit. Il a été estimé que le paludisme réduit le taux de croissance du produit national brut (PNB) des pays endémiques d’environ 1.3% par an.

En l'absence d'un vaccin efficace, le diagnostic précoce et la provision d’un traitement adéquat restent la principale stratégie de lutte contre le paludisme. Cependant, l'efficacité de cette stratégie est entravée par l'apparition et la propagation de la résistance des parasites face aux antipaludiques les plus couramment utilisés. Ce phénomène s’observe surtout chez *Plasmodium falciparum*, le plus virulent des parasites infectant l’Homme, et cela a pour principale conséquence une augmentation de la mortalité liée au paludisme. Un des plus grands défis pour les services de santé publique dans les pays endémiques est de décider quand et comment changer les traitements avant que la résistance ne se développe, pour permettre au patient d’avoir accès à un traitement efficace qui puisse non seulement résorber les symptômes, mais aussi le dégager de toute charge parasitaire.

Les mécanismes moléculaires induisant la résistance des parasites ont été élucidés pour les antipaludiques les plus couramment utilisés ; la sulfadoxine-pyrimethamine (SP) et la chloroquine (CQ). La résistance à la CQ a été associée à plusieurs mutations dans les gènes *Pfcrt* et *Pfmdr*, et la résistance à la SP associée à l'accumulation de plusieurs mutations dans les gènes *Pfdhfr* et *Pfdhps*. Ces mutations sont maintenant employées comme des marqueurs moléculaires pour la résistance à ces médicaments. Les facteurs influençant l'apparition et la diffusion de la résistance à ces médicaments au sein de la population sont loin d'être définis. L'utilisation abusive des médicaments est probablement l'un des facteurs principaux conduisant à la résistance, mais d'autres facteurs, tels que la pharmacocinétique, la pharmacodynamique, la pharmacogénétique, l'immunité acquise, le dosage, etc., jouent un rôle dans l’efficacité thérapeutique du traitement.

Une meilleure compréhension des facteurs déterminant l'apparition et la diffusion de la résistance dans la population est nécessaire; ces informations seront utiles pour la recherche sur les nouveaux traitements et pourront ainsi permettre de prolonger la durée de vie de la

prochaine génération des antipaludiques. Pour cela, une nouvelle approche est d'étudier l'évolution de la résistance en utilisant les marqueurs moléculaires de résistance dans des échantillons qui ont été récoltés avant et pendant le développement de la résistance pour mieux comprendre l'émergence et la propagation de la résistance.

Dans ce projet, nous avons étudié la dynamique des marqueurs moléculaires de résistance de *P. falciparum* dans deux régions où l'intensité de la transmission du paludisme et l'utilisation des antipaludiques sont différentes, en utilisant une nouvelle technique de puce à ADN qui permet d'analyser simultanément tous les marqueurs moléculaires de résistance connus.

Le premier objectif de cette étude était d'évaluer l'impact de l'utilisation de 3 doses de la SP sur le développement de la résistance, quand la SP a été introduite dans une région où la transmission du paludisme était élevée ; à Idete en Tanzanie. Nous avons utilisé les échantillons récoltés pendant l'essai clinique d'un vaccin antipaludique (SPf66) chez les enfants de moins de cinq ans en 1994. À l'époque, la chloroquine était le traitement officiel utilisé dans les accès palustres en Tanzanie. Cependant pour ne pas biaiser les résultats de l'efficacité du vaccin, chaque individu a été traité avec 3 doses de SP pour éliminer tous les parasites avant la vaccination. Nous avons constaté que l'utilisation de ces 3 doses de SP chez les enfants a induit une augmentation du taux de double et triple mutations dans les gènes *Pfdhfr* et *Pfdhps*, et ces doubles et triples mutations sont reconnues pour provoquer une augmentation de la résistance à la SP.

Le second objectif était d'étudier l'évolution de l'efficacité thérapeutique des traitements antipaludiques et celui des marqueurs moléculaires de résistance en relation avec l'utilisation des antipaludiques dans la région du Wosera en Papouasie Nouvelle Guinée (PNG), une région où la transmission du paludisme reste modérée. Les échantillons utilisés proviennent d'une étude qui s'est déroulée sur douze ans, entre 1991 et 2002, et dont l'objectif était d'évaluer la morbidité due au paludisme dans les centres de santé. En PNG, les antipaludiques de la classe des 4-aminoquinolines que sont la chloroquine (CQ) et l'amodiaquine (AQ) ont été les antipaludiques les plus utilisés dans le traitement de l'accès palustre jusqu' à la fin des années 90. Nous avons constaté une diminution de l'efficacité thérapeutique des traitements tout au long de cette période et en même temps une augmentation du taux des mutations dans les gènes *Pfcrt* et *Pfmdr* qui sont liés à la résistance aux antipaludiques de la classe des 4-aminoquinolines, en particulier à la CQ. Nous avons constaté également que la résistance à la SP se développait dans la région du Wosera, alors que la SP était très peu utilisée dans la population.

Pour récapituler, les deux études ont constaté que la résistance à la SP se développe rapidement malgré une faible utilisation du médicament dans la population, probablement en raison de la résistance croisée d'autres antifolates, comme le bactéricide largement utilisé dans les pays en voie de développement, la cotrimoxazole. Il a été démontré que le niveau d'utilisation des antipaludiques est un facteur important dans le développement de la résistance, même si d'autres facteurs sont susceptibles de jouer un rôle important. Il a aussi été démontré que des marqueurs moléculaires de résistance pourraient être utilisés comme des outils de détection précoce de la résistance, même s'ils ne peuvent pas être employés pour déterminer l'efficacité d'un traitement au niveau individuel. Une évaluation du taux de mutations dans les gènes liés à la résistance aux antipaludiques pourrait être d'une grande utilité quand on veut introduire un nouveau médicament dans une région donnée.

La technique de la puce à ADN employée dans cette étude pour analyser les marqueurs moléculaires de résistance s'est avérée être un outil très utile et pratique pour analyser des échantillons à grande échelle dans des études épidémiologiques.

Mots-clés : Paludisme, *Plasmodium falciparum*, antipaludéen, résistance, marqueurs moléculaires, Tanzanie, Papouasie Nouvelle-Guinée, dynamique, chloroquine, sulfadoxine-pyrimethamine

Introduction

I.1. Malaria parasite and its life cycle

The causative organisms of the disease malaria are Apicomplexa protozoan of the genus *Plasmodium,* family Plasmodiidae, suborder Haemosporidiidae, and order Coccidia. The 120 or so species of *Plasmodium* are found in blood of mammals, reptiles and birds. The great majority of malarial parasites are transmitted by mosquitoes, and the parasites of humans are exclusively transmitted by anophelines. There are numerous different species of anophelines in different areas of the world, with varying feeding preferences, breeding sites and efficiency of transmission. The parasites of humans are of two subgenera, *Laverania* and *Plasmodium* (Warrell & Gilles, 2002). The former subgenus includes *P. falciparum*, the most pathogenic form of malaria, and the closely related *P.reichenowi*, a parasite of the higher primates. The latter subgenus includes the remaining parasites of humans, namely *P.vivax*, *P. malariae* and *P. ovale*. Besides the four species of *Plasmodium* that commonly cause malaria in humans, simian malarias such as *Plasmodium inui*, *P.cynomologi* and *P.knowlesi* can also cause malaria in humans (Garnham, 1966), but this phenomenon was supposed to be extremely rare. However, *P.knowlesi* malaria in humans was reported recently to be common throughout Malaysian Borneo and other regions in South-eastern Asia, but is being incorrectly diagnosed as *P.malariae* (Cox-Singh et al., 2008). Moreover, *P. knowlesi* is likely to be virulent, and has been proposed to be the fifth human malaria parasite (White, 2008).

All the malaria parasites require two hosts to complete their life cycle, by definition, the definitive host (in which sexual development occurs) is the anopheline mosquito and the mammal is the intermediate host (Figure 1). The sporozoites are transmitted to humans by the bite of infected female mosquitoes; they circulate for a short time in the blood stream, and then invade liver cells, where they develop into exoerythrocytic schizonts during the next 5 to 15 days. *Plasmodium vivax* and *P. ovale* have a dormant stage, the hypnozoite that may remain in the liver for weeks or many years before the development of exoerythrocytic schizogony. This results in relapses of infection. *Plasmodium falciparum* and *P. malariae* have no persistent phase. An exoerythrocytic schizont contains 10000 to 30000 merozoites, which are released and invade the red blood cells (Warrell & Gilles, 2002). The entire invasion process takes about 30 seconds. The merozoite develops within the erythrocyte through ring, trophozoite and schizont (erythrocytic schizogony). The parasite modifies its host cell in several ways to survive. The erythrocyte containing the segmented schizont eventually ruptures and releases the merozoites, which invade new erythrocytes. In the course of infection, some merozoites become differentiated into sexual forms, which are macrogametocytes (female) and microgametocytes (male). Gametocytes may first be detected

in the blood within four days (*P. vivax*) to ten days (*P. falciparum*) after the first appearance of asexual parasites (Warrell & Gilles, 2002). The female anopheline mosquito must take blood meals on a regular basis to support the development of successive batches of eggs. When biting a malaria-infected vertebrate host, she will take up erythrocytes infected with gametocytes. Mature macrogametocytes taken into the midgut of the mosquito egress from the erythrocyte to form macrogametes. Microgametocytes in the midgut exflagellate, each forms 8 microgametes few minutes after infection. The microgamete moves quickly to fertilize a macrogamete and forms a zygote. Within 18 to 24 hours, the zygote elongates into a slowly motile ookinete. The Ookinete traverses the peritrophic membrane and the epithelial cells of the midgut, and then transforms into an oocyst beneath the basement membrane of the midgut epithelium. Between seven and fifteen days postinfection, depending on the *Plasmodium* species and ambient temperature, a single oocyst forms 10000 sporozoites. The motile sporozoites migrate into the salivary glands and accumulate in the acinar cells of the salivary glands. When an infected mosquito bites a susceptible vertebrate host, it will inject few sporozoites (usually less than twenty), and the *Plasmodium* life-cycle begins again (Warrell & Gilles, 2002).

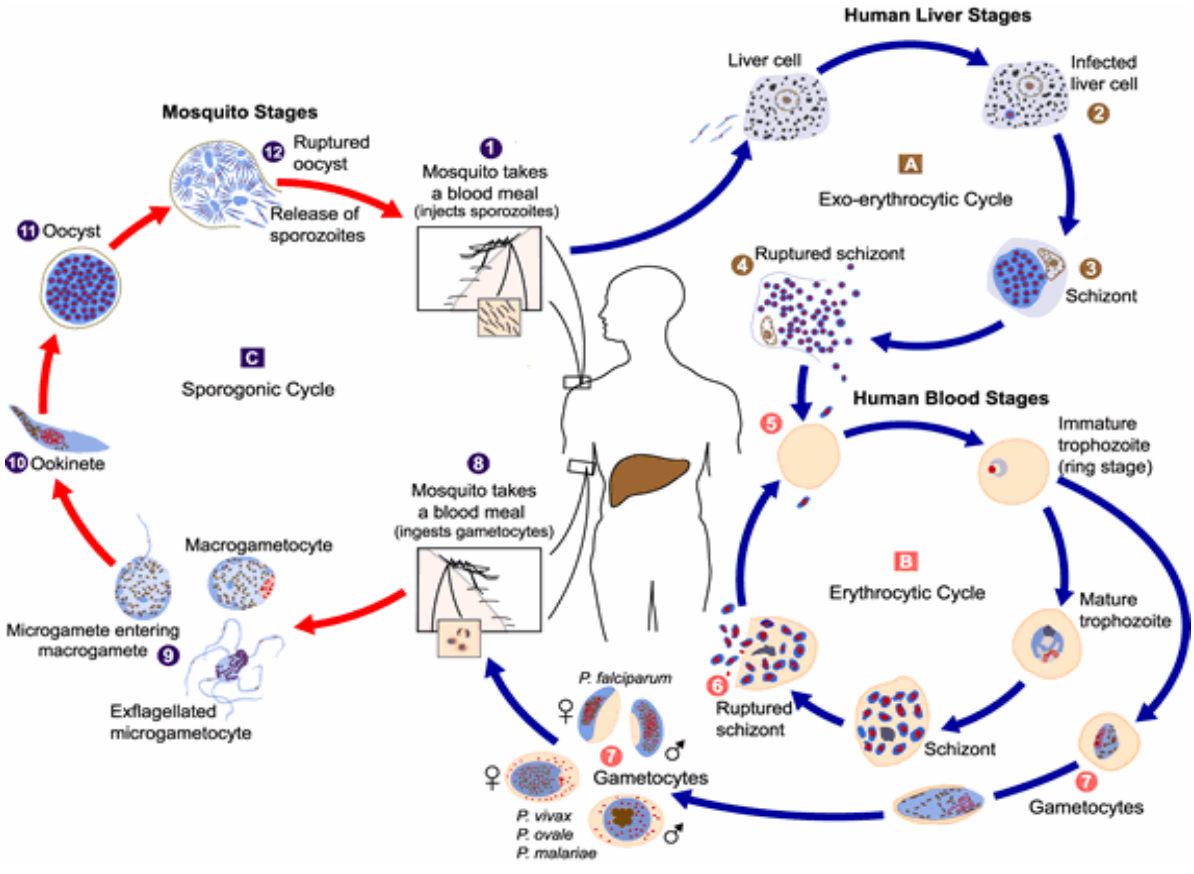


Figure 1: Life cycle of malaria (image from: www.cdc.gov/malaria/biology/life_cycle.htm)

I.2.The burden of malaria

Malaria is one of the most important infectious diseases in the world. In 2004, 107 countries and territories have reported areas under the risk of malaria transmission (WHO, World malaria Report, 2005). It is estimated that 40% of the world population live at risk of contracting the disease (Snow et al., 2005). Despite control efforts over the past decades, *falciparum* malaria remains a major cause of childhood morbidity and mortality, particularly in sub-Saharan Africa (Greenwood & Mutabingwa, 2002). It is very difficult to quantify the actual number of malaria episodes and deaths in the world. The reasons are multiple and relate not only to the way in which malaria is diagnosed and treated but also to the general weakness of systems in monitoring and reporting malaria cases and responses to treatment (De Savigny & Binka, 2004).

Between 350 and 500 million clinical episodes of malaria occur each year, of which 80 % are due to infections by *P. falciparum* (Nahlen et al., 2005). The global mortality is estimated at 1 to 3 millions cases each year, with the worst situation in children under 5 years and pregnant women (Wellems & Miller, 2003; Greenwood et al., 2005). Sub-Saharan Africa is by far the region with the greatest burden of malaria cases and deaths, most of it due to *P. falciparum* malaria (60% and 90% of the clinical episodes and deaths, respectively), but malaria in Asia contributes about 38% of the global burden of clinical malaria with an estimated 49% of its population at risk of malaria. About half of the malaria cases outside Africa is due to *P. vivax* malaria (Mendis et al., 2001).

Malaria, together with HIV/AIDS and Tuberculosis (TB), is one of the major public health challenges undermining development in the poorest countries of the world (Snow et al., 2001). In malaria-endemic areas, malaria infection in pregnancy is believed to account for up to a quarter of all cases of severe maternal anaemia and for 10–20% of low birth weight babies (Steketee et al., 2001; Guyatt & Snow., 2001a). In addition to its direct effect in infants, the disease could account for an additional 5–10% of neonatal and infant deaths based on its effect on birth weight (Guyatt & Snow, 2001b).

Malaria also has a direct impact on human resources. Not only does malaria result in loss of life and loss of productivity due to illness and premature death, but malaria also hampers children's schooling and social development through both absenteeism and permanent neurological and other damage associated with severe episodes of the disease (Fernando et al, 2003; Carter et al., 2005).

Malaria has been shown to be a major constraint to economic development (Sachs & Malaney, 2002). Economists believe that malaria is responsible for a growth penalty of 1.3%

per year in countries with intense transmission (Gallup & Sachs, 2001). The disease has been estimated to cost Africa more than US$ 12 billion every year in lost Gross Domestic Product (GDP) (Sachs, 2001). Today, malaria is understood to be both a disease of poverty and a cause of poverty (Barat et al., 2004; Jones & Williams, 2004).

I.3. Factors contributing to the burden of the disease

The prevalence of malaria is determined by multiple factors, accounting for a wide variation in the burden across the different regions of the world.

I.3.1. Level of transmission intensity

Malaria transmission intensity is defined as the rate at which people get inoculated with malaria parasites from mosquitoes. It is usually expressed as the average number of infective bites per person per unit time referred to as entomological inoculation rate (EIR). Transmission is related to the density of the vector, its survival and longevity, and the proportion of vectors carrying the parasite (Molineaux, 1988). Seasonal fluctuations in environmental factors such as rainfall, temperature and humidity affect the survival rate of anophelines and their population size (Warrell & Gilles, 2002).

Anopheles gambiae complex and *Anopheles funestus* are the most efficient vector for *P. falciparum* transmission, the highest rates of sporozoite development are in *An. gambiae*, the species that is widespread throughout tropical Africa (Breman, 2001). *An. gambiae* feeds preferentially on humans, is highly infected (sporozoite rate: 1-10%), and is long-lived, making it particularly effective at transmitting *falciparum* malaria from one person to another (Danis & Mouchet, 1991). Africa has substantially cross-continent variability in annual EIR (Craig et al., 1999) between countries, districts, and even in the same villages situated only a kilometre or two apart. This variation reflects sub-regional ecological heterogeneity, and year to year variation is also very important, particularly in seasonal areas (Hay et al., 2000).

Papua New Guinea (PNG) is a patchwork of different ecological zones, and this results in complex variations in vector ecology and malaria epidemiology. Because the country is located close to the equator, temperatures do not show much seasonal variations and depend mainly on altitude (Müller et al., 2003). In most parts of the lowlands there is perennial malaria transmission, with limited seasonality (Burkot et al., 1988; Genton et al., 1995). As altitude increases, transmission also decreases significantly, becoming unstable at an altitude of 1300-1600m. Intense transmission is then limited to local epidemics (Hii et al., 1997; Müller et al., 2005). All four *Plasmodium* species that infect human are present in PNG

resulting in a complex mechanism of cross-species regulation in semi-immune individuals (Bruce & Day, 2003). The main vectors of malaria in PNG are members of the *Anopheles punctulatus* group, which is composed of more than 10 species (Williams et al., 1995). These different species may vary in their ecological habitats, vectorial capacity, and malaria transmission (Benet et al., 2004), thus making a better knowledge of their biology a prerequisite to specific control measures.

Because entomologic parameters are often much more difficult to accurately measure than human infection, the parasite prevalence, or the fraction of hosts with detectable blood stage infections, is often used as measure of the intensity of malaria transmission (Guerra et al., 2007), and classified in four categories with different levels of parasite prevalence, referred to as endemicity (Lysenko et al., 1966): hypoendemic (<0.1); mesoendemic (between 0.11 and 0.5); hyperendemic (between 0.51 and 0.75); and holoendemic (>0.75). A number of studies have tried to quantify the relationship between prevalence rate and EIR (Charlwood et al., 1998; Beier et al., 1999; Smith et al., 2004), since this determines the public health impact of any intervention that works by reducing malaria transmission. But the clinical outcome of malaria infection depends also on the specific immunological and pathological interactions of parasite strains and individual humans (Gilbert et al., 1998; Stirnadel et al., 1999a; Stirnadel et al., 1999b).

A high EIR results in stable and intense transmission, with young children the most vulnerable for severe illness. Higher EIRs are, in general, associated with increased frequency and density of parasitemia, febrile episodes, anemia and cerebral malaria in heavily endemic areas (Beier et al., 1999; McElroy et al., 1997). The lower the EIR is, the greater the number of susceptible individuals who can develop severe infection and illness (Mbogo et al., 1995; McElroy et al., 1997). Recent climate-based mapping of malarious zones by endemicity and epidemic risk (Craig et al., 1999; Guerra et al., 2007) can help prevent medically catastrophic and disruptive malaria epidemics if realistic program objectives and indicators for action are established, periodic monitoring and supervision occur, and corrective steps are taken promptly when gaps are found (Bryce et al., 1994).

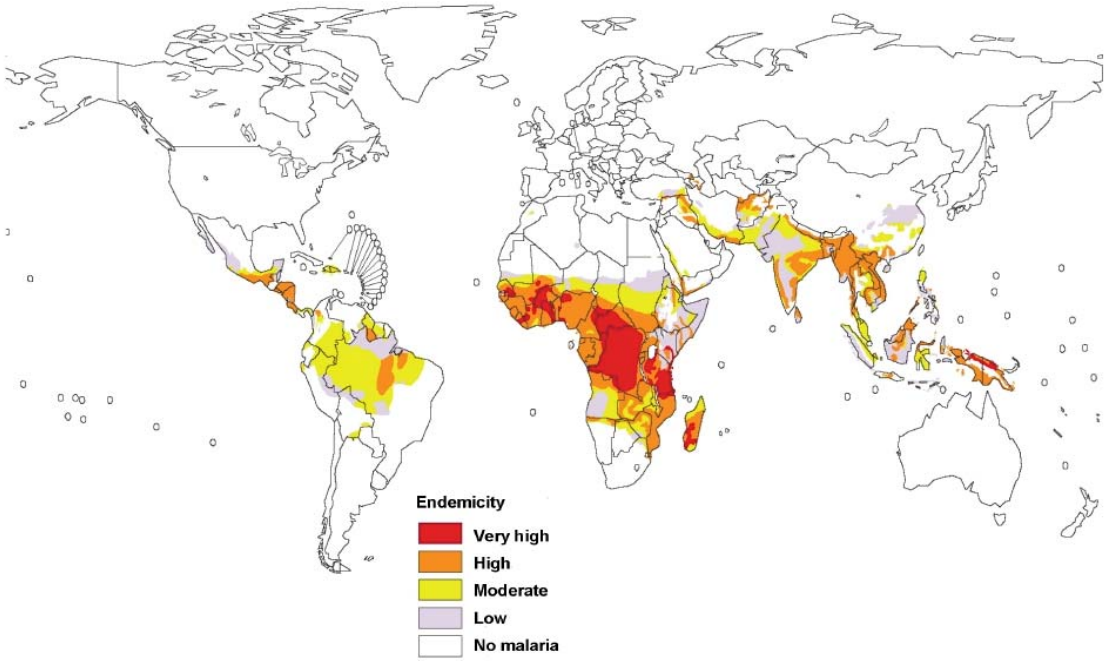


Figure 2: Malaria transmission risks map (From WHO,RBM, 2005)

Table 1: **Distribution (%) of *Plasmodium* species in following areas (Carter & Mendis, 2002)**

Species	**Sub-Saharan Africa**		**Asia**			**Central America and Caribbean**	**South America**
	West and Central	East and Southern	South central and Middle East	Western Pacific and Southeast Asia	Western Pacific (Vanuatu)		
P.falciparum	88.2	78.8	19.8	51.4	43.0	12.9	29.2
P. vivax	1.2	9.8	80.2	48.6	56.1	87.1	70.6
P. malariae	2.2	3.0	0.0	0.0	0.9	0.0	0.2
P. ovale	8.4	8.4	0.0	0.0	0.0	0.0	0.0

I.3.2. Meteorological and environmental changes

The world appears to have warmed over the past 100 years (Houghton, 2002), but the impact of the climate change on disease patterns is controversial. Global warming may have led to the spread of malaria in some previously malaria-free mountainous areas of Asia, South America and Africa, but other environmental factors are likely to be involved in this process (Hay et al., 2002).

Human engineering projects as constructions of dams, roads, and industrial and residential centres, can result in the disruption of the terrain, allowing increased mosquito breeding sites (Ghebreyesus et al., 1999).

The relationship between the amount of rainfall and the development of breeding sites is dependent on several factors, such as the slope of the land, run-off and soil type, and the suitability of these breeding sites will be further affected by the availability of shade, vegetation, salinity and predators (Keiser et al., 2005). Deforestation and the development of new breeding sites led to at least the doubling of malaria risk during the construction of the Trans-Amazonian Highway during the early 1970s (Warrell & Gilles, 2002).

Certain crops, notably rice, provide suitable environment for vector breeding, and such activities could have an effect on the malaria distribution; but evidence for this is mixed (Ijumba et al., 2001). In some rice systems, the density of *An. gambiae* was 10 times higher in the rice fields than the surrounding savannah, yet the sporozoite rate among the vectors was 10 times lower and the expected increase in malaria incidence was not observed (Robert et al., 1985).

I.3.3 Host factors and immune system

Human populations exposed to malaria infection may vary in their susceptibility to infection and severity of illness. It seems that human genetic polymorphisms, especially those affecting red blood cells (RBCs) have been selected because they confer selective advantage against malaria (Lin et al., 1994). These polymorphisms are found in elevated frequencies usually only in populations which have been exposed to malaria for at least several hundred years, namely in Africa, parts of the Middle East, Southern Asia, and Melanesia, and are associated with high protection against malaria in heterozygous combination but which also carry a high balancing cost in homozygous combination (Carter & Mendis, 2002).

The possible protective effects of sickle cell trait against *P. falciparum* malaria were first noted in patients from Zambia (Beet, 1946), where lower rates of malarial infection among carriers of the sickle cell trait than amongst non-sicklers were recorded. It has been fully

confirmed that this mutation give at least 90% protection to children against mortality of malaria infection (Gilles et al., 1967; Hill et al., 1991).

Outside of Africa, the highest malaria transmission intensities are found in Melanesia, including the island of Papua new Guinea (PNG), the Solomon Islands and the Islands of Vanuatu (Killeen et al., 2000), but the sickle cell trait does not seem to be present in Melanesian population (Cortes et al., 2003). However, a great variety of other red blood cell traits are found in PNG with geographical patterns paralleling those in malaria endemicity (Serjeantson et al., 1989), like the South Asian Ovalocytosis (SAO), which was found to offer moderate protection against malaria infection (Cattani et al., 1987), but complete protection against cerebral malaria (Genton et al., 1995b; Allen et al., 1999). Other red blood cell traits are found with different prevalence in PNG, such as the Gerbich blood group negativity, both α- and β-thalassemia, Glucose-6-phosphate dehydrogenase (G6PD) deficiencies and at lower level, Duffy polymorphism (Booth et al.,1982; Yenchitsomanus et al., 1985; Yenchitsomanus et al., 1986; Zimmerman et al., 1999) and have patchy distribution, suggesting separate and distinct evolution even within PNG, driven by the presence of malaria and aided by the relative isolation of PNG (Müller et al., 2003).

The immune status of the individual and population plays the most important role in the clinical response to infection and parasite transmission. Maternally derived antibody offers limited and short duration protection to the newborn (Bruce-Chwatt, 1952; Franks et al., 2001). In high endemic areas, over 30% children acquire parasites by 3 months of age (Slutsker et al., 1996). Repeated infections induce in most of people premunition. This is an age dependent condition in which the patient is infected with parasites, but does not have the symptoms (Smith et al., 1999). Many studies have suggested that the main determinant of clinical immunity is the duration of exposure to blood-stage parasites, and not the transmission intensity, defined as EIR (Smith et al., 1998; Gatton & Cheng, 2004; Bodker et al., 2006).

I.3.4. Population growth and urbanization

The rapid increase in the world's urban population has major implications for the transmission and epidemiology of malaria and other vector-borne diseases. The developing countries experience the highest urbanisation rates, often in the range of 2-6 % per year (Keiser et al., 2004). Higher human population densities may reduce biting rates, owing to the ratio of humans to mosquitoes (Robert et al., 2003). However many developing countries have declining economies and most cities are struggling to cope with the pace and the extent of

rapid urbanisation (Keiser et al., 2004). Inadequate water supply and sanitation infrastructure facilitate the creation of breeding sites for mosquitoes transmitting malaria and pose unique challenges for control (Breman; 2001). Furthermore, the adaptation of malaria vectors to urban areas has been well documented for at least two decades and local transmission has been conclusively demonstrated in most African cities (Keiser et al., 2004).

I.3.5. Health service provision

A high standard of care and treatment is crucial in reducing mortality from communicable disease. Little quantitative information exists on the state of public and private health service provision in most developing countries (Nolan et al., 2001). Many febrile illnesses in endemic areas mimic malaria, and confirmatory parasitologic diagnosis is not often available, prompt or reliable, particularly in rural areas (Amexo et al., 2004; Reyburn et al., 2004). Effective national malaria control programmes should be able to detect outbreaks, to monitor parasite and mosquito populations for changes in drug and insecticide resistance, and to make changes in treatment and control guidelines in a rapid and responsible way based on sound scientific findings. A major investment in staff recruitment, training and support after training is needed if the new tools that are being developed for malaria control are to be introduced and evaluated effectively in the areas where they are most needed.

I.3.6. Drug resistance

Because no effective vaccine is yet available, malaria prevention and control relies primarily on antimalarial drug and antimosquito measures such as insecticides and bed nets. Unfortunately, *P. falciparum* has developed resistance to nearly all classes of antimalarial drugs available. Widespread drug resistance in parasites aggravates the burden of malaria (Trape et al., 1998). The extent of the problem is due to the lack of effective drugs to treat the disease, and to the policies and practices constrained by limited resources. All drugs in use are affected by the phenomenon except artemisinin derivatives.

The resurgence of malaria in the past decade is in large part the result of increasing treatment failure associated with the development of drug resistance, particularly chloroquine resistance (Wernsdorfer & Noedl, 2003). The dramatic impact of chloroquine resistance on malaria mortality has long been underestimated because only a low proportion of malaria attacks are potentially lethal among older African children and adults exposed to high transmission (Snow, 1999; Trape, 2001). According to Trape et al., 1998 and Snow et al., 2001, malaria mortality has doubled or increased in most parts of Africa during the past two decades, where

drug resistance has also increased, and increased incidence of malaria-related anaemia has probably contributed to mortality from other diseases (Trape et al., 2001; Snow et al., 1999; Menendez et al., 2000). In Senegal, results of a 12-year community –based study showed that the onset of chloroquine resistance at least doubled childhood malaria death risk, and in some sites increased it up to 11-fold in the younger children (Trape et al., 1998). A major impact was also documented in the highlands of East Africa, where the spread of CQ resistance was the only factor likely to explain the changing epidemiology of malaria in areas of low transmission, despite initial claims that it could be attributed to global warming (Shanks et al., 2000).

I.4. Malaria control strategies

The global campaign to eradicate malaria, launched by WHO in 1955 and phased out by the end of 1960s did not come close to its headline objective of eradicating malaria. It did lead to enormous and sustained reductions in the burden of malaria in dozens of countries around the world. The eradication effort was abandoned when it became apparent that eradication was not possible in high transmission areas; resistance to DDT, the cornerstone of indoor residual spraying, appeared in mosquito vector species. Meanwhile, the malaria parasite was becoming resistant to chloroquine and other first-line drugs.

In the late 1990s, there was a renewed interest in malaria control. In 1997, the Multilateral Initiative on Malaria (MIM), an alliance of agencies, institutions, and governments, was formed to maximize the impact of scientific research on malaria through capacity building in Africa and through global collaboration. The following year, the World Health Organization (WHO), United Nations Children's Fund (UNICEF), United Nations Development Program (UNDP) and the World Bank, launched the Roll Back Malaria (RBM) Global Partnership to coordinate efforts in combating malaria. Several other major international initiatives have then been launched to tackle malaria. These include the Medicines for Malaria Venture (MMV), the Malaria Vaccine Initiative (MVI), and the Global Fund to Fight AIDS, TB and Malaria (GFATM), which supports the implementation of prevention and treatment programs.

There are a number of ways to decrease malaria transmission but none currently offers a complete block. Therefore new methods are urgently required. The three combined strategies of drug treatment, vaccination and vector control will ultimately be required to significantly reduce malaria transmission.

I.4.1 Vector control

Home-based vector and disease control interventions have been spectacularly successful in the past. Early efforts to control malaria during the 1950s and 1960s with spraying indoors with DDT and other insecticides achieved almost total eradication of the vector and the pathogen in many parts of the world. These efforts simultaneously reduced levels of transmission of dengue, leishmaniasis and filariasis (Hemingway et al., 2006). Despite attempts to ban DDT completely, the compound continues to be used, although arguments about issues as the effect on preterm births and the duration of lactation continue (Chen et al., 2003). However, in 2006, the World Health Organisation (WHO) announced the promotion of DDT for indoor spraying against malaria mosquitoes (WHO, 2006).

In some southern African countries (mainly those with unstable malaria), indoor house-spraying with DDT, carbamates, or pyrethroids, used alone or with Artemisinin-based combination therapies (ACTs) has substantially improved malaria control (Mabaso et al., 2004).

Some of the first studies worldwide of insecticide-impregnated bednets (ITNs) were carried out in Papua New Guinea (Müller et al., 2003). These demonstrated effects both on mosquito populations (Charlwood et al., 1987) and on the prevalence and incidence of *P. falciparum* in children (Graves et al., 1987), even if some studies tend to suggest a differential protective effect against *P.falciparum* and *P.vivax* (Bockarie et al., 1996; Prybylski et al., 1994; Burkot et al., 1990). This could be due to the biting behaviour of the mosquitoes infected with different malaria parasites; those infected with *P. vivax* biting earlier, at a time before people go to sleep under the protecting bednets (Bockarie & Dagoro, 2006).

More recently ITNs reduced morbidity and also mortality from all causes (Lengeler, 2004; Fegan et al., 2007). In African populations that use such nets consistently, parasitaemia in young children has been reduced by 62% and overall child survival has increased by 27% (Schellenberg et al., 2001b). This is a result of protection at the levels of the individual and the community (Lindblade et al., 2004). Regular re-treatment of nets with insecticides has proved difficult to sustain on a large scale, especially if users have to pay for it (Killeen et al., 2007). This problem should be overcome by the development of long-lasting insecticidal nets (LLINs), in which insecticide is incorporated into the nets fibres. Despite the proven benefits and cost-effectiveness of ITNs, achievement of widespread use has proved difficult (Teklehaimanot et al., 2007). Full cost recovery for nets makes them unavailable to the most vulnerable groups. Part cost recovery, often combined with social marketing (Schellenberg et al, 2001b) solves the problem to some extent. Free mass distribution of LLNs, sometimes

linked to other initiatives such as vaccination or attendances to antenatal clinics, has strong advocates (Curtis et al., 2003) and is gaining support (Teklehaimanot et al., 2007), but this approach needs a major and sustained commitment from international donors (Sachs & Mc Arthur, 2005).

Repellents could provide useful protection against malaria (Rowland et al., 2004), especially in places where vector mosquitoes bite early in the evening. In such situations, they might add benefits to ITNs.

Environmental management (including drainage of breeding sites), improvements to house design, use of larvivorous fish, and zooprophylaxis have proved effective in some specific epidemiological situations, but must be based on detailed behavioural knowledge of the main vector (Breman et al., 2001).

The development of novel tools to improve our understanding of the biology of the mosquito vectors is providing a unique opportunity for targeted interventions to fight the disease. The genome of *Anopheles gambiae* has now been sequenced (Brey, 2003), providing opportunities for new, targeted measures for control. For example, use of olfactory cues by female mosquitoes to find their human hosts and identification of odorant-binding proteins in the antennae of mosquitoes that respond to components of human sweat could lead to development of new types of insect traps and repellents (Hallem et al., 2004). In recent years, researches on the development and release of mosquito that are fully refractory to *Plasmodium* have been launched (Riehle et al., 2003; Catterucia, 2007). Although refractory strains have been produced in laboratories, the challenge is to find ways to drive resistance mechanisms into the wild mosquito population, without that the genetically altered mosquitoes are at a selective disadvantage and therefore quickly eliminated (Coleman et al., 2004). Alternative genetic modifications that are lethal to female mosquitoes could be more effective (Alphey et al., 2002).

I.4.2. Accurate diagnosis and effective treatment

Rapid, accurate diagnosis is fundamental to effective management and control of malaria. However conclusive diagnosis in endemic countries still relies predominantly upon clinical presentation and microscopy. Diagnosis by clinical syndrome alone is not reliable (Chandramohan et al., 2002). Light microscopy, the “gold standard” in highly trained hand can provide good sensitivity and specificity while providing parasite density, stage and species differentiation (Coleman et al., 2002). However light microscopy is labour-intensive, requires significant technical skills and good equipment.

The consequences of these diagnosis limitations are that many patients treated for mild or severe malaria do not actually have the disease, and conversely many cases of malaria are not diagnosed (Amexo et al., 2004, Reyburn, 2004). Over-diagnosis and consequent over-prescription of antimalarials has led to the increasing resistance of *P. falciparum*. Alternatively, several rapid diagnostic tests (RDT) based on antigen-capture techniques have been developed that have high sensitivity and specificity for *falciparum* malaria and that could contribute greatly to improving malaria diagnosis in some situations (Craig et al., 2002; Murray et al., 2003). However, these tests have limitations. In highly endemic areas, many healthy individuals have parasitaemia. Thus, although a negative test rules out malaria, a positive test does not prove that an illness is caused by malaria (Moody, 2002). Another limitation of RDTs is their cost, but the introduction of artemisinin-based combination therapies (ACTs) financed by Global Fund initiative probably will make RDTs more cost-effective (Greenwood et al., 2005).

Although chloroquine is still the first-line treatment for uncomplicated *falciparum* malaria in some countries it now fails almost everywhere (WHO, World malaria Report, 2005). Resistance to sulfadoxine-pyrimethamine has occurred in most countries where the drug has been introduced to replace chloroquine (EANMAT, 2003), although not in all (Plowe et al., 2004). Some form of combination treatment is now clearly needed for the first-line treatment of *falciparum* in Africa and Asia (Kremsner & Krishna, 2004). There is a considerable interest in the use of multiple drugs, with different mechanisms of action (White, 1999). Such combination treatment is likely to extend the useful lifetime of the constituent drugs by reducing the rate at which resistance develops (White, 1999).

I.4.2.1. Artemisinin-based combination therapies (ACTs)

Artemisinin-based combination therapies (ACTs) are now the best available treatment for uncomplicated multidrug-resistant *falciparum* malaria (WHO, 2006). Artemisinin enhances efficacy and has the potential of lowering the rate at which resistance emerges and spreads (White & Olliaro, 1996). Because of their rapid reduction of parasite biomass and the complete absence of documented resistance despite over 2000 years of use, the artemisinin derivatives are components of many candidate combinations (White, 1997; Nosten & Brassuer, 2002). Artemisinin derivatives (artesunate, artemether, dihydroartemisinin) are well tolerated antimalarials and produce the fastest therapeutic response of all drugs (White et al., 1999; Ashley & White, 2005). Another advantage is that ACTs may also reduce malaria transmission and spread of resistance through their gametocytocidal properties (Price et al.,

1996, Nosten et al., 2000). Widespread deployment has been associated with a reduction in the incidence of *falciparum* malaria both on the northwestern border of Thailand and in Kwa-Zulu Natal province, South Africa (Barnes et al., 2005; Muheki et al., 2004; Nosten et al., 2000). The World Health Organization (WHO) now recommends treating uncomplicated malaria with artemisinin-based combination therapies (ACTs) (WHO, 2006).

Even if ACTs are generally accepted, many operational questions remain. The major obstacle to large-scale use of ACTs is their cost; they are up to ten times more expensive than current monotherapy, which is unrealistic in many settings unless subsidies are introduced (Whitty et al., 2004). Other limiting factors are limited knowledge on safety of ACTs in pregnancy (Dellicour et al., 2007), operational issues such as inappropriate drug use, lack of suitable drug formulations (Mutabingwa, 2005), lack of post-marketing surveillance systems (Hetzel et al., 2007), and the imbalance between demand and supply (Kindermans et al., 2007). The current choice of ACTs is restricted; the combination of artemether and lumefantrine, known by the trade name Coartem®, is currently the only commercially available co-formulated artemisinin-containing combination therapy for malaria (Greeenwood et al., 2005).

However, the Global fund has now agreed to subsidize ACT in most sub-Saharan Africa (GFATM, 2008), and several new drugs are at different stages of development (MMV, 2007). Two have considerable promise. First the fixed-dose combination dihydroartemisinin-piperaquine (Artekin™) is already being used in much of Southeast Asia, has proven very efficacious, and is cheaper than Coartem® (Myint et al., 2007). The second, treatment is the combination of artesunate and chloroproguanil-dapsone. The combination is cheap, well-tolerated, and works in areas where SP has failed (Mutabingwa et al., 2001, Alloueche et al., 2004).

I.4.3. Intermittent preventive treatment (IPT)

The discovery that sulfadoxine-pyrimethamine (SP) given on two or three occasions during pregnancy was more effective at preventing infection of the placenta than chemoprophylaxis with chloroquine was a breakthrough (Schultz et al., 1994). Subsequent studies in endemic areas have shown that this approach, now known as intermittent preventive therapy in pregnancy (IPTp) reduced the incidence of severe maternal anaemia and improved birth-weight (Vallely et al., 2007). Its use in areas of medium to high transmission is recommended by World Health Organization (WHO, 2007), even if its efficacy is reduced in HIV-positive women (Van Eijk et al., 2004).

In children, intermittent preventive therapy (IPTi) lowered mortality, morbidity and severe anaemia from malaria substantially (Schellenberg et al., 2001; Massaga et al., 2003), but it is difficult to sustain over long periods, could encourage drug resistance, and could impede the development of natural immunity (Greenwood, 2006).

An important issue is the increasing resistance of *P. falciparum* to SP, which makes the search more urgent for a safe and effective alternative therapy. Dihydroartemisinin-piperaquine may be the most promising candidate, it is well tolerated and piperaquine is slowly eliminated. It has not yet been evaluated in pregnancy, so more information is needed on safety and pharmacokinetics in this context (White, 2005).

I.4.4. Malaria vaccines

Vaccine development against both *falciparu*m and *vivax* is ongoing (Moorthy et al., 2004; Aide et al, 2007). The decision on which parasite antigens are to undergo clinical development is difficult. Criteria for development include evidence showing that the antigen serves a function critical to the parasite, is associated with naturally acquired immunity, or is protective in animal models (Greenwood & Alonso, 2002). So far, most efforts have been directed at the development of pre-erythrocytic stage vaccines designed to prevent invasion of hepatocytes by sporozoites or to destroy infected hepatocytes.

RTS,S/AS02A is the most advanced pre-erythrocytic vaccine, and is a hybrid molecule in which the circumsporozoite protein of *P. falciparum* is expressed with hepatitits-B surface antigen (HBsAg) in yeast (Stoute et al., 1997). The vaccine is given with AS02, a complex three-component adjuvant. RTS,S/AS02A has provided substantial (70%) but only short-term protection in naturally exposed, semi-immune adults from the Gambia (Bojang et al., 2001). Some protection was restored by one booster dose vaccine given the next year. In a subsequent trial in Mozambican children, RTS,S/AS02A gave 30% protection against the first clinical episode of malaria and 58% protection against severe malaria (Alonso et al., 2004). More recently, a formulation with another adjuvant (RTS,S/AS02D) proved to be safe, well tolerated and immunogenic in a phase I/IIb trial in Mozambican infants with efficacy of 35% in protecting against clinical episode of malaria (Aponte et al., 2007).

Multicomponent vaccines will probably be needed to cope with the problem of antigenic polymorphism. An alternative approach is whole “organism vaccines” with sporozoites or blood stage forms (Druilhe & Barnwell, 2007). A combination of very low doses of infection

and drug treatment gave complete protection against malaria in volunteers (Pombo et al., 2002).

Transmission-blocking vaccines, which are designed to prevent mosquitoes that feed on vaccinated individuals from becoming infected, thus reducing transmission and providing indirect protection to the entire population, are well in advance and phase I trials are taking place (Malkin et al., 2005, Aide et al., 2007).

I.5. Antimalarial chemotherapy

I.5.1 Antifolates

This large group interferes with the DNA synthesis by inhibiting the folate biosynthesis pathway resulting in decreased pyrimidine synthesis, hence reduced DNA, serine and methionine formation. Activity is exerted at all growing stages of the asexual erythrocytic cycle and on young gametocytes (Gregson & Plowe, 2005).

Prokaryotic and eukaryotic cells require reduced folate cofactors for the biosynthesis of many cellular components. The malarial parasite can either synthesize or salvage folate precursors, whilst mammals have no *de novo* synthesis and must rely on exogenous (dietary supplied) folate (Dieckmann & Jung, 1986b; Kicska et al., 2003).

In the folate synthesis pathway, which is unique to microorganisms and plants, *p*-aminobenzoate (pABA) condenses with 2-amino-4-hydroxy-6-hydroxymethyl-7,8 dihydropteridine pyrophosphate (DHPPP), a reaction catalyzed by dihydropteroate synthetase (DHPS) to form dihydropteroate (DHP). DHP is the substrate for the next enzyme, dihydrofolate synthase (DHFS), which adds glutamate to produce dihydrofolate (DHF). DHF is then reduced by the enzyme dihydrofolate reductase (DHFR) to form tetrahydrofolate (THF). THF and its derivatives are used as cofactors in several biosynthetic reactions involved in the synthesis of amino acids such as methionine, serine, glycine and histidine, in addition to purine and thymidylate biosynthesis (Patel et al., 2004).

The antifolates are mainly used in combination, most commonly sulfadoxine and pyrimethamine (SP or Fansidar). Sulfadoxine is a member of the group of sulfonamides and sulfones that mimic p-aminobenzoic acid (pABA). They prevent the formation of dihydropteroate (DHP) from DHPPP catalysed by dihydropteroate synthase (DHPS) by competing for the active site of DHPS (Dieckmann & Jung, 1986a). Pyrimethamine, biguanides and triazine metabolites, quinazolines inhibit dihydrofolate reductase (DHFR).

DHFR inhibitors mimic the pteridine ring of the natural substrate DHF, and compete with it for the active site of the enzyme (Arav-Boger & Shapiro, 2005).

I.5.2. 4-aminoquinolines

This class includes chloroquine (CQ), pyronaridine (PY) and amodiaquine (AQ). Despite years of use, the mechanism of 4-aminoquinoline action and resistance is still a matter of investigation. Various mechanisms have been proposed for the action of chloroquine and related compounds, either extra- or intravacuolar (O'Neill et al., 1998; Basco et al., 1994). Some of the proposed mechanisms would require higher drug concentrations than those that can be achieved *in vivo,* and hence, are not generally regarded as convincing options. These include inhibition of protein synthesis (Surolia & Padmanaban, 1991), inhibition of food vacuole lipase (Ginsburg & Geary, 1987) and aspartic proteinase (Van der Jagt et al., 1986), and inhibition of DNA and RNA synthesis (Cohen &Yielding, 1965; Meshnick, 1990).

The commonly accepted hypothesis is that quinoline-containing drugs act primarily on heme disposal, a process whereby intraerythrocytic stage malaria parasites detoxify hem in the food vacuole. Where most of theories appear to concur is that 4-aminoquinolines selectively affect stages of the parasite life cycle after haemoglobin digestion and that they accumulate in the digestive vacuole of the parasite (Yayon et al., 1984), where they are supposed to interfere with the heme detoxification (Slater and Cerami, 1992) or modifying aspartic and cysteine protease activity (Goldberg et al., 1991) or intravascular pH (Krogstad et al., 1985).

To produce amino acids (Rudzinska et al., 1965; Krugliak et al., 2002), and possibly, to regulate osmotic pressure within the host erythrocyte (Lew et al., 2004), malaria parasites use their endolysosomal system to feed on hemoglobin. The latter is acquired by endocytosis, and then the hemoglobin-laden endocytic vesicles mature into digestive vacuoles, which are probably the equivalent of acidic late endosomes or lysosomes in other eukaryotic cells (Aikawa and Seed, 1980; Bannister et al., 2000). As hemoglobin-laden endocytic vesicles mature, they not only lose their inner membranes, they become acidic (Yayon et al., 1984a; Krogstad et al., 1985) and acquire proteolytic enzymes (Levy et al., 1974; Shenai &Rosenthal, 2002; Banerjee et al., 2002), their cargo of hemoglobin is denatured and degraded, and peptides and ferriprotoporphyrin IX (ferric heme, FP) are released (Goldie et al., 1990; Kolakovich et al., 1997; Banerjee et al., 2002).

The peptides are degraded to amino acids probably by cytoplasmic proteases (Krugliak et al., 2002, Kolakovich et al., 1997), some of the FP also may be degraded (Ginsburg et al., 1998;

Loria et al., 1999), but most of it is detoxified and sequestered in hemozoin (Fitch and Chevli, 1981; Egan et al., 2002). The amino acids are believed to undergo protonation and exported to the cytosol where they are utilized for protein synthesis and parasite growth. The heme is membrane-toxic; it rapidly intercalates with lipid bilayers and interferes with electron transport chains, leading to peroxidative change to unsaturated lipids and or membrane-embedded proteins (Zhang et al., 1999). Undimerized FP is detoxified by conversion to β-hematin (Fitch and Kanjananggulpan, 1987; Slater et al., 1991). β-hematin is comprised of FP monomers reciprocally linked through coordination complexes between the carboxyl group of a propionate side chain of one monomer and an iron atom in the porphyrin ring of another monomer (Slater et al., 1991; Pagola et al., 2000).

In the absence of an enzyme, other candidates to promote FP dimerization have been evaluated, and fractionation studies have demonstrated that lipid is the only substance that could promote the dimerization (Fitch et al., 1999). Furthermore, studies of commercially available purified lipids showed that arachidonic, linoleic, oleic, and palmitoleic acids, and 1-mono- and di-oleoylglycerol are able to promote FP dimerization (Fitch et al., 1999). Apparently, the unsaturated fatty acids and their mono- and diglycerides serve to concentrate monomeric FP and keep it in a state favourable for dimerization (Fitch et al., 1999, 2000), perhaps through an oxidative mechanism (Fitch et al., 1999; Tripathi et al., 2002). Since malaria parasites are enriched with linoleic acid, it is probable that linoleate or derivative of linoleate is the principal lipid that promotes FP dimerization (Fitch et al., 2000).

It seems that chloroquine binds to undimerized ferriprotoporphyrin IX, delaying its detoxification, finally causing it to accumulate and allowing it to exert its intrinsic biological toxicities (Fitch, 1986). When susceptible parasites are exposed to chloroquine, endocytosis of erythrocyte cytoplasm continues, but the processing of endocytic vesicles does not (Macomber et al., 1967; Zhang, 1987; Fitch et al., 2003a). Consequently, hemoglobin-laden endocytic vesicles with double membranes pile up in the parasites, clump together, and provoke autophagic vacuole formation (Macomber et al., 1967; Fitch et al., 2003a). In addition to morphologic changes, the accumulation of FP-chloroquine complex has other several effects, including impairment of the membrane function (Chou and Fitch, 1980; Dutta and Fitch, 1983), peroxidation of the lipids (Tappel, 1955; Sugoika and Suzuki, 1991), inhibition of proteolytic enzymes (Vander jagt et al., 1987), destruction of glutathione (Ginsburg et al., 1998), release of calcium from the acidic store (Passos and Garcia, 1998), redistribution of a neutral aminopeptidase (Fitch et al., 2003a), and masking of the lipid that promotes FP dimerization (Fitch et al., 2003b).

I.5.3. Quinoline-4-methanols

This class includes quinine, mefloquine, lumefantrine and halofantrine. Similarly, the precise mode of action of these quinolines is not known. Like chloroquine, it seems that the quinoline-4-methanol act primarily on the intraerythrocytic stages (Schmidt, 1978; Geary et al., 1986), but the available data suggest that mefloquine and quinine interfere with a different step in the parasite feeding process than chloroquine (Geary et al., 1986; Fitch, 2004).

When exposed to mefloquine or quinine, chloroquine-susceptible malaria parasites, do not exhibit accumulation and aggregation of hemoglobin-laden endocytic vesicles (Ladda, 1966, 1969; Jacobs et al., 1987; Olliaro et al., 1989), increased masking of the lipid that promotes FP dimerization or excess accumulation of undimerized FP (Chou and Fitch, 1993). On the contrary, quinine and mefloquine prevent and partially reverse chloroquine-induced abnormalities in susceptible malaria parasites, by inhibiting and reversing vesicular docking in the endolysosomal system, either directly or indirectly by inhibiting calcium release for the acidic store (Osisanya and Warhurst, 1981; Fitch et al., 2003a, b). Mefloquine and quinine still kill the parasite by inhibition of membrane recycling, causing an excess membrane to accumulate in the form of multilamellar bodies (Jacobs et al., 1987; Olliaro et al., 1989).

A second hypothesis is that quinine and mefloquine inhibit the hemoglobin digestion (Famin and Ginsburg, 2002), which could explain the chloroquine-antagonist effect. It seems also that mefloquine binds with high affinity to membrane and purified phospholipids (San George et al., 1984), and quinine to phospholipids (Ginsburg and Demel, 1983; Porcar et al., 2003), supporting the hypothesis that malaria parasites have one or more phospholipid targets for quinoline-4-methanol antimalarials (Fitch, 2004).

I.5.4. Artemisinins

Artemisinin is a potent antimalarial compound extracted form plant material (*Artemisia annua*). Artemether, artesunate and dihydroartemisinin are semi-synthetic derivatives of artemisinin that are in common clinical use (Dollery, 1999). All members of this drug group have activity throughout the phases of the asexual intra-erythrocytic schizogonic cycle, and also act on young gametocytes.

The mechanism of action is incompletely understood. Originally the mode of action of artemisinins was considered to be similar to chloroquine; inhibition of heme polymerization (Pandey et al., 1999). However, it was later shown that artemisinin kills parasites by heme-depended activation of the endoperoxide bridge (Meshnick, 1994). The prevailing hypothesis is that reductive cleavage of the intact peroxide by ferroheme ferrous –protoprophyrin IX (Fe

(II) PPIX) generates C-entered radicals, which, in turn, would alkylate biomolecules, leading to the death of the parasite (Winstanley and Ward, 2006). It was also proposed that antimalarial activity of artemisinin is conferred by the 1,2,4 trioxane pharmacophore within artemisinin (Olliaro et al., 2001). The trioxane structure is now being exploited in developing synthetic peroxide antimalarials (Vennerstrom et al., 2004).

However, localization of artemisinin to parasite cytoplasm and not to food vacuole membranes, and killing of tiny rings lacking hemozoin argue against the food vacuole being a major site for drug action (Terkuile et al., 1993).

A number of putative targets have been suggested and may be specific (PfATP6, heme, gluthathione) or indiscriminate (membranes, proteins, DNA) (Creek et al., 2007). The various *in vitro* studies reported for these targets all require Fe (II) iron, either in the form of reduced heme or as an inorganic Fe (II) salt (Meshnick, 2002). The importance of iron-mediated activation has also been shown by the inhibitory effect of iron chelators on artemisinin activity in cultures of *P. falciparum* (Meshnick et al., 1993).

I.6. Molecular basis of parasite resistance to antimalarial drugs

I.6.1 Resistance to antifolates

The molecular basis of resistance to sulfadoxine-pyrimethamine is the best characterised of all antimalarials. Specific mutations in *P. falciparum* that lead to resistance to both sulfadoxine and pyrimethamine have been identified. Sulfadoxine and pyrimethamine act synergistically. The former inhibits dihydropteroate synthetase (DHPS) and the latter inhibits dihydrofolate reductase (DHFR).

Resistance to SP *in vitro* is associated with a series of substitutions within the active site of target enzymes: DHPS and DHFR. This has been demonstrated through laboratory-based *in vitro* sensitivity tests and transfection experiments with *Pfdhfr* with respect to pyrimethamine (Wu et al., 1996; Zolg et al., 1989) and *Pfdhps* with respect to sulfadoxine (Triglia et al., 1997; Triglia et al., 1998; Wang et al., 1997). For *Pfdhps*, several point mutations are associated with in vitro resistance to sulfadoxine and other sulfas and sulfones: Ser→Ala at codon 436, Ala→ Gly at codon 437, Lys→Glu at codon 540, Ala→Gly at codon 581 and Ser→Phe at codon 436 coupled with either Ala→Thr or Ala→Ser at codon 613 (Plowe et al., 1997; Nzila et al., 2000).

Serine in position 108 of *Pfdhfr* is found in sensitive strains, whereas a mutation to asparagine at that position is associated with resistance to pyrimethamine and a moderate loss of response

to cycloguanil. A mutation to threonine at position 108, together with an alanine to valine change at position 16, appears to confer resistance to cycloguanil with only slight loss of response to pyrimethamine.

A modulation towards higher levels of pyrimethamine resistance appears to be facilitated by mutations of asparagines to isoleucine at position 51 and of cysteine to arginine at position 59 when associated with the asparagine-108 mutation. A mutation from isoleucine to leucine at position 164 in combination with the asparagine-108 and one or both of the isoleucine-51 or arginine-59 mutations has been found in *P. falciparum* strains that are highly resistant to both drugs (Foote et al., 1990; Peterson et al., 1990; Nzila et al., 2000). Accumulation of the mutations, in a stepwise manner is incriminated for increased resistance to antifolates (Plowe et al., 1998; Plowe et al., 1997).

The relative importance of mutations in *Pfdhfr* and *Pfdhps* to sulfadoxine-pyrimethamine resistance in vivo has been debated (Triglia et al., 1998; Watkins et al., 1999). Parasites with fewer than 3 *Pfdhfr* mutations Asn-108, Ile-51 and Arg-59 may be cleared by sulfadoxine-pyrimethamine, regardless of *Pfdhps* genotype (Wang et al., 1999). In the presence of this *Pfdhfr* triple mutation form, the *Pfdhps* mutant genotype increases the likelihood of treatment failure. This model is consistent with field studies that show an association between the prevalence of mutations in both genes and sulfadoxine-pyrimethamine treatment failure rates and that demonstrate selection for mutations in both genes after sulfadoxine-pyrimethamine treatment (Plowe et al., 1997; Kublin et al., 1998; Curtis et al., 1998; Wang et al, 1997). Nzila and colleagues showed that patients infected with parasites carrying the DHPS double mutant Gly-437/Glu-540 and the *Pfdhfr* triple mutant had a higher relative risk of treatment failure than did those infected with parasites carrying the *Pfdhfr* triple mutant alone (Nzila et al., 2000).

I.6.2. Resistance to 4-aminoquinolines

Resistant parasites to chloroquine are characterised by a reduced amount of CQ accumulated in the food vacuole as compared to sensitive parasites (Fitch et al., 1970).

Plasmodium falciparum resistance to CQ has been linked to mutations in the *P. falciparum* multidrug resistance (*pfmdr1*) gene and the *P. falciparum* chloroquine related transporter (*pfcrt*) gene (Wellems et al., 1991; Fidock et al., 2000; Reed et al., 2000; Sidhu et al., 2002). The *pfcrt* gene is located on chromosome 7 and codes for PfCRT, a vacuolar membrane transporter protein. Accumulation of mutations in the *Pfcrt* gene at position 72 Ser, 74 Ile, 75 Glu, 76 Thr, 220 Ser, 271 Glu, 326 Ser, 356 Thr and 371 Ile (Fidock et al., 2000) are

associated with chloroquine resistance, but the substitution of threonine for lysine in codon 76 was shown *in vitro* to associate absolutely with resistance in isolates from Africa, South America, Asia and Papua New Guinea (Djimde et al., 2001a; Fidock et al., 2000).

Most of the studies reporting an association note that, although the Thr76 mutation may be essential for the resistant phenotype, it is also present to a lesser degree in CQ-sensitive strains, suggesting that other polymorphisms in *pfcrt* are necessary or that several genes are involved. Djimde and colleagues found that the lack of Thr76 mutation was highly predictive of chloroquine treatment success, whereas its presence accounted for only a third of treatment failures (Djimde et al, 2001b).

Another gene, *pfmdr1*, which is located on chromosome 5 and codes for P-glycoprotein homologue 1 (Pgh1) (Reed et al., 2000), 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; in Mali (Djimde et al., 2001b); in Gambia (Von Seideln et al., 1997); in Sudan (Babiker et al., 2001); Uganda (Flueck et al., 2000); in Thailand (Price et al., 1999); in Brasil (Zalis et al., 1998), but not in others: in Uganda (Dorsey et al., 2001), in Laos (Pillai et al., 2001), in Thailand (Chaiyaroj et al., 1999) and in Brazil (Povoa et al., 1998). Several other *pfmdr1* polymorphisms, notably Phe184, Cys1034, Asp1042, and Tyr1246, have been implicated to varying degrees in chloroquine resistance (Flueck et al., 2000; Zalis et al., 1998; Povoa et al., 1998).

Although evidence for the association of *pfmdr1* with chloroquine resistance has not been as convincing as for *pfcrt*, Reed and colleagues showed in a parasite transfection experiment that polymorphisms in the *pfmdr1* gene modulate the susceptibility to chloroquine, as well as to mefloquine and the structurally related compounds quinine and halofantrine (Reed et al., 2000). An association between *pfcrt* Thr76 and *pfmdr1* Tyr86 has been noted in Nigeria (Adagut et al., 2001) and Sudan (Babiker et al., 2001) and provides support for a convergence of polymorphisms necessary for a resistance phenotype. The slow development of chloroquine resistance may signal such a multifactorial mechanism (Wellems et al., 2001).

I.6.3. Resistance to Quinoline-4-methanols

Mefloquine resistance in field isolates of *P.falciparum* is associated with amplification of the *pfmdr1* gene (Peel et al., 1993; Cowman et al., 1994) and over expression of its protein pgh-1 (Cowman et al., 1994). The P-glycoprotein pump (PgP) that is coded by *Pfmdr1* affects the intraparasitic concentration of several important antimalarials. It has been suggested that PgP indirectly mediates substrate accumulation by regulating the plasma pH gradient and/or

membrane potential (Roepe et al., 1996). It is likely that in some cases indirect mechanisms contribute to resistance while in other cases there is direct transport of the drug (Duraisingh et al., 2005). Single nucleotide polymorphisms in *Pfmdr1* that alter sensitivity in vitro to structurally unrelated antimalarials have been identified (Reed et al., 2000; Foote et al., 1994). Increase in *Pfmdr1* copy number was associated with up to 40-fold decrease in the in-vitro susceptibility to mefloquine (Price et al., 2004). In contrast to chloroquine, mutations in *Pfcrt* have been associated with increased susceptibility to mefloquine and quinine (Sidhu et al., 2002).

I.5.4. Resistance to artemisinins

Artemisinins show structural similarities to thapsagargin, which is a highly specific inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and it has been proposed that (SERCA)-type PfATP6 protein could be the target of artemisinin, because PfATP6 is the only SERCA-type Ca^{2+}-ATPase sequence in the parasite's genome (Eckstein-Ludwig et al., 2003). The interaction of artemisinins with thapsagargin-binding cleft of susceptible SERCAs was confirmed (Uhlemann et al., 2005), pointing out that a single amino acid in transmembrane segment 3 of SERCAs can determine susceptibility to artemisinins *in vitro*.

An *in vitro* study with field samples showed that S769N, E431K, and A623E mutations were related to *P. falciparum* with elevated IC_{50} values for artemisinins (Jambou et al., 2005), but these findings need to be confirmed.

Increased *Pfmdr1* copy number has been associated with elevated IC_{50} for artemisinins in SE Asia (Pickard et al., 2003; Price et al., 2004), but it should be also related to mefloquine, as in this part of the world artemisinin derivatives are always used in combination with mefloquine. As for arylaminoalcohols, *Pfmdr1* single nucleotide polymorphisms (SNPs) are generally associated with artemisinin hypersensitivity in laboratory studies (Duraisingh et al., 2000), transfections (Reed et al., 2000; Sidhu et al., 2005), and field isolates from Africa (Duraisingh et al., 2000), and SE Asia (Pickard et al., 2003), but these are not uniform findings (Anderson et al., 2005; Price et al., 2004). Similarly, *Pfcrt* seems to have a minor modulating effect on artemisinin IC50, this effect is either slight (Sidhu et al., 2002) or non-existent (Lakshmanan et al., 2005), depending on the parasite background.

A recent study in the rodent malaria model *P. chabaudi* showed that increasing resistance to artesunate and artemisinin was associated with single nucleotide polymorphisms in a gene coding for an ubiquitin-specific protease (*UBP-1*), but these results need to be confirmed in *P. falciparum* (Hunt et al., 2007).

I.7. Emergence and spread of *P. falciparum* resistance

I.7.1 Chloroquine

The introduction of chloroquine (CQ) for malaria treatment and prevention in the late 1940s was a great advance on existing drugs. Quinine, the mainstay of therapy for malignant tertian malaria caused by *P. falciparum* was not reliable as prophylactic and had unpleasant side effects. Chloroquine, cheap and better tolerated became the mainstay of therapy and prevention. However, resistance eventually developed in SE Asia and S. America by the end of 50s, in Papua New Guinea in the 70s, and reached E. Africa by late 70 (Talisuna et al., 2004).

Efficacy data collected in southern Africa (Tanzania, Uganda, Kenya and Rwanda) showed high rates of clinical failure rates to CQ, ranging from 10% to 71% (EANMAT, 2003), with most of the regions being above the critical value total treatment failure=25% (Talisuna et al., 2004). In contrary, in the same period, CQ was efficacious in western Africa (Nigeria, Mali, Senegal, Ghana, Ivory Coast, and Gambia). Only Ghana had CQ treatment failure rate above the critical value (Talisuna et al., 2004), even though the drug has been the most commonly used for uncomplicated malaria in this region (Evans et al., 2005; Happi et al., 2005).

In Papua New Guinea (PNG), chloroquine (CQ) or amodiaquine (AQ) were used for many years as standard treatment for uncomplicated malaria. However, chloroquine resistant P. *falciparum* has become highly prevalent after its first emergence in 1976 (Han and Grimmond, 1976). By the early 1980's, *in vivo* studies indicated that CQ resistant *P. falciparum* was present in ~50% of the children, while *in vitro* assays revealed that ~80% of the isolates were CQR (Cattani et al., 1986; Dulay et al., 1987). Recently, efficacy data from a rural area of PNG showed a clinical failure rate of 16% and 26%, respectively for CQ and AQ (Genton et al., 2005), and molecular studies have shown that CQR-associated mutations had spread throughout PNG by the mid 1980's (Mehlotra et al., 2001; Mehlotra et al., 2005).

The migration of drug resistance can be traced by the analysis of microsatellite markers closely linked to the genes conferring resistance; it provides a powerful approach to determining the numbers of origins of spread of resistance and complements epidemiological and clinical data describing the emergence of resistance. Examination of point mutations in *pfcrt* and microsatellite demonstrate at least four origins of resistance alleles, including a single origin in SE Asia and Africa, two independent origins in South America and an additional independent origin in Papua New Guinea (Fidock et al., 2000). All alleles conferring CQ resistance have the K76T mutation critical for resistance. Resistant alleles in

Asia and Africa have an additional seven to eight amino acid changes, those in South America have four to five other amino acid changes, while those in Papua New Guinea have five changes. Microsatellite data from chromosome 7 confirm these four independent origins (Wootton et al., 2002) and demonstrate that identical coding changes evolved independently in Papua New Guinea and South America (Mehlotra et al., 2001).

I.7.2. Sulfadoxine-pyrimethamine (SP)

The combination of pyrimethamine and sulfadoxine (SP) known as Fansidar is a cheap and effective agent against chloroquine resistant *falciparum* malaria that is widely used in Africa. SP has now replaced chloroquine as the first-line curative antimalarial in most parts of Africa (Pearce et al., 2003). Around 2000, most southern African countries replaced CQ with SP monotherapies, artemisinin combination therapies (ACTs) or non-ACTs as first line malaria treatment drugs (EANMAT, 2003). Unfortunately, the clinical efficacy of SP is diminishing as a consequence of the development of *P. falciparum* resistance to SP, rendering a limited lifespan of SP (Alifrangis et al., 2003). SP efficacy is already low in parts of East Africa. In Kilifi, Kenya coast, parasitological failure following SP treatment is 20% at day 7 (Nzila et al., 2000), and at Muheza, Tanzania, the day 7 failure rate is 45% rising to 60% on second treatment (Mutabingwa et al., 2001). In 2001, Tanzania replaced chloroquine with SP as interim first-line antimalarial drug (EANMAT, 2003). Two years later, treatment failure at day 28 was already 42.3% (Mugittu et al., 2005).

In PNG, SP was introduced in combination with CQ or AQ as standard treatment for *P. falciparum* malaria in 2000 after having been used in combination with quinine for a long time as second line treatment, but resistance developed rapidly. In Wosera, treatment failure at day 28 was already 28% in 2003 (Marfurt et al., 2007). Molecular data confirmed that reduced sensitivity to SP has developed in PNG after introduction of SP in combination with CQ or AQ (Mita et al., 2006).

Recent studies of microsatellite loci surrounding *dhfr* from areas of low and moderate transmission of malaria found a common haplotype for triple-mutant genotypes in sites 4000km apart in Africa and single haplotype for highly resistant genotypes in Southeast Asia (Nair et al., 2003; Roper et al., 2003). Further analysis of these haplotypes suggested that they originated in Southeast Asia, subsequently spread to Africa today (Roper et al., 2004; Maïga et al., 2007). Another study, also using microsatellites markers demonstrated that there was an independent origin of pyrimethamine resistance in *P. falciparum* in Melanesia (PNG, Vanuatu, Solomon Island) (Mita et al., 2007). It seems that resistance in Africa has spread in

part due to sporadic acquisition of resistance-mediating mutations, but expansion of highly resistant clones appears to have played a major role (Roper et al., 2003).

In a study conducted in Kenya, McCollum et al. found that SP-resistant *dhfr* genotypes may have multiple origins, and demonstrated also that the mutations in *dhfr* do not always occur in a predictable, ordered, stepwise manner (McCollum et al., 2006), meaning that local evolutionary history, as well as gene flow, is important to consider when one tries to understand the dynamics of drug resistance.

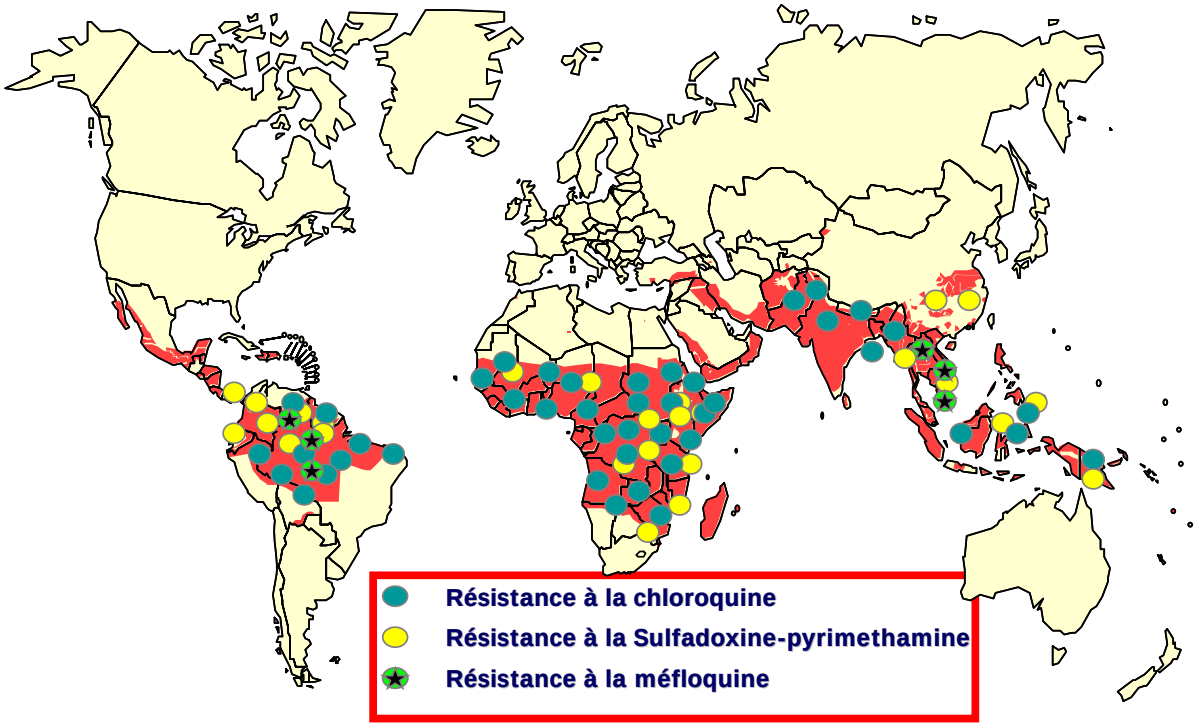


Figure 3: Drug resistance to *P. falciparum* (from WHO, RBM, 2005)

I.8. Factors contributing to drug resistance

Although the molecular mechanisms for parasite resistance have been elucidated for SP and more or less for CQ; the factors influencing the appearance and the spread of drug resistance within communities are far form being clear. Intuitively antimalarial drug use ought to be a critical factor and it has been postulated that widespread drug use and misuse will result in a faster spread of resistant parasites (Wongsrichanalai et al., 2002).

The emergence of resistance can be considered in two parts: first the initial genetic event which produces the resistant mutant, and second the subsequent selection process in which the

survival advantage in the presence of the drug leads to preferential transmission and the spread of resistance (White et al., 2003).

The genetic events which confer antimalarial drug resistance (whilst retaining parasite viability) are spontaneous and rare. They are thought to be independent of the drug (White et al., 2003). These can be mutations or changes in the copy number of genes relating to the drug's target. A single genetic event may be all that is required, or multiple unlinked events may be necessary.

However, drug resistance in malaria does not usually arise through a single mutational step, but more commonly arises as the end of a longer process during which parasites accumulate mutations and become ever more tolerant of the drug (Hastings & Watkins, 2006). These changes can help the parasite to resist the effects of the drug, but mutation away from a presumed evolutionary-optimal biochemical structure is likely to be disadvantageous in the absence of the drug (Hastings & Donnelly, 2005). Although the natural proportion of such mutants in the parasite population is low, and malaria isolates from populations and individuals show heterogeneity, selection of the most "fit" parasites occurs under drug pressure (Walliker & Babiker, 2005). One of the explanations for this is that some mutations reduce greatly fitness, so they are eliminated by natural selection and the successful one is associated with a second "compensatory" mutation that partially, or fully, compensates for the metabolic defect of the original mutation, and those resistant parasites are maintained by drug pressure (Nair et al., 2003). This is supported by another observation made in Malawi, where a study made 8 years after the cessation of CQ use, showed that CQ-sensitive parasites have re-emerged and are now predominant (Kublin et al., 2003).

However, other phenomenons have been suggested to increase chloroquine sensitivity. Primaquine has been shown to reverse chloroquine resistance for *P. falciparum* in culture (Bray et al., 2005), and it has been suggested to combine both drugs to extend the life of chloroquine (Egan, 2006; Egan & Kaschula, 2007). On the other hand, the combination of artemether and lumefantrine (Coartem®) has been suggested to select *Pfmdr1* 86N, which is the form found in chloroquine sensitive parasite both *in vitro* (Duraisingh et al., 2000) and *in vivo* (Sisowath et al., 2005), probably selected by lumefantrine.

The development and spread of drug resistance involve the interaction of drug-use patterns, characteristics of the drug itself, human host factors, parasite characteristics, and vector and environmental factors (Wernsdorfer et al., 1991; Wernsdorfer; Wernsdorfer et al., 1994 et al., 1994; Wongsrichanalai et al., 2001).

I.8.1. Pharmacodynamics and pharmacokinetics

The development of resistance depends in part on the pharmacokinetic and pharmacodynamic characteristics of drugs. Antimalarial drugs with long terminal half-lives are particularly vulnerable to the development of resistance. There is an increased chance that a new and unrelated infection may be acquired whilst drug concentrations following treatment have fallen below those sufficient to prevent parasite multiplication, and radically cure the new infection (White, 1997). If the originally infection is not radically cured, surviving parasites will be subjected to drug pressure as asexual cycles are exposed to sub-therapeutic blood concentrations (Watkins & Masobo, 1993). The transition from low to high-level resistance is slow for the quinoline antimalarials (chloroquine, amodiaquine, mefloquine), but may be more rapid for SP combinations (White, 1999).

I.8.2. Host immunity and genetics

Host immunity and genetics affects also the development of drug resistance in a number of ways, including a direct influence on the likelihood that an infected person will be symptomatic and will therefore seek treatment (Yeung et al., 2004). More potent immune responses increase the efficacy of the chemotherapy. A semi-immune patient might be cured by a drug despite the fact that his parasites are partially resistant. Individuals who are naïve to malaria have no specific immune response that is not effective as the specific immunity elicited by repeated infections (Wongsrichanalai et al., 2002). In a study, conducted in Mali, the ability to clear chloroquine-resistant parasites was strongly associated with age, which is the most consistent correlate of protective immunity in areas endemic for *P. falciparum* malaria (Djimdé et al., 2003).

The resistant parasite clearance phenotype was independently associated with ethnic background. These results suggest that there may be genetic characteristics that are important in the host's ability to mount a protective immune response to malaria infection (Modiano et al., 1996; Djimdé et al., 2003).

I.8.3. Transmission intensity

Transmission intensity does not directly affect the evolution of resistance; it exerts its influence through clinical/epidemiology mediators such as clonal multiplicity, level of immunity, and the level of the drug use in the population (Hastings & Watkins, 2005). It seems that where the transmission is low, the high use of the drug (because of the higher probability of an infection evolving towards a clinical attack) would select resistant strains

that would spread rapidly because the lower genetic complexity of the parasite population in infected individuals would increase the probability of self-fertilization (White et al., 2003).
In areas of high transmission intensity, the situation is more complex, as disease controlling immunity results in frequent asymptomatic parasitaemia and transmission from untreated healthy individuals and thus lower drug pressure. In addition, sexual stage specific immunity gradually develops in those living in areas of high intensity malaria transmission. The probability of cure of drug resistant parasite is greater and the selective advantage of resistant parasites is less (Barnes & White, 2005). These factors reduce both *de novo* emergence and spread of resistance (White & Pongtavonpinyo, 2003; White, 2004).
These differences may explain why historically multi-drug resistance has generally developed in areas with low transmission intensity and unstable malaria (Wongsrichanalai et al., 2002).

I.9. Assessment of drug resistance

Drug resistance has been defined as 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 those usually recommended but within the limits of tolerance of the subject (WHO, 1973).
Shortly after the first report of chloroquine resistance in 1965, standardized *in vivo* antimalarial drug efficacy testing systems were developed, used, and updated in 1972. These protocols remained in use until 1996, when a specific protocol for intense transmission areas (WHO, 1996) was developed. In this protocol, in vivo treatment responses were assessed for 14 days and classified on the basis of either clearance of clinical signs/symptoms as adequate clinical response (ACR), early treatment failure (ETF), and late treatment failure (LTF) or parasite resistance as resistance level 1 (RI), level 2 (RII), level 3 (RIII). Experience gained showed that 14 days protocol underestimates treatment failure rates. This led to the suggestion that post-treatment follow up should be long enough to detect recrudescent infections emerging later after initial parasite clearance.
The protocol was again revised in 2002 incorporating *in vitro* parasite susceptibility testing and drug resistance molecular marker assessment protocols as supporting methods (WHO, 2002). This new protocol recommends that assessment of response should be done for 28- 63 days, depending on the half life of the drug under study. In addition, the protocol combines clinical and parasitological observations in assessing treatment responses. Therefore, treatment outcomes are classified as adequate clinical and parasitological (ACPR); ETF, late failure (LCF) and late parasitological failure (LPF). Thus it is redundant now to report clinical and parasitological responses separately (Ringwald, 2004). However, longer follow-up

periods pose difficulties in interpreting drug efficacy outcomes, particularly in high transmission areas, because new infections occurring during follow up may be wrongly interpreted as treatment failures. Therefore, the WHO 2002 protocol emphasizes that molecular genotyping must be used to distinguish between new and recrudescent infections. Distinction of recrudescence from new infection is usually done by genotyping the highly polymorphic *P. falciparum msp1*, *msp2* and *glurp* genes using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis and subsequent comparison of genotypes from admission (Day 0) and the day of recrudescence (Snounou and Beck, 1998; Viriyakosol et al., 1995; Beck, 1999; Greenwood, 2002). Recently, analysis of immunological neutral microsatellite markers has been suggested to complement MSP (Nyachieo et al., 2005) whereas fluorescent-labelled PCR and sizing of fragments by Genescan was found to be more precise than PCR-RFLP and bears the potential for high throughput (Falk et al., 2006).

In vitro efficacy tests and molecular genotyping of resistance markers (mutations in genes conferring resistance) are supplementary methods used in the assessment of *P. falciparum* resistance to antimalarial drugs. The former involves testing the susceptibility of parasite to drug in culture whereas the latter measures the single nucleotide polymorphisms (SNPs) at various positions in resistance-associated genes. If reliable evidence on their *in vivo* resistance predicative value is established, the two methods may replace the former method. These *in vivo* tests provide valuable information on which to base changes in malaria treatment policy, since they are closer to the real life situation, than *in vitro* assays and molecular markers. But they are also demanding in terms of personnel, equipment, costs and time. Moreover, the lack of standardized protocols and procedures applicable to different epidemiological settings makes comparability between studies difficult.

Though the advantages of the molecular tests are similar to those of *in vitro* assays (i.e., detection of true parasite resistance without interfering host factors, ability to perform multiple analyses with a single patient sample), they are limited with regard to many aspects. Most of the work has been focused on CQ and SP, both available and cheap drugs widely used in malaria endemic areas worldwide. Therefore, molecular markers are only available for a limited number of drugs. Furthermore, the role of molecular markers in predicting *in vivo* therapeutic outcome has been controversial and the correlation has not been fully established (Plowe, 2003). In order to be able to fully exploit molecular markers of antimalarial drug resistance we need to get a better understanding of how drugs work and how resistance come about.

I.10. Rationale of the study

P. falciparum has developed clinically significant resistance to all classes of antimalarial drugs, with the exception of artemisinin (White, 2004). A consensus has begun to emerge that the development of drug-resistant malaria should be delayed through a strategy of routinely employing combinations of drugs containing artemisinin derivatives (ACT) (WHO, 2007). But what will happen when resistance to artemisinin compounds will emerge? In recent years, there has been a great progress in understanding the molecular basis of drug resistance in *P. falciparum*; the mode of action and mechanisms of resistance to antifolate and quinolines have been studied, but remain to be partially characterized (Foote et al., 1994; Olliaro; 2001; Woodrow & Krishna, 2006). This has led to the advocacy for molecular monitoring of parasite resistance as a supplementary tool to *in vivo* drug efficacy studies (Plowe, 2003; WHO, 2003). However, on an individual level, an association of specific molecular markers with *in vitro* resistance does not allow prediction of resistance *in vivo* or of the therapeutic response. A number of other parameters, such as the use of drug combinations, the level of prior immunity which is closely linked to transmission intensity, compliance to treatment, etc., play a role in clearing symptoms and parasites (White, 2004).

We need more information about the process determining the emergence and spread of resistance. Lessons from the history of the antimalarials and the modes by which resistance arose and disseminated may help prolong the life span of the next generation of chemotherapies for this recalcitrant disease. It seems that resistance emerges *de novo* through spontaneous mutations or gene duplications, which are thought to be independent of drug pressure (Barnes & White, 2005), but these mutants are then selected for and spread as a result of the drug pressure which provides a selective advantage to resistant parasites (D'allessandro & Buttiëns, 2001; Hastings & Watkins, 2005). More information on the genetics of drug resistance will help in designing the novel, improved molecular based tools for early detection and interventions that aim at limiting the extent of established multidrug resistance and preventing new foci of drug resistance from emerging may become possible.

The cost implications for changing a first line antimalarial are enormous (Shretta et al., 2000) and the design of an effective sentinel for the choice and deployment of alternative therapy is a vital public health tool, and for that kind of study we need to better understand the emergence, the development and the spread of drug resistance. Important inferences about the rate of drug resistance mutation can be gleaned from laboratory selection experiments. However, these experiments cannot provide information on the subsequent survival of resistant mutants in nature. An alternative retrospective approach is to investigate the

evolutionary history of drug resistance mutations using molecular markers that are closely linked to resistance alleles. The study of the correlation between drug resistance in natural parasite populations and genetic polymorphisms may allow the development of molecular tools to help predict responses to drugs.

In this study, we propose to assess the dynamics of CQ and SP resistance using molecular markers in historical samples. We will study these dynamics in two different settings with different malarial transmission intensities, and different patterns of drug pressure. We will also investigate the relationships between the dynamics of the treatment effectiveness and frequency of molecular markers and drug pressure to determine the impact of drug pressure on the development of drug resistance.

I.11. Goal of the study

To study the dynamics of CQ and SP resistance in historical samples and over time in two areas of different transmission intensity (Idete in Tanzania and Wosera in Papua New Guinea) determining the molecular markers of resistance.

I.12. Specific objectives

1. To assess the impact of 3 doses of SP in 600 children under 5 years of age on SP resistance markers in an area where SP has rarely been used (Idete, Tanzania).

2. To study the dynamics of SP and CQ molecular markers of resistance in the Wosera area of Papua New Guinea between 1991 and 2002.

3. To study the relationships between the dynamics of theses resistance markers in Wosera with treatment effectiveness and to evaluate the impact of drug pressure on the development of resistance.

Material & Methods

II.1. Study areas

II.1.1. Idete

The United Republic of Tanzania has a population of 34.5 million, all of whom are at risk of malaria. However, endemicity and risk of transmission varies and have recently been mapped by the MARA collaboration (Le Sueur et al., 1998). This GIS-based analysis reveals that 75% of the population is subject to stable perennial or stable seasonal malaria transmission; 8% to unstable highly seasonal transmission; and 17% to no malaria transmission in the average year, but still at risk of epidemic malaria. Tanzania has the third largest population at risk of stable malaria in Africa after Nigeria and the Democratic Republic of Congo (De Savigny et al., 2004).

The village of Idete is located in the Kilombero valley, in the Morogoro region, southern Tanzania. It lies south of the Udzungwa Mountains, at the northern edge of the alluvial plain of the Kilombero River at 270m. The Kilombero valley is an area of intense and perennial malaria transmission. The climate is marked by a rainy season from November to May and a dry season from June to October with annual rainfall ranging between 1200 and 1800 mm (Tanner et al., 1991). *Anopheles gambiae* sp and *Anopheles funestus* are the two main vectors. Although mosquito densities and exposure are seasonal, the prevalence of asymptomatic *Plasmodium falciparum* parasitaemia is high and shows no marked seasonality. 80% of infants are infected by age 6 months (Kitua et al., 1996) and data from an adjacent village indicates that, on average, everyone receives more than 300 infective bites per year (Smith et al., 1993). Transmission of the other three species of human malarias is low and unstable. Most of population depends on subsistence farming of rice, maize and cassava, with some fishing. Malaria (predominantly *P. falciparum*) is the leading cause of morbidity and mortality (Tanner et al. 1991) and the most frequent single diagnosis at the Saint Francis Designated District Hospital in Ifakara (SFDDH). It accounts for 30% of all admissions, 29% of hospital deaths in the under-fives, and 13% in older age groups (Kitua et al., 1996).

N.B: The description here is the epidemiology of malaria in Idete at the time of the vaccine trial in 1994, the epidemiology of malaria in this area is likely to have changed due to the implementation of many programs on vector control (Schellenberg et al., 2004).

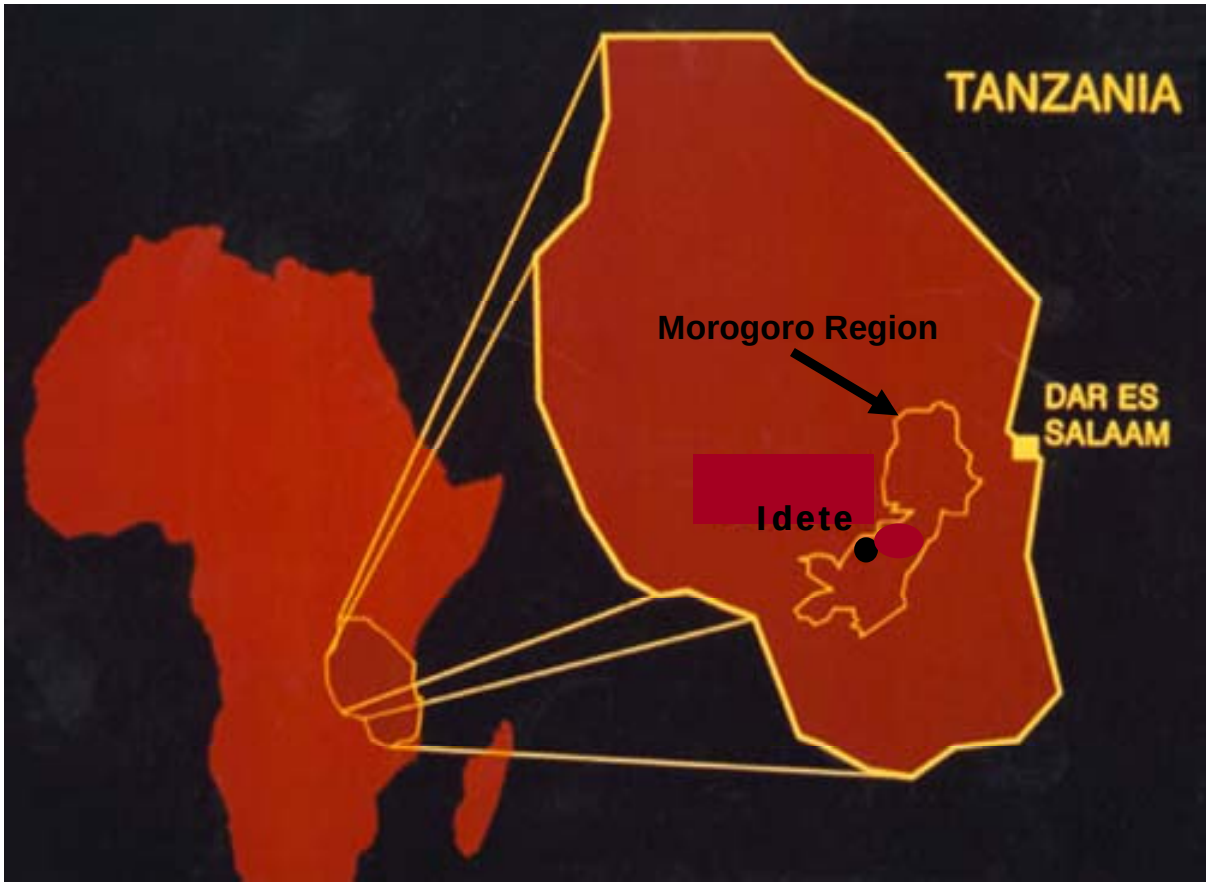


Figure 1: Map of Tanzania presenting the location of study site

II.1.2. Wosera

Papua New Guinea (PNG) which is a patchwork of different geographical and ecological zones and inhabited by a population of approximately 5.7 million people characterized by exceptional cultural and linguistic diversity. PNG features complex variations in vector and malaria epidemiology. All four *Plasmodium* species that infect humans are found in both, lowland and highland areas, with *P. falciparum* and *P. vivax* being the predominant species. Malaria intensity ranges from unstable low-level endemicity where outbreaks are common to high transmission comparable with most endemic regions in Sub-Saharan Africa.

The Wosera region (Eastern Sepik Province, Papua New Guinea) is characterized by a tropical wet climate. Rainfall is the main climatic feature which shows some seasonality, with a wet season from October to April and the dry season from May to September. The inhabitants are predominantly subsistence farmers living in villages subdivided geographically into hamlets with little socio-economic stratification (Genton et al., 1995a).

The area is highly endemic for malaria, with a mean parasite prevalence of 52%, *P. falciparum* is the predominant specie, but *P. vivax* and *P. malariae* are also common there (Genton et al., 1995a; Mehlotra et al., 2002). Transmission is perennial, and the number of *P. falciparum* infective bites per year is around 50-100 (Genton et al., 1995a; Benet et al., 2004). Malaria is the commonest cause of outpatient presentation and accounts for 27% of all attendances at health facilities (Müller et al., 2003). Awareness of the cause of malaria and methods of treatment is high due to the relatively good access to the health services and to regular information sessions delivered in the area. No drug circulates in the community, as antimalarials are received at health centre on a daily basis and no commercial outlet is present in the area (Genton et al., 1995a).

Figure 2: Map of Papua New Guinea presenting the location of study site

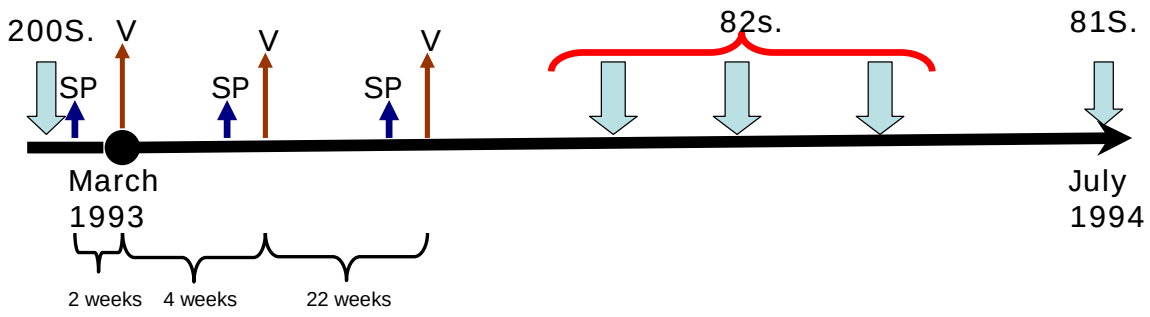
II.2. Samples

II.2.1. SPf66 vaccine trial

The samples were collected during a vaccine (SPf66) efficacy trial in children under 5 years between March 1993 and July 1994 in Idete (Alonso et al., 1994). The study was a randomised double blind, placebo controlled trial, and the objective was to determine whether three doses of SPf66 reduce the incidence of clinical episodes attributable to malaria or the prevalence and intensity of parasitaemia in children aged 1-5 years, and to estimate the protection achieved. Six hundred children were recruited for this study.

At that time, the first line antimalarial treatment was chloroquine, but in order to maximize the immune response to the vaccine by avoiding the transient immuno-suppression associated with malaria parasitaemia, every individual was treated with a single curative dose of sulfadoxine-pyrimethamine (SP or Fansidar) 2 weeks before the administration of each vaccine dose. The children received 3 vaccine doses; the second dose was given 4 weeks after the first one, and the last dose 22 weeks later.

To study the dynamics of drug resistance markers during this vaccine trial, 351 parasite positive samples in total confirmed by microscopy were selected from the placebo group: 200 samples from the baseline, which were collected 2 weeks before the first dose of vaccine, 82 samples from cross sectional surveys during the trial and 81 collected at the end of the vaccine trial were selected for this study.



V: administration of vaccine

SP: administration of a dose of sulfadoxine-pyrimethamine

S: blood samples collected

Figure 3: Study design: Spf66 vaccine trial

II.2.2. Morbidity surveillance in Wosera

The samples were collected over a 12 years period during a health-facility based morbidity surveillance between 1991 and 2002 conducted in Wosera.

Health services in the area are provided through a government health centre in Wombisa and two church health centres in Kaugia and Kunjingini. A demographic surveillance was set up in Wosera in 1990 as part of the Malaria Vaccine Epidemiology and Evaluation Project (Alpers et al., 1992; Genton et al., 1995a). It started with baseline investigation into malaria epidemiology and immunology investigations in 9 villages surrounding the Kunjingini Health Centre (Genton et al., 1995a; Genton et al., 1995b); leading to a phase IIb trial of a 3 component malaria vaccine in 1999 (Genton et al., 2003). The coordinates of all houses in these villages were recorded in 1993 using a global positioning system (Hii et al., 1997). A complete census of these villages was carried out annually and each individual was registered and given an identity number (ID). In each village, one or more villagers were employed to record vital events, including pregnancies, births, deaths and migration in or out the village. A morbidity surveillance system is maintained at both health centres within the DSS. All patients seen by the health centre staff and their diagnosis were recorded with details of age, sex, place of residence, ID number, presumptive diagnosis and treatment. From patients with symptoms of presumptive malaria, the patient was physically examined by the IMR (Institute of Medical Research) surveillance nurse, blood slides were collected for parasitological assessment, haemoglobin levels measured and treatment prescribed. Diagnostic procedures at the health centres followed the guidelines of the Papua new Guinea Department of Health, which recommend malaria treatment for all patients with fever (PNG, department of Health, 1993). From 1991 to 1993, all the patients coming to the health facility were recorded in the IMR database used for this study. Later on, only patients with a presumptive diagnosis of malaria were recorded.

The first line antimalarial treatment was chloroquine or amodiaquine, and the second line, quinine and SP till 2000. The combination of SP with either chloroquine or amodiaquine was adopted then as first line antimalarial treatment, with SP + artesunate as second line treatment.

To study the dynamics of resistance molecular markers over this 12 years period, samples were randomly selected from the database of this morbidity surveillance, from patients with >1000 *P.falciparum* parasites/µl [>25 asexual parasites /200 white blood cells (WBCs)]. Six hundred samples were analysed, 50 from each year.

II.3. Laboratory methods

II.3.1. Analysis of single nucleotide polymorphisms (SNPs) on DNA- microarray

The point mutations in *Pfcrt*, *Pfmdr*, *PfATPase*, *Pfdhfr* and *Pfdhps* genes were analysed using a microarray-based technique developed by the Swiss Tropical Institute (Crameri et al., 2007). Briefly, DNA was extracted from 200µl blood pellet using the QIAamp® 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. Using gene-specific primers, the five different genes were amplified by primary and nested PCR. 10µl of each nested PCR product were diluted 1:10 with sterile water and SAP-digested in duplicate. Single base primer extension reactions were carried out using 20-30bp locus-specific extension primers, which are designed that the last bases on their 3' end precedes the SNP site. The extension over the SNP site is performed using Cy3 or Cy5-labelled ddNTPs and sequenase. The extended primers (whose last base on their 3' ends are now labelled with a fluorochrome) were denatured by heating (2 minutes at 94°C) and hybridised to specific antisense probes anchored onto aldehyde-coated microscopic slide in a humid chamber at 94°C for 1 hour. Slides were washed three times with saline-sodium citrate (SSC) and sodium dodecyl sulphate (SDS), dried with compressed air and scanned with an Axon 4100a GenePix Personnal scanner, arrays were analysed by Genepix to detect SNPs.

The method allows analyzing multiple single nucleotide polymorphisms (SNPs) in different genes at the same time:

- five SNPs in *Pfdhfr*: dhfr16, dhfr51, dhfr59, dhfr108, and dhfr164;
- 8 SNPs in *Pfdhps*: dhps436, dhps437, dhps540, dhps581, dhps613, dhps640, dhps645;
- 5 SNPs in *Pfmdr*: mdr86, mdr184, mdr1034, mdr1042, mdr1246;
- 10 SNPs in *Pfcrt*: crt72, crt76, crt97, crt152, crt163, crt220, crt271, crt326, crt356, crt371;
- 5 SNPs in *PfATPase*: ATPase538, ATPase574, ATPase623, ATPase683, ATPase769.

II.3.1.1. DNA extraction

DNA was isolated from frozen EDTA blood by the "*QIAamp* 96 DNA Blood Kit" from Qiagen according to the manufacturer's instructions.

II.3.1.2. Polymerase Chain Reaction (PCR)

The primary and the nested PCR were performed in a 96-well PCR plate. A set of 10 primers for the different genes or parts of genes were used (see appendix C).

- Primary PCR reaction mix

Reagents	**Volume (1 reaction)**
H_2O	30.00 µl
10x PCR reaction buffer (without $MgCl_2$) Buffer B (Solis BioDyne, Tartu, Estonia)	5.00µl
dNTPs mix (200µM) Amersham Biosciences	5.00µl
$MgCl_2$ (25mM) Solis BioDyne, Tartu, Estonia	6.00µl
Primary PCR primer mix (10µM each) Operon	1.00µl
Taq polymerase (Firepol®; 5 U/µl) Solis BioDyne, Tartu, Estonia	0.50µl
Master mix	**47.50µl**
DNA	**2.50µl**
Total	**50.00µl**

- Reaction conditions

Initial Denaturation	96°C	180 sec	
Denaturation	96°C	30 sec	25cycles
Annealing	52°C	90 sec	
Extension	72°C	90 sec	

- Nested PCR reaction mix

Reagents	**Volume (1 reaction)**
H_2O	60.00 µl
10x PCR reaction buffer (without $MgCl_2$) Buffer B (Solis BioDyne, Tartu, Estonia)	10.00µl
dNTPs mix (200µM) Amersham Biosciences	10.00µl
$MgCl_2$ (25mM) Solis BioDyne, Tartu, Estonia	12.00µl
Nested PCR primer mix (10µM each) Operon	2.00µl
Taq polymerase (Firepol®; 5 U/µl) Solis BioDyne, Tartu, Estonia	1.00µl
Master mix	**95.00µl**
Primary PCR product	**5.00µl**
Total	**100.00µl**

- Reaction conditions

Initial Denaturation	96°C	180 sec	
Denaturation	96°C	30 sec	25cycles
Annealing	52°C	90 sec	
Extension	72°C	90 sec	

The amplifications were performed on a Perkin Elmer DNA Thermal Cycler 480.

The PCR products were checked on an Agarose gel (1.5%) and kept at 4°C (Fig.4).

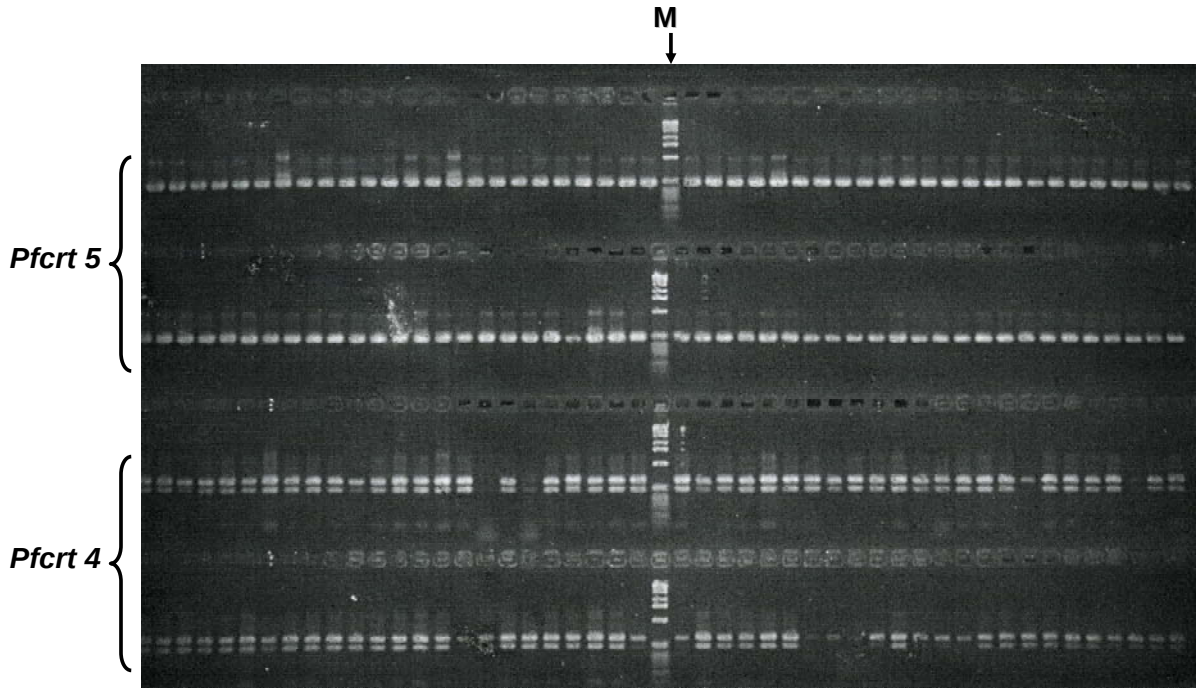


Figure 4: Typical agarose gel showing PCR products of 96 samples of *Pfcrt 4* and *Pfcrt 5* amplifications. M: DNA standard 1Kb ladder (Invitrogen®), *Pfcrt 5 =626 kb; Pfcrt 4 = 697 kb* and *630 kb***.**

II.3.1.3. SAP-digestion

Shrimp Alikaline Phosphatase (SAP) was used to eliminate free dNTPs in the nested PCR product. 10µl of each nested PCR product were pooled into a new plate briefly mixed and centrifuged. 10µl of this mix were transferred into a new plate, and diluted 1:10 with sterile water, briefly mixed and centrifuged. The SAP-digestion was done in duplicate, because 2 extension reaction mixes per sample were needed due to the availability of only two different fluorochromes. 5µl of the 1:10 dilution PCR product were transferred to a new plate in duplicate.

- SAP digest reaction mix

Reagents	Volume (1 reaction)
H_2O	4.00 µl
10x SAP buffer Amersham Biosciences	1.00µl
Shrimp Alkaline Phosphatase (SAP) 1U/µl Amersham Biosciences	2.00µl
Master mix	**7.00µl**
Nested PCR product diluted 1:10	**5.00µl**
Total	**12.00µl**

The SAP- digestion was performed on a Perkin Elmer DNA Thermal Cycler 480 at 37°C for 1 hour and the inactivation of SAP at 90°C for 15min.

II.3.1.4. Primer extension

Single base primer extension reactions were carried out using 20-30bp locus-specific extension primer (see appendix C), thermo sequenase and Cy3- or Cy5-labelled ddNTPs. The extension primers were designed such that the last base on their 3' end precedes a polymorphic site. There were two combinations mixes of primers and ddNTPs.

- Extension primer mixes

Genes	Combination 1	Combination 2
Pfdhps	437, 540, 581, 613, 640	436, 613B, 645
Pfdhfr	16, 51, 59, 108, 164	108B, 164B
Pfcrt	72, 152, 271, 326, 326B, 356, 356B	74, 76, 97, 163, 220, 371
Pfmdr	86, 184, 1034, 1042,	1246
PfATPase6	538, 769, 769B	574, 623, 683

- ddNTPs mixes

Combination 1	Combination 2
ddATP Cy3	ddUTP Cy3
ddCTP Cy3	ddCTP Cy3
ddGTP Cy5	ddATP Cy5
ddUTP Cy5	ddGTP Cy5

- Primer extension mix 1 or 2

Reagents	Volume (1 reaction)
H_2O	1.60 µl
10x Sequenase buffer (Amersham Biosciences)	2.00µl
ddNTPs mix combination 1 or 2 (2.5µM) (Perkin Elmer)	2.00µl
Extension primer mix combination 1 or 2 (62.5nM each) (Operon, HPLC-purified)	2.00µl
Thermo sequenase (Termipol®; 5 U/µl) Solis BioDyne, Tartu, Estonia	0.40µl
Total	**8.00µl**

8 µl of extension reaction mix were added to SAP digestion product.

- Reaction conditions

Initial Denaturation	94°C	60 sec	
Denaturation	94°C	10 sec	35 cycles
Extension	50°C	40 sec	

The primer extension was performed on a Perkin Elmer DNA Thermal Cycler 480.
Extension reaction mixes combination 1 and combination 2 of each sample were pooled together. 6 µl of denaturing solution (0.5 µl of 0.5M EDTA pH8, 2.0µl of 10% SDS and 3.5 µl of H_2O) were added. The plate was incubated at 94°C for 60 sec, and chilled on ice for 2 min.

II.3.1.5. Hybridisation of extended oligos on chip

The extended primers were hybridised to specific oligonucleotides (see appendix C) probes spotted onto aldehyde-coated microscopic slides (Genetix, Munich, Germany). Each oligonucleotide was spotted in triplicates, and pre-labelled Cy3 and Cy5 anchor oligonucleotides as well as four oligonucleotides with a random sequence were added as positive and negative controls, respectively. 23µl of the extension reaction mix were added to the microarray together with 6µl of 20x SSC to each well, incubated in a humid environment at 50°C for 60 min. The slide was then washed with 2x SSC+0.2% SDS for 10 min., followed by 2x SSC for 10 Min, and with 2x SSC+2% EtOH for 2 min. Finally, the slide was dried with compressed air and scanned with the GenePix personal 4100A scanner (Axon, Bucher Biotec AG, Basel, Switzerland). The slides were scanned at 635 nm and 532 nm. Cy3 and Cy5 images were acquired and analysed using the Axon GenePix® Pro (version 6.0) software (www.axon.com). This software generates data points using pixel intensity after background subtraction. After the first reading, the slide was washed again and read a second and third time. Data from the second scan were used for further analysis. In-house generated software was developed for further analysis of raw data. Each signal was classified either as wild type, mutant, or mixed, based on the ratios of intensities of the scanned image. The grouping was done according to the following algorithm: Fluorescence intensities below 9000 (Cy3) or 10000 (Cy5) units (mean intensities minus background) were regarded as negative. For measures above these threshold values, the ratio Cy5 to Cy3 intensity was considered to discriminate wild-type, mutant, or mixed.

Sample	Gene	Id	Mean Ratio	Cy3	Cy5	Genotype	Dominant
21523	CRT	76	8.20520337	2346	25309	mut	mut
21623	CRT	76	6.37E-02	30196	1868	wt	wt
21776	DHFR	59	0.95353627	49593	49329	wt mut	wt
21776	DHPS	437	57.3371226	796	43534	wt	wt
12964	MDR	86	1.14808023	18365	21361	wt mut	mut

Figure 5: Final excel Output (example). In the first column, there is the ID number of the sample analyzed, in the second the gene and the third the mutation analyzed. The mean ratio is the ratio of Cy5 and Cy3 intensities. The column 5 and 6 represent the mean Cy3 and Cy 5 fluorescence intensities derived from the three triplicates. The threshold values were determined empirically: for Cy5 to Cy3 ratios below 0.7 the sample was classified according to whether wild-type or mutant were labelled with Cy3. Ratios between 0.7 and 2.4 were assigned to mixed genotypes, and ratios above 2.4 to the Cy5 labelled genotype. The dominant represents the form with the highest intensity.

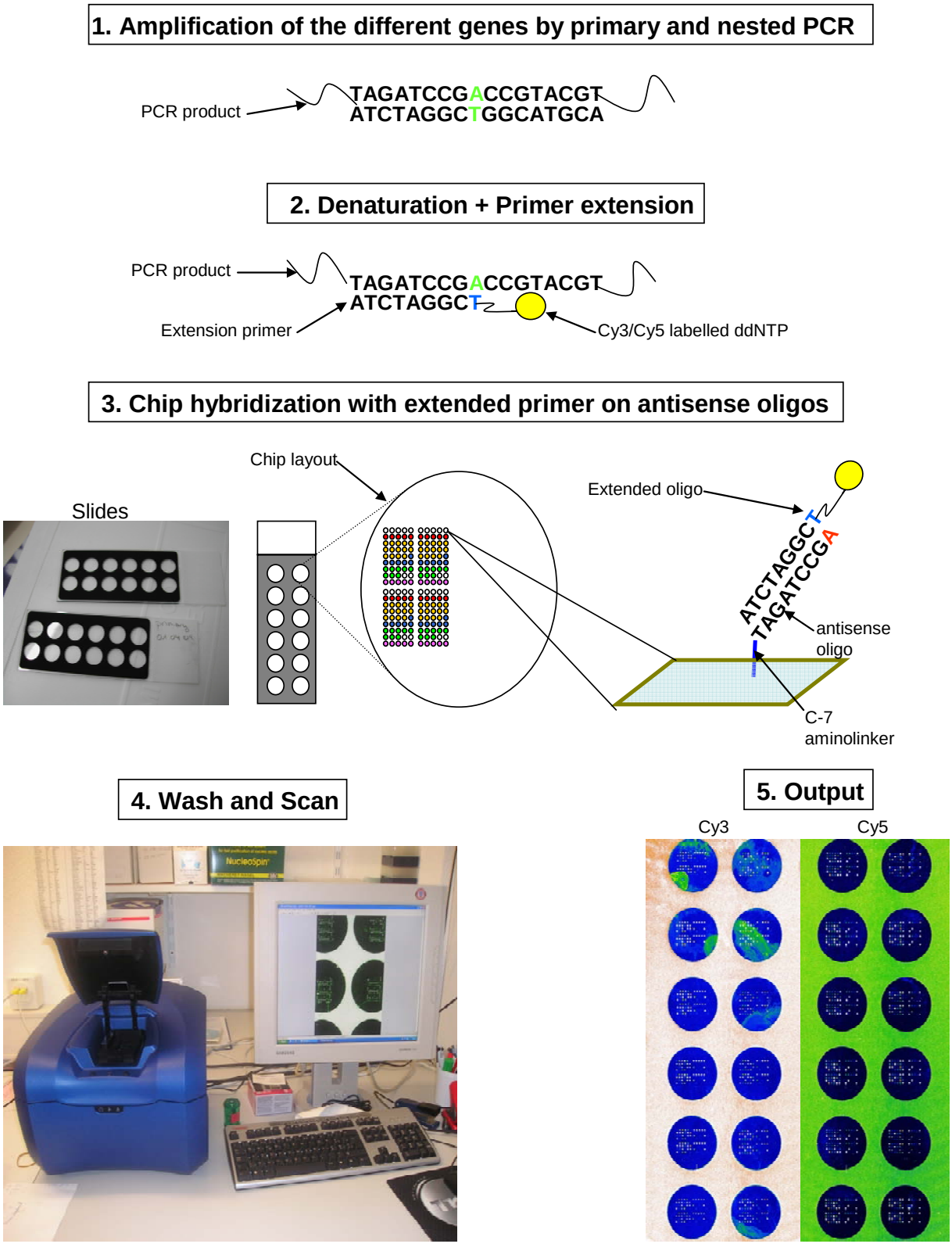


Figure 6: Schematic representation of the DNA-microarray technique

II.3.2. Genotyping of *Pfmsp2*

To determine the multiplicity of infection (MOI), the genotyping was done using the polymorphic gene *Pfmsp2*, which codes for the merozoite surface protein 2 (MSP2).

Two techniques were used, a PCR-RFLP-based technique (Felger et al., 1993, Felger & Beck, 2002), and a genescan-based technique (Falk et al., 2006).

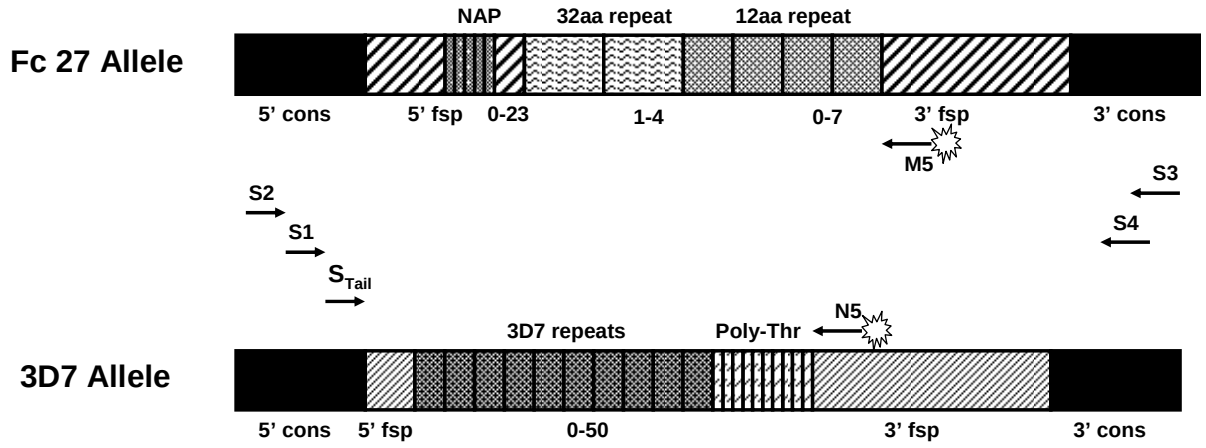


Figure 7: Schematic representation of MSP2 and primers location

II.3.2.1. Genotyping of MSP2 by PCR-RFLP

Briefly, DNA was extracted from 200µl blood pellet using the QIAamp® 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. DNA was amplified by PCR, using the primers S2/S3 and S1/S4 for Primary and nested PCR, respectively. The restriction digest was done overnight at 37°C, in a total volume of 20µl, containing 7µl of nested PCR product, 10.5µl of dH_2O, 2µl of 10x buffer NEB, and 0.5µl (5 units) of HinfI. Digest products were then run on 10% polyacrylamide at 200V for 2 hours. Gels were stained 15 minutes in ethidium bromide solution, and visualised under UV light and photographed. Distinction and counting of different genotypes was achieved by analysis of the restriction fragments pattern (Felger & Beck, 2002).

II.3.2.1.1. DNA extraction

As prepared for SNP analysis.

II.3.2.1.2. Polymerase Chain Reaction (PCR)

- Primary PCR reaction mix

Reagents	Volume (1 reaction)
H_2O	33.50 µl
10x PCR reaction buffer (without $MgCl_2$) Buffer B (Solis BioDyne, Tartu, Estonia)	5.00µl
dNTPs mix (200µM) Amersham Biosciences	5.00µl
$MgCl_2$ (25mM) Solis BioDyne, Tartu, Estonia	3.00µl
S2 primer (50µM) Operon	0.50µl
S3 primer (50µM) Operon	0.50µl
Taq polymerase (Firepol®; 5 U/µl) Solis BioDyne, Tartu, Estonia	0.50µl
Master mix	**48.00µl**
DNA	**2.00µl**
Total	**50.00µl**

- Reaction conditions

Initial Denaturation	94°C	120 sec	
Denaturation	94°C	30 sec	30 cycles
Annealing	45°C	45 sec	
Extension	70°C	90 sec	
Final elongation	70°C	10 min	

- Nested PCR reaction mix

Reagents	**Volume (1 reaction)**
H_2O	34.70 µl
10x PCR reaction buffer (without $MgCl_2$) Buffer B (Solis BioDyne, Tartu, Estonia)	5.00µl
dNTPs mix (200µM) Amersham Biosciences	5.00µl
$MgCl_2$ (25mM) Solis BioDyne, Tartu, Estonia	3.00µl
S1 primer (50µM) Operon	0.50µl
S4 primer (50µM) Operon	0.50µl
Taq polymerase (Firepol®; 5 U/µl) Solis BioDyne, Tartu, Estonia	0.30µl
Master mix	**49.00µl**
Primary PCR product	**1.00µl**
Total	**50.00µl**

- Reaction conditions

Initial Denaturation	94°C	120 sec	
Denaturation	94°C	30 sec	30 cycles
Annealing	50°C	45 sec	
Extension	70°C	90 sec	
Final elongation	70°C	10 min	

The amplifications were performed on a Perkin Elmer DNA Thermal Cycler 480.

The PCR product were checked on an Agarose gel (1.5%) and kept at 4°C (Figure 8).

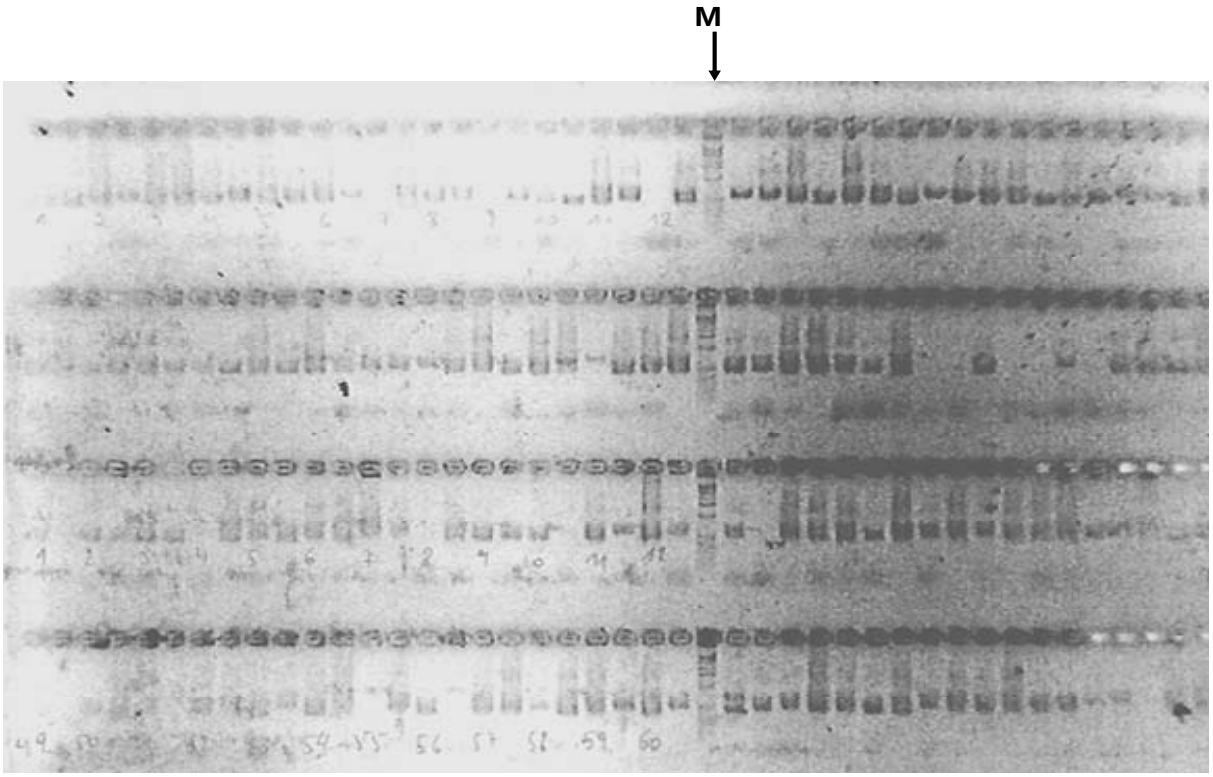


Figure 8: Typical agarose gel showing *Pfmps2* PCR products of 96 samples. M: DNA standard 1Kb ladder (Invitrogen®). The sample sizes vary from 378 bp to approx. 740 bp.

II.3.2.1.3. Restriction Fragment Length Polymorphism (RFLP)

- RFLP reaction mix

Reagents	Volume (1 reaction)
H_2O	10.50 µl
10x NEB buffer 2	2.00µl
Hinf I (10.000U/ml) NEB	0.50µl
Master mix	**13.00µl**
Nested PCR product	**7.00µl**
Total	**20.00µl**

The mix was incubated overnight at 37°C. The day after, 15µl of blue juice (Bromophenol blue and xylencyanol) were added, and the 35µl were loaded on a 10% polyacrylamide (PAA) gel. The gel was run at 200V for 2 hours, and stained 15 minutes in ethidium bromide staining solution, and a picture was taken using a UV gel imager.

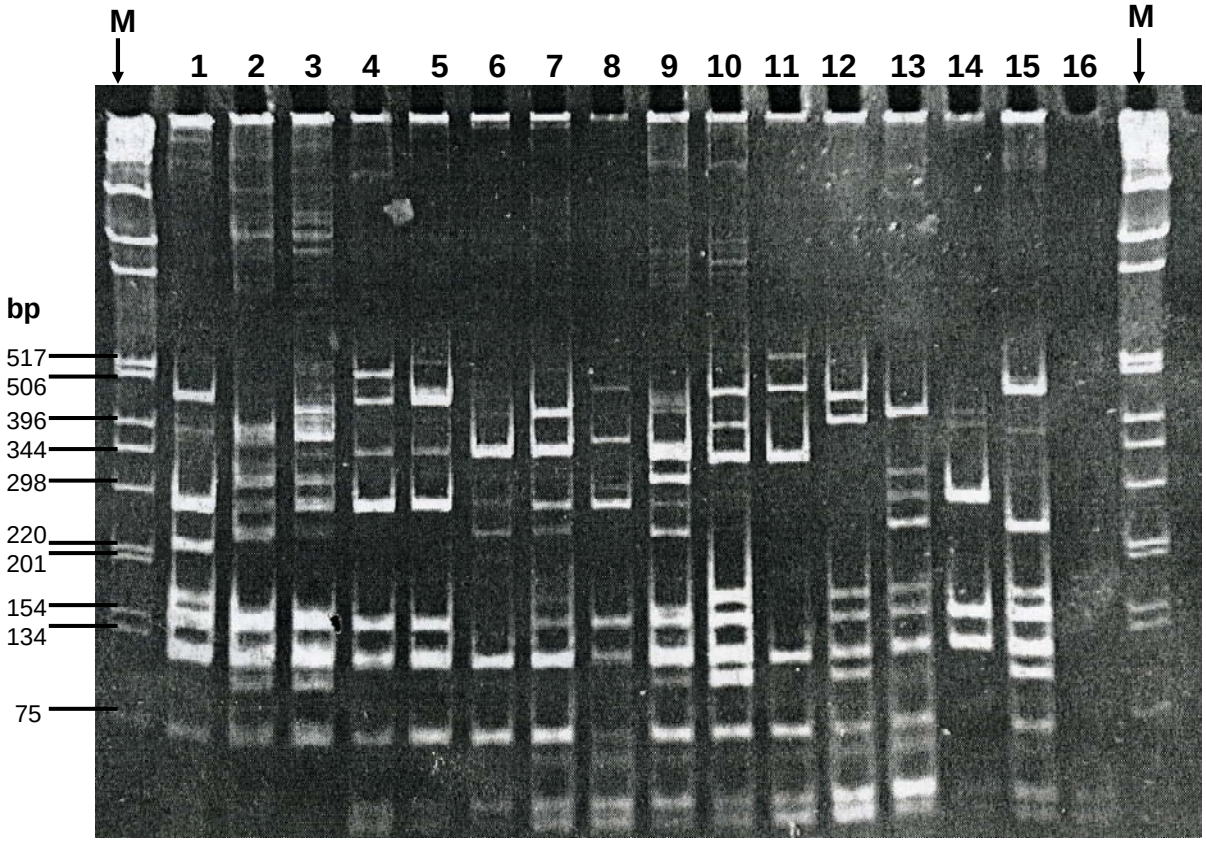


Figure 9: RFLP picture of *Pfmsp2* PCR product after HinfI digest. M: DNA standard 1Kb ladder (Invitrogen®).

II.3.2.1.4. RFLP pattern analysis

The allele of *Pfmsp2* fall into 2 allelic families: the FC 27- and 3D7- types alleles. The HinfI fragments at the end 5' and 3' are family specific and conserved (Figure 9).

The FC27-type has two conserved fragments, one at 137 bp and the other at 115 bp (line 14). The presence of one of these fragments indicates the presence of at least of 1 allele of the FC27-family. The 3D7-type alleles have 2 conserved HinfI fragments: 70 bp and 108bp (line 6 and 7). In multiple infections, a clear indication for the presence of at least 1 allele of the 3D7-family is the 70bp HinfI fragment, because in the 10% PAA gel, the 108 bp fragment runs as a double-band together with 115 bp long FC27-family-specific fragment. The presence of both 137 and 70 bp bands indicates a mixed infection of the two families (lines 1 to 3), and the multiplicity of infection (MOI) is two if no other bands are present above 137 bp. All the other fragments between 137 and 517 bp are new alleles belonging to one of the two families.

II.3.2.2. Genotyping of MSP2 by Genescan

Genescan can be used as alternative and slightly precise fragment sizing method. Primary PCR product is amplified using two family specific fluorochrome labelled primers spanning the polymorphic region.

Briefly, DNA was extracted from 200µl blood pellet using the QIAamp® 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. DNA was first amplified by primary PCR, using the S2/S3 primers. 1µl of primary PCR product was amplified in nested PCR with the fluorescent-labeled family-specific primer M5 (FC27-specific), and N5 (3D7-specific). The non-fluorescent–labeled forward primer S_{Tail} was modified at the 5' end by adding a 7-bp tail. Nested PCR product was diluted 1:10 with sterile water and 2.5µl were combined with 10 µl ROX-labeled size standard (diluted 1:40 with dH_2O). Samples were dried and sent to the Genomics Core Laboratory of the MRC Clinical Science Center in London. Highly deionized formamide was added, and after denaturation, samples were analyzed on an ABI PRISM 3700 genetic analyzer. An in-house–generated computer program was used to process the output of the GeneScan analyzer.

II.3.2.2.1. DNA extraction

As prepared for SNP analysis.

II.3.2.2.2. Polymerase Chain Reaction (PCR)

- Primary PCR reaction mix

Reagents	Volume (1 reaction)
H_2O	33.50 µl
10x PCR reaction buffer (without $MgCl_2$) Buffer B (Solis BioDyne, Tartu, Estonia)	5.00µl
dNTPs mix (200µM) Amersham Biosciences	5.00µl
$MgCl_2$ (25mM) Solis BioDyne, Tartu, Estonia	3.00µl
S2 primer (50µM) Operon	0.50µl
S3 primer (50µM) Operon	0.50µl
Taq polymerase (Firepol®; 5 U/µl) Solis BioDyne, Tartu, Estonia	0.50µl
Master mix	**48.00µl**
Primary PCR product	**2.00µl**
Total	**50.00µl**

- Reaction conditions

Initial Denaturation	94°C	120 sec	
Denaturation	94°C	30 sec	25cycles
Annealing	45°C	45 sec	
Extension	70°C	90 sec	
Final elongation	70°C	10 min	

1µl of primary PCR product was amplified in nested PCR with the fluorescent-labeled family-specific primer M5 (FC27-specific), and N5 (3D7-specific). The non-fluorescent–labeled forward primer S_{Tail} was modified at the 5' end by adding a 7-bp tail to serve as a template for A addition at the 3’ end of the labeled forward strand. Indeed, there is a tendency of Taq polymerase to add a non-template nucleotide (usually an A) to the 3’ end of the double-

stranded DNA. This addition is not absolute, and only a certain fraction of amplicons receive the additional nucleotide, causing ambiguity in allele calling. The tail promotes the A addition, thus reducing the ambiguity in allele calling.

- Nested PCR reaction mix

Reagents	**Volume (1 reaction)**
H_2O	31.50µl
10x PCR reaction buffer (without $MgCl_2$) Buffer B (Solis BioDyne, Tartu, Estonia)	5.00µl
dNTPs mix (200µM) Amersham Biosciences	5.00µl
$MgCl_2$ (25mM) Solis BioDyne, Tartu, Estonia	3.00µl
M5 primer (10µM) Applied Biosystems	1.00µl
N5 primer (10µM) Applied Biosystems	1.00µl
S_{Tail} primer (10µM) Applied Biosystems	2.00 µl
Taq polymerase (Firepol®; 5 U/µl) Solis BioDyne, Tartu, Estonia	0.50µl
Master mix	**49.00µl**
Primary PCR product	**1.00µl**
Total	**50.00µl**

- Reaction conditions

Initial Denaturation	94°C	120 sec	
Denaturation	94°C	30 sec	25cycles
Annealing	50°C	45 sec	
Extension	70°C	90 sec	
Final elongation	70°C	10 min	

The amplifications were performed on a Perkin Elmer DNA Thermal Cycler 480. The PCR products were checked on an Agarose gel (1.5%) and kept at 4°C (Figure 10).

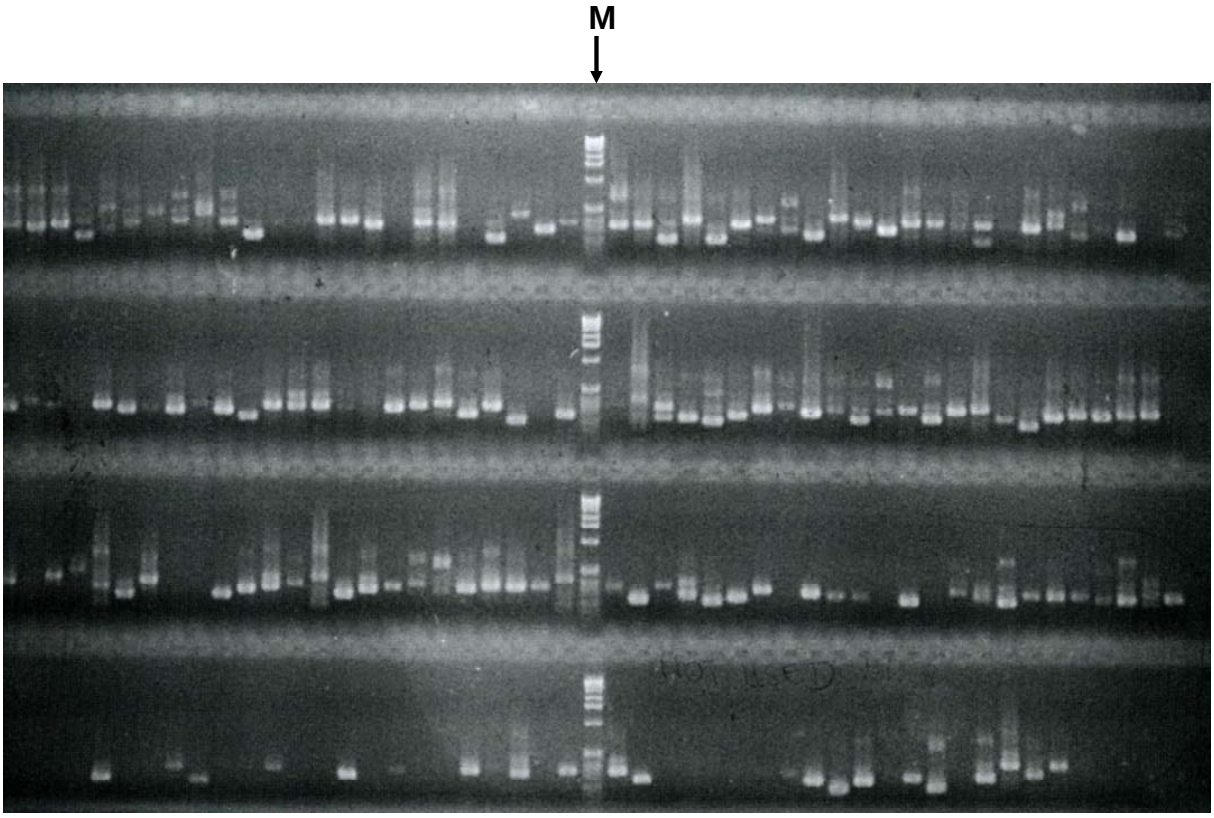
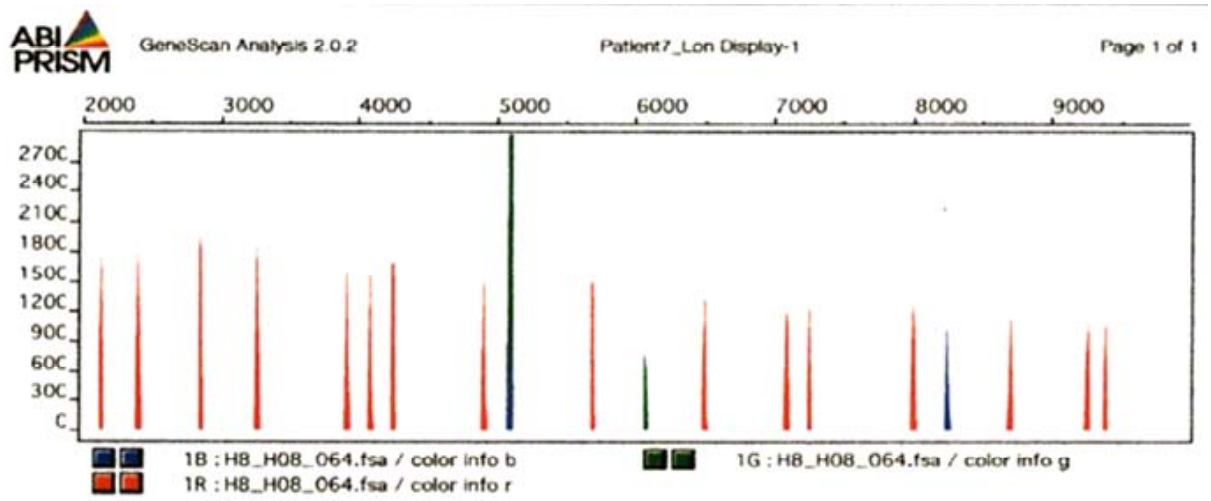


Figure 10: Typical agarose gel showing PCR products of 96 samples amplified with *Pfmsp2* allelic-family specific primers. M: DNA standard 1Kb ladder (Invitrogen®)

II.3.2.2.3. Genescan sample preparation

Nested PCR product was diluted 1:10 with sterile water and 2.5µl of each sample were combined with 10 µl ROX-labeled size standard (Applied Biosystems) in a 96-well plate. The size standard was previously diluted 1:40 with dH_2O. The plate was briefly vortexed and centrifuged. Then the samples were dried over night in the dark (the plate was let open overnight in a box), and sent to the Genomics Core Laboratory of the MRC Clinical Science Center in London the day after. Highly deionised formamide was added (12 µl), and after denaturation, samples were analyzed on an ABI PRISM 3700 genetic analyzer. Analyzed Genescan samples were returned by email in an fsa-format.



Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
1B, 1	42.98	211.57	1217	13191	5107
1B, 2	69.52	417.08	1106	12489	8260
1G, 1	42.86	210.66	239	1992	5092
1G, 2	42.98	211.57	6622	68059	5107
1G, 3	51.19	272.52	798	8257	6082
1R, 1	18.03	35.00	1845	18874	2142
1R, 2	20.31	50.00	1888	19227	2413
1R, 3	24.11	75.00	1933	18131	2865
1R, 4	27.61	100.00	1939	18734	3280
1R, 5	33.06	139.00	1740	16387	3928
1R, 6	34.47	150.00	1717	16155	4096
1R, 7	35.85	160.00	1708	16106	4259
1R, 8	41.38	200.00	1617	15864	4917
1R, 9	42.98	211.57	429	4635	5107
1R, 10	47.95	248.14	1500	14812	5697
1R, 11	54.78	300.00	1443	14939	6508
1R, 12	59.79	340.00	1236	13321	7104
1R, 13	61.16	350.00	1357	14227	7266
1R, 14	67.47	400.00	1332	14605	8016
1R, 15	73.40	450.00	1239	13929	8721
1R, 16	78.07	490.00	1125	13098	9276
1R, 17	79.13	500.00	1151	13373	9402

Figure 11: Genescan Output. The red peaks indicate the 16 defined fragments of the size standard (35, 50, 75, 100, 139, 150, 160, 250, 300, 350, 400, 450, 490, 500 bp). The blue peaks indicate the presence of Fc27 alleles; the green peaks indicate 3D7 alleles. Thus, this sample contained three infections, on Fc27 and two 3D7 type infections.

In-house generated software has been designed to transform the data into an excel file.
The software was designed to determine a cut off using the size standard for each sample and each allelic family, and also to calculate the size of each fragment, to identify known alleles (within a database previously created).

Patient ID	Family	Name	Size	Round 1
12964				3D7:4591 FC27:287
12964	Fc 27	Fc27genotype372.3	372.3	S: 372.13 H: 2246.0
24908				3D7:2126 FC27:133
24908	3D7	3D7genotype248	247.5	S: 247.24 H: 2409.0
24908	3D7	3D7genotype359	359.5	S: 359.52 H: 21362.0
24908	Fc27	Fc27genotype336.3	336	S: 335.98 H: 1576.0

Figure 12: Final excel output (example). The patient ID represents the number of the sample analyzed. The sample is classified in one of the two families: Fc27 and 3D7. The name and size columns represent the known alleles to which the sample corresponds. The round 1 represent the first result from the same patient as consecutive samples from the same patients can be analyzed over time. S represents the size of the allele found and H the height corresponding to the intensity of the fluorescence.

Comparative efficacy and safety of Artekin, Coartem and Sulfadoxine-Pyrimethamine in the treatment of uncomplicated *Plasmodium falciparum* malaria in Tanzanian children under 5 years

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Proposal fro a PhD project

Nota bene (N.B)

I started to work on this project in January 2004 when I went to Ifakara centre (IHRDC) and met some local scientists and discussed with them what kind of project would be interesting for the centre and me. It came out at that moment that Tanzania wanted to switch from sulfadoxine-pyrimethamine (SP) to another antimalarial as first line treatment, and the country needed information on safety and efficacy on the available antimalarials to choose the next first line drug in the treatment of uncomplicated malaria. Artekin was an excellent candidate, because it was cheap, and from the few studies conducted in Southeast Asia, the drug was safe and efficacious against multidrug resistant parasites, but there was no data on safety and efficacy on Artekin in African settings.

I worked on the project from January 2004 till April 2005. I went to Ifakara a second time in September 2004 to revise the project following the recommendations of the IHRDC ethic committee and submitted the project to the national ethic committee.

I got the permit in April 2005, but by this time I had to stop the project because we could not get the drug.

I. Summary

Every year, 1.5-2.7 million people worldwide die from malaria, while 300-400 million become infected. 90% of all deaths occur in Africa. The groups suffering most are young children and pregnant women. People who survive a severe malaria attack often suffer permanent development deficits and neurological abnormalities. Malaria's economic impact is huge, reducing economic growth rate by an estimated 1.3% per year.

Malaria has doubled or increased in most parts of Africa during past two decades because of the widespread resistance of *Plasmodium falciparum* to conventional drugs, such as chloroquine, sulfadoxine-pyrimethamine (SP) and amodiaquine.

Resistance to chloroquine, the cheapest and most widely used antimalarial, is common throughout Africa. Resistance to SP, often seen as the first and least expensive alternative to chloroquine, is also increasing in east and southern Africa. As a result of the trends, many countries are changing their treatment policies and use drugs which are more expensive; including combinations of drugs, hopping will slow the development of resistance.

A consensus has begun to emerge that the development of drug-resistant malaria will be delayed through a strategy of routinely employing combinations of drugs. Such combination treatment is likely to extend the useful lifetime of the constituent drugs by reducing the rate at which resistance develops.

Over the last decade, a new group of antimalarials have been deployed on an increasingly large scale: the artemisinin compounds, especially artesunate, artemether and dihydroartemisinin. The compounds produce a very rapid therapeutic response (rapid reduction of the parasite biomass and reduction of symptoms), are effective against multi-resistant *P. falciaprum* malaria, are well tolerated by the patients and reduce gametocyte carriage, thereby having the potential to reduce transmission of malaria. Studies in South East Asia have shown that combinations of artemisinin compounds with another drug yield high cure rates with treatment lasting only three days. Furthermore there is some evidence that such combinations can greatly retard development of resistance to partner drugs.

Because of the diminishing efficacy of SP in East Africa, we need to develop new drugs and implement strategies to protect them against resistance. Alternative drugs must be evaluated for the potential challenges of treating this life-threatening disease in the most vulnerable groups of the African population. Many studies with artemisinin combination therapy (ACT) have already started in Africa with SP + Artesunate, Amodiaquine + Artesunate and Artemether + Lumefantrine (Coartem). Coartem is the only commercially available co-

formulated artemisinin-containing combination therapy for malaria and is the most likely choice for replacing SP in Tanzania. Several other ACTs are in mid or late phase of development, like Artekin, a combination of piperaquine and dihydroartemisinin. This drug has been shown to be highly efficacious against multi-drug resistant parasites and no serious adverse drug reactions have been noted or reported. Moreover this new two-drug fixed combination antimalarial is quite inexpensive, costing about $ 1 for an adult treatment.

The proposed work will compare the clinical efficacy and safety of Artekin and Coartem compared to SP in children under 5 years with symptomatic malaria. Artekin pharmacokinetics and its in vitro activity will be also investigated.

The study will take place in Mkuranga in eastern Tanzania on the coastal region, about 50 km south of Dar es Salaam, the capital of the country.

Children eligible for enrolment will be randomly assigned to the regimens and will be followed for 56 days during which their level of peripheral parasitemia as well as haemoglobin level will be routinely monitored. These parameters will be used to measure drug performance. Clinical indicators will also be incorporated.

II. Introduction

Malaria is one of the most important diseases in the world (WHO, RBM, 2004). Despite control efforts over the past decades, *falciparum* malaria remains a major cause of childhood morbidity and mortality in sub-Saharan Africa (Greenwood et al., 1991). The World Health Organization (WHO) estimates that more than 90 % of the annual incidence of malaria (300 to 400 millions) occurs in Africa. The global mortality is estimated at 1.5 to 2.7 million cases each year, with the worst situation in children under 5 years living in Sub-Saharan Africa (WHO, RBM, 2004).

Malaria, together with HIV/AIDS and TB, is one of the major public health challenges undermining development in the poorest countries of the world. In Africa, malaria is the leading cause of mortality in children under 5 years (20%) and constitutes 10% of the continent's overall disease burden. It accounts for 40% of public health expenditures, 30-50% of inpatient admissions and up to 50% of outpatients visit in areas of high malaria transmission (WHO, RBM, 2004).

Malaria has been shown to be a major constraint to economic development (WHO, RBM, 2004). The disease has been estimated to cost Africa more than US$ 12 billion every year in lost GDP. Economists believe that malaria is responsible for a growth penalty of 1.3% per year in some African countries (WHO, RBM, 2004). In Africa today, malaria is understood to be both a disease of poverty and a cause of poverty. Malaria also has a direct impact on Africa's human resources. Not only does malaria result in lost of life and lost productivity due to illness and premature death, but malaria also hampers children's schooling and social development through both absenteeism and permanent neurological and other damage associated with severe episodes of the disease.

Unfortunately, an increasing prevalence of drug resistant strains exacerbates the situation (Trape et al., 1998; Snow et al., 2001). Resistance is primarily seen in *Plasmodium falciparum*, the most virulent human parasite (Rosenthal, 2003). The mainstay of malaria control remains early, effective treatment of clinical cases (WHO, RBM, 2004), yet the effectiveness of this policy is likely to decrease as resistance to many of the available and affordable antimalarial drugs increases.

The introduction of chloroquine (CQ) for malaria treatment and prevention in the late 1940s was a great advance on existing drugs. Quinine, the mainstay of therapy for malignant tertian malaria caused by *P. falciparum* was not reliable as prophylactic and had unpleasant side

effects. Chloroquine, cheap and better tolerated became the mainstay of therapy and prevention. However, resistance eventually developed in SE Asia and S. America by the end of 50s, and reached E. Africa by late 70s. In spite of its reduced efficacy, chloroquine is still widely used in Africa (Warhurst, 2001). The dramatic impact of chloroquine resistance on malaria mortality has long been underestimated because only a low proportion of malaria attacks are potentially lethal among older African children and adults exposed to high transmission (Snow, 1999; Trape, 2001). Malaria mortality has doubled or increased in most parts of Africa during the past two decades (Trape et al., 1998; Snow et al., 2001) where drug resistance has also increased, and increased incidence of malaria-related anaemia has probably contributed to mortality from other diseases (Trape et al., 2001; Snow et al., 1999; Menendez et al., 2000). In West Africa, results of a 12-year community –based study showed that the onset of chloroquine resistance at least doubled childhood malaria death risk, and in some sites increased it up to 11-fold in the younger children (Trape et al., 1998). A major impact was also documented in the highlands of East Africa, where the spread of CQ resistance was the only factor likely to explain the changing epidemiology of malaria in areas of low transmission, despite initial claims that it could be attributed to global warming (Shanks et al., 2000).

The combination of pyrimethamine and sulfadoxine (SP) known as Fansidar is a cheap and effective agent against chloroquine resistant *falciparum* malaria that is widely used in Africa (Wang et al., 1997). SP has now replaced chloroquine as the first-line curative antimalarial in much of East Africa (Pearce et al., 2003). Unfortunately, the clinical efficacy of SP is diminishing as a consequence of the development of *P. falciparum* resistance to SP, rendering a limited lifespan of SP (Alifrangis et al., 2003).

SP efficacy is already low in parts of East Africa. At Kilifi, Kenya coast, parasitological failure following SP treatment is 20% at day 7 (Nzila et al., 2000), and at Muheza, Tanzania, the day 7 failure rate is 45% rising to 60% on second treatment (Mutabingwa et al., 2001).

Resistance to SP *in vitro* is associated with a series of substitutions within the active site of target enzymes of the folate biosynthesis pathway, dihydropteorate synthase (*dhps*) (Brooks et al., 1994; Triglia et al., 1994) and dihydrofolate reductase (*dhfr*) (Cowman et al., 1988; Peterson et al., 1988; Snewin et al., 1989). This has been demonstrated through laboratory-based in vitro sensitivity tests and transfection experiments with *dhfr* with respect to pyrimethamine (Wu et al., 1996; Zolg et al., 1989) and *dhps* with respect to sulfadoxine (Triglia et al., 1997; Triglia et al., 1998; Wang et al., 1997).

The development of resistance depends in part on the pharmacokinetics and pharmacodynamic characteristics of drugs. Antimalarial drugs with long terminal half-lives are particularly vulnerable to the development of resistance. There is an increased chance that a new and unrelated infection may be acquired whilst drug concentrations following treatment have fallen below those sufficient to prevent parasite multiplication, and radically cure the new infection. If the originally infection is not radically cured, surviving parasites will be subjected to drug pressure as asexual cycles are exposed to blood concentrations (Watkins and Masobo, 1993; White, 1997).

The transition from low to high-level resistance is slow for the quinoline antimalarials (chloroquine, amodiaquine, mefloquine), but may be more rapid for pyrimethamine-sulfadoxine combinations (White, 1999). WHO now recognizes that resistance to SP is also widespread, its use will soon have to be discontinued, and persistent use of ineffective treatments may lead to an increase in the overall incidence of *falciparum* malaria (WHO, CDS, 2003).

While we wait for a malaria vaccine, case management with prompt effective treatment remains the mainstay of malaria control (Plowe et al., 2003).

A consensus has begun to emerge that the development of drug-resistant malaria should be delayed through a strategy of routinely employing combinations of drugs.

There is a considerable interest in the use of multiple drugs, with different mechanisms of action, for the treatment of malaria cases (White et al., 2000). Such combination treatment is likely to extend the useful lifetime of the constituent drugs by reducing the rate at which resistance develops. The principle is simple: resistance arises from mutations. The chance that a mutant will emerge that is simultaneously resistant to two different antimalarial drugs is the product of the mutation rates per parasite for the individual drugs, multiplied by the number of parasites in an infection that are exposed to the drugs. For example, if one in 10^9 parasites are resistant to drug A and one in 10^{13} are resistant to drug B, and the genetic mutations that confer resistance are not linked, only one in 10^{22} parasites will be resistant simultaneously to drug A and B. Most malaria patients have between 10^8 and 10^{12} parasites at presentation, and biomass of more than 10^{13} parasites in a single person is impossible, only approximately 1 in 100 millions treated patients would have surviving drug resistant parasite (White et al., 1999).

Currently there is general agreement that combination treatment with two differently acting drugs should be considered (Peters, 1987). One drug should be fast-acting with short half –life to reduce parasite numbers quickly; the other should be slow-acting and possess a long half-

life (White & Olliaro, 1996). The use of the two drugs in conjunction both provides effective treatment and reduces the chance of recrudescence.

Because of their rapid reduction of parasite biomass, and the complete absence of documented resistance despite over 2000 years of use, the artemisinin derivatives are components of many candidates combinations (White et al., 1997; Nosten & Brassuer, 2002). Artemisinin derivatives may also reduce malaria transmission and spread of resistance through their gametocytocidal properties (Price et al., 1996, Nosten et al., 2000). Another advantage is that the artemisinin derivatives (artesunate, artemether, dihydroartemisinin) are well tolerated antimalarials and produce the fastest therapeutic response of all drugs (White et al., 1999). There is evidence that combination of artesunate and mefloquine may have played a role in both slowing down the development of resistance to mefloquine as well as reducing malaria transmission in an area of high mefloquine-resistance in Thailand (Price et al., 1997). A meta-analysis carried out by the International Artemisinin Study Group to measure the effect of adding an artemisinin derivative to existing treatment regimens on patients with acute uncomplicated *P. falciparum* malaria showed that the addition of Artesunate was associated with shorter parasite clearance times. The median parasite clearance was about a day shorter than the obtained with standard treatment (International Artemisinin Study Group, 2004).

Despite some obstacles to implement effective combination therapies, programs evaluating the efficacy and effectiveness of new combinations are now underway in Africa (Von Seideln et al., 2000; Doherty et al., 1999; Adjuk et al., 2002). Among the challenges facing combination antimalarial therapy in Africa are cost and safety issues.

The combination of an artemisinin derivative with a long-acting schizontocidal drug is currently advocated as the optimal strategy for the treatment of malaria (WHO, RMB, 2001). Existing candidates are relatively expensive (mefloquine and atovaquone-proguanil), have unpleasant side-effects (mefloquine), or face high levels of pre-existing parasite resistance (chloroquine, amodiaquine, sulfadoxine-pyrimethamine).

Because of the diminishing efficacy of SP in East Africa, there is an urgent need to find new molecules with novel structures and targets to circumvent resistance. But we also need to develop and implement strategies to protect drugs against resistance. Resistance to single-drug therapies will inevitably occur. Alternative drugs must be evaluated for the potential challenges of treating this life-threatening disease in the most vulnerable groups of the African population. Drug combinations, which have been standard practice for viral and bacterial diseases, are now being adopted for malaria as well. Many studies with combination

therapy have already started in Africa with SP plus Artesunate, Amodiaquine plus Artesunate and Artemether plus Lumefantrine (Myint et al., 2004; Olliaro & Taylor, 2003).

The combination of artemether and lumefantrine, known by the trade name Coartem®, is currently the only commercially available co-formulated artemisinin-containing combination therapy for malaria. To date, Coartem has been approved in more than 60 countries, mainly in Africa, Asia, South America, and Europe (Lefèvre et al.,2002). Artemether/lumefantrine has been shown to be an efficacious and safe formulation when used for treatment of acute uncomplicated malaria in China, Africa and Thailand (CDS, RBM, 2002), and it seems to be the most likely choice for replacing SP in Tanzania. Although lumefantrine is similar to halofantrine, an antimalarial drug that has been shown to produce QTc-interval prolongation that may be fatal, extensive studies have failed to demonstrate QTc-prolongation or any other cardiotoxicity following the administration of of Artemether/lumefantrine (Van Vugt et al., 1999; Ezzet et al., 2000; Hassan Alin et al., 1999). There are two regimens of Coartem being promoted by the drug company; a 4-dose regimen taken over 2 days recommended for semi-immune patients, and a 6-dose regimen taken over 3 days for non-immune patients. While the efficacy of both preparations is high, the 4-dose regimen (cure rates reported from 69.3% to 95.4%) is clearly less efficacious than the 6-dose regimen (cure rates reported from 95.5% to 99.1%).

Several other drug combinations are in mid or late phase of development, among them there is Artekin™, a combination of piperaquine and dihydroartemisinin (DHA).

Piperaquine is a member of the 4-aminoquinoline group that includes chloroquine. The first human studies of piperaquine were carried out in the 1970s and involved its prophylactic use in several thousand adults and children. Piperaquine proved to be effective and well tolerated and no cross-resistance with chloroquine was observed (Chen L et al., 1982). More recently, piperaquine has been used as part of short-course artemisinin-based combination oral therapies designed to have high cure rate, to have few side effects, and to reduce malaria transmission.

Three combinations of DHA with piperaquine exist at present: CV8 (piperaquine, DHA, trimethoprim and primaquine), Artecom (piperaquine, DHA, trimethoprim) and Artekin (piperaquine and DHA). Artekin seems to be the simplest and least expensive drug of these three combinations (Hien et al., 2004). Recommended Artekin doses regimens have been developed empirically, because little is known about the pharmacokinetic properties of piperaquine and DHA alone, and when used in combination.

Few studies were published on efficacy and safety for the use of Artekin in humans, but all those studies reported that Artekin was highly efficacious against multi-drug resistant parasites and no serious adverse drug reactions were noted or reported (Karunajeewa et al., 2003; Denis et al., 2002; Hien et al., 2004). The efficacy of dihydroartemisinin-piperaquine was equivalent to that of artesunate-mefloquine, the current treatment of choice for multidrug-resistant *falciparum* malaria in southeast Asia (Hien et al., 2004). This new two-drug fixed combination antimalarial is quite inexpensive; in 2003, it costed about $ 1 for an adult treatment. Other treatments against multidrug-resistant *Plasmodium falciparum* malaria are much more expensive ($ 6 for artesunate-mefloquine and $ 2.40 for artemether-lumefantrine). Cost is a major factor deterring countries and international donors from providing artemisinin-based combinations to combat the worsening global malaria transmission (Nosten et al., 2002; WHO/CDS/RBM, 2001). There is an urgent need for alternative combination antimalarial drug in fixed doses that are effective, safe, and affordable.

Because of its efficacy and relatively low cost, the use of Artekin is likely to increase in future, but we need more information about its efficacy in area of intense malaria transmission and its safety in children. More information about its pharmacokinetic properties are also needed to adjust dose regimens before the optimum combination of this antimalarial drug is found.

To assess the efficacy of dihydroartemisinin with piperaquine (Artekin), we propose to carry out a three arm, randomized, open –label safety and efficacy study of Artekin, Artemether with lumefantrine (Coartem), Sulfadoxine/pyrimethamine; the actual first-line drug in the treatment of uncomplicated malaria in many countries in Africa, and also to assess the in vitro activity and the pharmacokinetic properties of Artekin.

III. Goal of the study

The aim of the present study is to compare the clinical efficacy and safety of Artekin with sulfadoxine/pyrimethamine and Artemether plus Lumefantrine in children under 5 years with symptomatic *falciparum* malaria. Techniques applying PCR and restriction fragment length polymorphism (RFLP) for polymorphic locus will allow identifying recrudescent or new infections after drug treatment and evaluating Artekin and SP resistance using molecular markers. Artekin pharmacokinetics and its in vitro activity will be also investigated.

IV. Specific Objectives

- To compare the safety and efficacy of the three drugs (SP, Coartem and Artekin) in children under five years.
- To compare their capacity to reduce gametocyte carriage.
- To distinguish recrudescences from new infections by PCR-RFLP analysis of MSP2 in a comparative trial of SP, Coartem and Artekin in children under five years.
- To evaluate whether selection of mutants *dhps* and *dhfr* occurs after SP treatment and *Pfcrt* and *Pfmdr* mutants occurs after Artekin treatment by comparing pre and post-treatment isolates.
- To assess baseline in vitro response of African clinical isolates to Artekin and to evaluate the possible cross-resistance between chloroquine and piperaquine.
- To carry out a population pharmacokinetic study on Artekin in African patients.

V. Collaborations and responsibilities

This work is collaboration between the University of Neuchâtel, the Swiss Tropical Institute and the Ifakara Health Research and Development Centre.

The main partners will be:

Switzerland:

- University of Neuchâtel (UNINE), Neuchâtel
  - Prof. Dr. Bruno Betschart — Main supervisor, director of the thesis, lab work supervisor.
  - Harriet Babalanda — Scientific collaborator Laboratory analysis.
- Swiss Tropical Institute (STI), Basel
  - Prof. Dr. Marcel Tanner — Main supervisor in STI, study development, design and proposal development, coordinator of the project.
  - Dr. Timothy Haley — collaborator in STI study design and statistical analysis.

Tanzania:

- Ifakara Health Research and Development Centre (IHRDC), Ifakara

- Dr. Hassan Mshinda	Main contact in Ifakara, field work supervisor, study development.
- Dr. MD. Salim Abdulla	Field work supervisor, study design.
- Dr. Boniface Idindili	Main collaborator in Ifakara, in charge of patients recruitment and clinical follow up.
- Dr. Ayoub Shekimweri	Director of Mkuranga's district hospital.

Australia:

- Victorian College of Pharmacy, Monash University, Parkville

- Prof. William Charman	Collaborator in Australia for the Pharmacokinetic and pharmacodynamic studies.

VI. Methodology

VI.1. Study area

The study will be conducted in Mkuranga district. The place is situated in the east of Tanzania on the coastal region, about 50 km south of Dar es Salaam, the capital of the country. The district has an estimated population of 186 927 (2002, national census). The area has a hot and humid tropical climate with two rainy seasons: an intense one observed during the months of March and May, and a mild one occurring in November and December. The average temperature ranges from a maximum of 31.5°C-32°C to a minimum of 18.1°C-18.6°C, and the average annual rainfall is 1.115mm. Mkuranga is an area with perennial malaria transmission, however there is a peak in transmission between May and November. Health services are offered in a through a system of distinct hospital, health centre and dispensary. The study will be implemented at the district hospital.

VI.2. Assessment of drugs efficacy and safety

- Recruitment of patients

Children will be recruited when they will come to the hospital for medical care.

A rapid screening procedure will be used to identify those children who will be eligible for enrolment.

Children under 5 years with an axillary temperature > 37.5°C will be screened for *P. falciparum.* The study will be explained to the parents, their understanding will be assessed with a set of standard questions and written informed consent will have to be obtained. Blood smears will be collected from the patients using a finger stick procedure. Giemsa-stained thick film blood smears will be prepared and examined by an experienced microscopist. Speciation will be determined using thin film microscopy. All children with blood smears of > 1000 parasites/ µl and below 250 000 parasites/µl, with no clinical evidence of complications will be assigned a consecutive, unique number for further assessment.

If the child meets the enrolment criteria and takes part in trial, the screening number will become the patient's study number and will be used to identify all forms and blood samples from the child.

A card containing demographic and clinical information will be started on each febrile child, thus allowing for appropriate tracking of each patient through the screening procedure.

Exclusion criteria will include concomitant illness, previous antimalarial therapy (receipt of quinine or an artemisinin drug within 7 days, of a 4-aminoquinoline within 14 days, or of pyrimethamine, sulfonamide, or both within 28 days), severe malaria, severe malnutrition, patients who are not able to come for stipulated follow up visits and have not easy access to the hospital, patients who had history of skin rashes after sulphonamide drug intake.

- Sample size

To be able to compare the efficacy of Artekin and Coartem to SP, that the proportions day 28 cured are 56%, 80% and 85% for SP, Coartem and Artekin respectively (Unpublished non-PCR corrected results from the area), using alpha=0.025 (to allow for multiple comparisons), and 90% power; the required sample size of evaluable patients is 93. A seven percent lost to follow is expected in this area. Hence, 100 patients will be recruited in each arm making a total of 300.

- Antimalarial therapy

The following drugs will be tested:

- Sulfadoxine/pyrimethamine (Fansidar®)
- Artemether plus Lumefantrine (Coartem®)
- Dihydroartemisinin plus piperaquine (Artekin™)

All the patients will be randomly assigned to receive one of these treatments:

- Sulfadoxine/pyrimethamine will be given in a standard dose of 25mg/kg.
- Artemether plus Lumefantrine will be given in the regimen recommended by WHO (see annex I) in accordance with age-based doses at times 0, 8, 24, 36, 48 and 60 hours.
- Artekin will be given in the form of sachets (120mg piperaquine phosphate plus 15 mg DHA) in accordance with the manufacturer's instructions in age-based doses (see annex II) given at times 0, 6, 24 and 32 hours.

All the patients will be observed for 1 hour after treatment with the first dose for adverse reactions or vomiting. Those vomiting during the study will be re-treated and excluded from the study.

- Clinical procedures

Clinical procedures and follow-up will be selected on the basis of the WHO's 28-day test extended to 56 days (WHO, 2002).

After enrolment, each subject will be weighed, a full physical examination will be performed and vital signs (axillary temperature, heart rate, respiratory rate, blood pressure) will be recorded. In addition to a blood smear, each patient will have vacutainer blood taken for white cell count, plasma glucose and haemoglobin.

Blood smears will be taken at 0, 8, 24, 48 and 72h. The temperature of each patient will be measured orally at 0, 8, 24, 48 and 72h. All patients will be followed up from day 0 to day 56 (day 0, 1, 2, 3, 7, 14, 21, 28, 35, 42, 49 and 56). At each daily assessment, symptoms and adverse effects will be recorded; a blood smear and filter paper will be taken. If patients cannot come to the hospital, they will be followed up at their home.

Parasite resistance will be categorized as RI (prompt and sustained asexual parasite clearance through day 7, but reappearing before day 28), RII (>75% decrease in asexual parasitemia by 48h, but no clearance and persistent parasitemia on day 7), or RIII (< 25% decrease in asexual parasitemia by 48h and persistent on day 7).

Clinical and parasitological data will be used to categorize therapeutic response as early treatment failure, late clinical failure, late parasitological failure or adequate clinical and parasitological response (see the definitions in annex III).

- Laboratory methods

Giemsa-stained blood smears will be examined by a skilled microscopist. For positive slides, parasite density will be calculated from the number of asexual forms per 200 leukocytes and from the whole blood leukocyte count. The slides will also be examined for the eventual presence of gametocytes per 1000 leukocytes. Haemoglobin levels, plasma glucose concentrations will be also measured.

- Safety assessment

The safety profile will be evaluated by documenting the number and timing of deaths, admissions, and outpatient attendances in study patients. Skin reactions and evidence of bone marrow suppression will be assessed.

The proportion of patients with haematological and biochemical parameters falling outside the normal range at day 56 will be also documented.

- Data analysis

The efficacy of different regimens in terms of treatment outcome (see annex III) will be done. Furthermore, resolution of symptoms present at baseline will be assessed by comparing the total duration of symptoms in the different groups. All comparisons will be made relative to SP because this is the standard treatment.

VI.3. Distinction of recrudescence from new infections by PCR-RFLP analysis

Before the treatment (day 0) and at each daily assessment, blood samples will be blotted on filter paper from a subset of parasitological failures, and will be used for PCR analysis. The filter papers will be dried at room temperature and placed into separate plastic bags, sealed, and stored at -20°C.

- Extraction of DNA, PCR amplification, and restriction digest

Extraction and purification of DNA will be performed using the Chelex method (Plowe et al., 1995). 5 µl of DNA will be used for the primary reaction and 2 µl of PCR product will be used for the nested reaction in a 100 µl reaction composed as follows: 78 µl of dH_2O & DNA template, 10 µl of 10x PCR buffer, 10 µl of dNTPs 2mM, 1 µl of primer S2 50µM, 1 µl of primer S3 50µM and 0.3 µl of taq polymerase (5U/µl). Primer pairs S2 and S3 will be used for the primary reaction and S1 and S4 for the nested reaction as published by Foley et al., 1992. Both PCR reactions will be performed with the following profile:5 min at 94°C and 30 cycles: 30 sec at 94°C, 2 min at 55°C, 2 min at 70°C. Negative controls will be included with each set of PCR reactions.

The different MSP2 alleles will be genotyped as previously described (Felger et al., 1993). 20 µl of nested PCR product will be subjected to restriction digests with the restriction enzyme HinfI, and run on a 10% polyacrilamide gel. All samples of the same patient will be loaded side by side and sizes will be calculated using a standard commercial marker. RFLP patterns will be visualized by ethidium bromide staining and documented electronically.

- Data analysis

Patients will be included in the efficacy analysis as parasitological failures if they are parasite-positive by microscopy or if rescue medication (quinine) is required at or before the respective time point. They will be included as treatment successes if an aparasitaemic sample (by microscopy) is available for the time point and no other parasites is recorded between

completion of treatment and collection of that sample. Genotypes found by PCR either in samples from day 0 or day 3 will be considered to be present initially and considered as baseline. Recrudescence will be deemed to have occurred if the RFLP pattern of an allele seen at baseline (day 0 and/or day 3) is detected by PCR on any consecutive follow-up day. New infections will be defined as having completely different RFLP patterns in follow-up samples from those seen at baseline. Hence, new infections with the same MSP2 genotype should be considered as recrudescence. Estimates of recrudescence and new infection rates will be made separately for days 7, 14, 28 and 56. For each day, the recrudescence rate will be estimated as the proportion of tested samples found by PCR to contain recrudescent parasites, multiplied by the failure rate (failures/total patients included) for that day. A similar calculation will be carried out for the patients by PCR as being newly infected.

VI.4. Comparing the selection of *dhps* and *dhfr* mutants after SP treatment and *Pfcrt* and *Pfmdr* after Artekin treatment

Paired samples from patients with either late treatment failure (between day 4 and 28) or adequate clinical response with parasitological failure (on day 28) will be included in the study.

- Extraction of DNA, PCR amplification, and restriction digest

Extraction and purification of DNA will be performed using the Chelex method (Plowe et al., 1995).

The *pfcrt* gene will be analysed as described elsewhere by Djimde et al., 2001. Genomic DNA from the samples will be amplified using primers flanking residue 76 in 2 rounds of PCR analysis by use of outer primers TCRP1 and TCRP2 and nested primers TCRD1 and TCRD2. The 145-bp nested PCR product then will be digested with restriction enzyme ApoI. The enzyme cuts the *Pfcrt-K76* (wild type) but not the *Pfcrt-T76* (mutant).

Detection of *Pfmdr1* alleles will be done as described elsewhere by Duraisingh et al., 2000. Genomic DNA will be amplified with primers flanking codon 86. First, an outer PCR will be done using primers MdrA1 and MdrA3 and then nested PCR will be done by using primers MdrA2 and MdrA4. *Pfmdr1* alleles will be identified by using ApoI, which cuts allele *N86* (wild type) but not *Y86* (mutant). Positive and negative controls will be included in all amplifications procedures.

Detection of *dhfr* and *dhps* alleles will be done as described elsewhere by Duraisingh et al., 1998. In the first round, a 648-bp portion of *dhfr* gene will be amplified by use of the primers M1 and M5. Similarly, a 710-bp portion of *dhps* gene will be amplified by use of R2 and R/. For the second round of the *dhfr* gene, primers F and M4 will be used to detect 59-arginine, 108-serine, and 108-threonine or M3 and F/ to amplify the fragments containing 16-alanine, 51-asparagine, 108-asparagine, and 164-leucine. Similarly, for the *dhps* gene, primers K and K/ will be used to detect 436-serine, 436-alanine, 437-glycine, 437-alanine, 540-glutamic acid, or primers L and L/ for 582-alanine, 581-glycine, 613-threonine, 613-alanine. The primer pair K/ and J will be used to detect 436-phenylalanine. Positive and negative controls will be included in all amplifications procedures. The 326-bp product of the primer pair F-M4 will be cut by AluI at codon 108. In the same PCR product, BstNI detects the 108-threonine mutation and XmnI detects 59-arginine. The amplified product of the M3-F/ primer pair will be cut by DraI to detect 164-leucine and by BsrI to detect 108-asparagine. NlaIII will detect the 16-alanine/valine mutation and Tsp509I will detect the 51-asparagine/isoleucine mutatuion. For the *dhps* gene the 438-bp product of K-K/ will be cut by MnlI or MSPAI to identify 436-serine/alanine mutation, by AvaII or MwoI for 437-glycine/alanine, and Fok I for the 540-glutamic acid. The 161-bp product of L-L/ will be cut by BstUI or BslI to detect 581-alanine/glycine mutation, by MwoI or AgeI to detect 613-alanine/serine.

All product digest will be separated by electrophoresis on a 10% polyacrilamide gel.

To determine whether the patient had recrudescent parasites or new infections, MSP-2, size polymorphisms will be compared between the pre- and post-treatment samples by PCR-RFLP analysis as described above.

- Data analysis

The comparison of pre- and post-treatment *P. falciparum* isolates my present several possibilities. Infection with isolates carrying *pfcrt*, *pfmdr-1*, *dhfr* and *dhps* both before and after Artekin or SP treatment possibly implies that reinfection occurred, drug absorption was not adequate, or these genes are not the only involved in Artekin or SP resistance.

Mixed infection before the treatment and selection of mutant parasites at recrudescence seem to be the best available evidence for the possible role of *pfcrt* and *pfmdr-1* in *in vivo* Artekin resistance and of *dhfr* and *dhps* in *in vivo* SP resistance.

These interpretations may be subject to error in individual patients because of various intervening host factors but may be useful to assess the possible role of these genes in determining in vivo SP and Artekin resistance if there are a sufficient number of observations.

VI.5. *In vitro* activities of Artekin against clinical isolates of *P. falciparum*

- Clinical procedures

Children who are recruited in the safety and efficacy study will be asked to participate in this study. Children from this cohort will sampled (excluding those in the pharmacokinetic study) until 100 cultured samples are obtained. The clinical procedure will be explained to the parents and they will be asked venous blood can be taken from their children at Recruitment and the day of failure if they come back with a malaria episode during follow-up.
If the parent gives his consent, venous blood sample (3 ml) will be taken on EDTA-coated tubes and kept in the fridge not later than 24h.

- In vitro assay

Venous blood samples will be washed with RPMI 1640 culture medium three times by centrifugation (2'000 rpm x 10 min) within 2h after blood collection. The *in vitro* drug sensitivity assay will be performed according to the isotopic microtest assay described by Desjardins et al., 1979. The culture plates will be incubated for 42 h at 37°C in 5% CO_2 in an incubator. The incorporation of [^{3}H]hypoxanthine will be measured by a liquid scintillation counter. The *in vitro* activities of test compounds will be expressed as the 50% inhibitory concentration (IC_{50}) defined as the drug concentration at which 50% of the incorporation of [^{3}H]hypoxanthine is inhibited compared with the incorporation in the drug-free wells. Parasite growth will be plotted against drug concentration, and the best-fitting sigmoid curve will be traced using software to determine the IC_{50} value.

- Data analysis

The *in vitro* activity of Artekin will be expressed as the geometric mean of the IC_{50} of all isolates. To measure the possible difference in the levels of activity against the chloroquine-sensitive and the chloroquine- resistant isolates, the mean logarithmic IC_{50} values of Artekin and chloroquine will be compared by two-tailed unpaired Student t test. The potential for in vitro cross-resistance will be evaluated by linear regression. For all statistical tests, the significance level (P) will set at 0.05.

VI.6. Pharmacokinetic study

- Clinical procedures

Children will be recruited from the efficacy and safety study. A selection of children recruited into the Artekin Arm will be asked to participate. The selected children will not take part in the *in vitro* sub-study and must be over two years of age. 50 children will participate in this study as with previous studies (Te-Yu Hung et al., 2004).

The clinical procedure will be explained to the parents, and they will be asked if their child can be admitted to the hospital for 3 days and if 4 venous blood samples can be taken from their children during that period and a further 5 collections in the next two months period.

If the parent gives his consent, the child will be admitted to the hospital for 3 days.

Artekin will be given to the patients in the form of tablets (320mg piperaquine phosphate plus 40 mg DHA) or (120mg piperaquine phosphate plus 15 mg DHA) in accordance with the manufacturer's instructions in age-based doses (see annex II) given at times 0, 6, 24 and 32 hours.

Serial blood samples (3 ml) will be taken for each child at four of these times: 0, 0.5, 1, 1.5, 2, 3, 4, 6, 12, 24, 32, 48h and then at day 7, 14, 28, 42 and 56. The times of sampling were chosen according to the results from a previous study (Te- Yu Hung et al., 2004).

A measured volume of blood (100 uL) will be aliquoted on to a filter paper for dihydroartemisinin quantification in the blood samples by spectrometry.

With each patient, the following observations will be made at 0, 12, 24, 48 and 72h hours from the time of the beginning of the treatment until the complete clearance of parasitemia: axillary temperature, pulse blood pressure, liver and spleen size.

Blood smears will be taken at the same times. Haemoglobin levels and glucose concentrations will be also measured.

- Laboratory methods

Samples will be centrifuged immediately at 3000rev/ min for 5 min. The plasma will be removed after and stored at -20°C.

A combination of HPLC and UV methods will be used to determine the quantity of piperaquine in plasma (Te-Yu Hung et al., 2003).

Plasma (1 ml) will be aliquoted into 10 ml polypropylene tubes, with artemisinin as internal standard (I.S.) (20 ng) and 0.1 ml of 1 M NaOH. Dichloromethane (8ml) will be added and piperaquine extracted by shaking vigorously for 10min. After centrifugation at 1300 g for 10

min, the supernatant will be aspired to waste, and the remaining dichloromethane transferred to a clean polypropylene tube. Piperaquine will then be back-extracted into 0.3 ml of 0.01 *M* KCl (buffered to pH 2.4 with 0.01 *M* HCl) by shaking vigorously for 5 min. After centrifugation as above, dichloromethane layer will be aspired to waste and the remaining acidic aqueous extract will be transferred to a clean round-bottomed borosilicate glass tubes and centrifuged at 1300 g for 20 min to evaporate any traces of dichloromethane that remained. Aliquots (100 µl) of this final extract will be injected onto HPLC column.

Analytes will be detected by their UV absorbance at 340 nm.

A combination of HPLC and mass spectrometry methods will be used to determine the quantity of Dihydroartemisinin in plasma (Souppart et al.,2002).

Appropriate volume of I.S. will be added the samples following by 0.125 ml of NaCl saturated solution and 2.5 ml of 1-chlorobutane-isooctane (55:45, v/v) in silanized glass tube. The tubes will be placed on a horizontal shaker for 5 min at a velocity of 250 rpm. After a 15-min centrifugation at 3650 g at 15°C, the organic layer will be transferred into another silanized conical tube. The solvent will be evaporated to dryness at 30°C under nitrogen stream. The residue will be dissolved in 100 µl of ethanol 0.1% glacial acetic acid (50:50, v/v) by vortex mixing and the solution will be transferred into a conical polypropylene insert inside amber glass microvials.

The mass spectrometer will be operated in positive ion mode, single ion monitoring with a maniford temperature set at 70°C, the dynode at 15 kV and the electron multiplier at 1400 V. Nitrogen will be used as nebulizer gas at pressure of 586 kPa with a vaporizer temperature at 400°C.The capillary temperature will be set at 300°C and the corona needle discharge at 5 µA. Nitrogen will also be used as auxiliary gas at 15 units on the flow meter.

- Data analysis

Pharmacokinetics indices will be calculated using the appropriate methods.

The terminal elimination rate, the elimination half-life, the area under the curve, the oral clearance, and the steady state volume of distribution will be calculated.

The time at which maximum concentrations will be attained and the maximum concentrations will be obtained directly from the plasma concentration-time data.

Parasite clearance time will be defined as the time from starting therapy to the first 2 consecutive negative thick films.

The time for parasite reduction to 50% of initial parasitemia will be determined by linear interpolation from plots of parasite densities versus time. Sublingual temperature will be

monitored at the same times as thick films will be made. Fever subsidence time will be taken as the time required for the temperature to fall below 37.5 and remains so far 2 consecutive readings.

The relationship between the pharmacokinetics and therapeutic response will be evaluated.

VII. Dissemination of results

Parents/ guardians of children participating in the study will be notified immediately of their child's results regarding parasitemia and haemoglobin levels, as well as overall treatment outcomes.

The overall study results will be disseminated to the NMCP (National Malaria Control Programme), EANMAT (East African Network for Monitoring Antimalarial Treatment) and other bodies within tanzanian Ministry of health. Results will also be submitted to peer-reviewed scientific journals and presented at national and international meetings.

VIII. Ethical Considerations

There is minimal physical risk associated with the study. The child may experience a brief moment of physical discomfort and/or fear during the finger stick or venous blood collection procedure. All children will be treated for their initial parasitemia and any additional parasitemia that presents with clinical symptoms during study period.

As discussed earlier (introduction) existing data suggest that these combination therapies are safe and the efficacy is high. Coartem is registered product in Tanzania. Artekin is only registered in China and Cambodia, but a development programme has been developed between Holleykin Pharmaceuticals and Guangzhou University (China), the University of Oxford, MMV and WHO/TDR to support the international registration of the drug. However further understanding of drug safety and effectiveness will allow for better treatment of malaria, thus reducing malaria-related mortality and morbidity, and in the case of Artekin, this study should be an important step in the international registration of the drug.

In addition, the children will be examined and treated for other concurrent illness. At each visit, parent/guardian will be informed as to the status of their child's health and regarding procedures and/or treatments that will occur during that visit. They will have the right to refuse a procedure or treatment or to withdraw form the study. Children enrolled in this study will benefit in several ways. Each patient's health will be closely monitored over 56 days, and he/she will receive appropriate treatment for malaria. In addition, parents/guardians are

encouraged to bring the child back to the health facility for further assessment and/or treatment at any point at which the child is perceived to be ill. Haematological response to therapy will be assessed and anaemia diagnosed hence treated. Children will benefit from having anaemia diagnosed because they can be referred for further treatment and iron supplementation, as needed, and as per national guidelines. The frequent follow-up procedures will allow for rapid identification of adverse effects of therapy, if any.
As discussed earlier, parents/guardians of children who fulfil the entry criteria will be asked for their informed written consent. Details about the trial and its benefits and potential risk will be explained to the parents in Swahili. All information regarding patients will remain confidential to the extent legally possible.

IX. Data Safety and Monitoring Board

The safety data will be evaluated during this clinical trial by a DSMB (Data safety and monitoring board).

The DSMB will be composed by following persons:

- Prof. Dr. Zulfilcar Premji, Parasitologist
  Muhimbli University College of Health Sciences
  Chairman of the DSMB

- Dr. Makwuya, Epidemiologist, Biostatistician
  Muhimbili University College of Health Sciences

- Dr. Renata Mandike
  National Malaria Control Program
  Ministry of Health

- Dr. Alizeus Kahigwa
  WHO country office
  Tanzania

X. Proposed study timeline

This study will be implemented over 3 years, from January 2004 to December 2006.
The field work should be done between January and May/June 2005.

- Time schedule

Time	Place	Activity
January 2004	Neuchâtel	- Preparation of the journey to Ifakara
February 2004	Ifakara	- First contact with local collaborators - Development of the project
March - May 2004	Neuchâtel	- Writing of the proposal
June –August 2004	Basel/Neuchâtel	- Training in STI to learn PCR-RFLP techniques and in vitro assay. - Submission of the project to the IHRDC ethic committee
September 2004	Ifakara	- Revision of the project with the IHRDC ethic committee and finalization - Submission to MRCC (national ethic committee)
October- December 2004	Neuchâtel	- Organization of the field work
January – May/June 2005	Mkuranga/ Dar es Salaam	- Field work - Collect of samples - Efficacy and safety assessment - In vitro study
June –December 2005	Ifakara	- Lab work - PCR-RFLP analysis - Data analysis
January - June 2006	Neuchâtel	- Lab work - HPLC analysis - Data analysis
July – December 2006	Neuchâtel	- Data analysis - Writing up and presentation of the thesis

XI. The Budget (Amount in Swiss Francs)

	1st year	2nd year	3rd year	Paid by
1. Costs in Switzerland				
Personnel				
Salary→ Assistant employed by the University of Neuchâtel with 50%	40'284	42'036	45'540	University of Neuchâtel
Laboratory costs				
HPLC			36'000	University of Neuchâtel
Sub-Total Switzerland	**40'284**	**42'036**	**81'540**	
2. Field Costs				
Travels				
Trip Switzerland-Tanzania	3'100	1'800		
Travels inside the country		9'000		
Personnel				
2 clinical officers → 6 months		5'100		
1 microscopist → 6 months		2'700		
1 nurse → 6 months		2'700		
1 fieldworker → 6 months		2'700		
Materials				
Drugs and rescue drugs		6'000		
Materials for samples collection and field analysis (blood slides, filter papers, PCV tubes, EDTA tubes, vacutainer needle…)		12'000		
Hospitalization costs		2'000		
Papers, copies, fax, phone		2'000		
Laboratory costs				
Lab bench fees for Ifakara centre and Muhimbili University labs		7'000		
PCR-RFLP		14'400		
In vitro assay		3'600		
Accommodation & subsistence	3'000	6'000		
Sub-total field costs	**6'100**	**77'000**		
Total Budget each year	**46'384**	**119'036**	**81'540**	

The total budget for 3-year study is about **246'960**SFr.

- Justification of the budget

The salary of a clinical officer or a microscopist is about 450 SFr./ month in Tanzania. Laboratory costs are estimated at 40 SFr. per HPLC, 12 SFr. per PCR-RFLP and 30 SFr per in vitro assay, for approximately 500 samples for HPLC analyses, 1200 samples for PCR-RFLP analyses and approximately 120 samples for in vitro test analyses. Travels inside the country include the renting of a motorbike to allow to the field-worker to follow the patients at home when they cannot come to the hospital, the travels from Mkuranga to Dar es Salaam at Muhimbili University to do the in vitro assay and the travels from Mkuranga to Ifakara for the other lab analyses. We will need a nurse to take care of the children who will be hospitalized during 3 days for the pharmacokinetic study, and we will deal with all the hospitalization's costs. Accommodation &subsistence costs are about 1000 sFr./month in Dar es Salaam.

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XIII. Annexes

Annex I: The Coartem regimen recommended by WHO (CDS/RBM, 2002).

	Number of Tablets				
	Children <10 kg	Children 10-14 kg	Children 15-24 kg	Children 25-34 kg	Adults ≥ 35 kg
0 hrs	NR	1	2	3	4
8 hrs	NR	1	2	3	4
24 hrs	NR	1	2	3	4
36 hrs	NR	1	2	3	4
48 hrs	NR	1	2	3	4
60 hrs	NR	1	2	3	4
Total	NR	6	12	18	24

NR: not recommended

Annex II: Recommended Artekin (dihydroartemisinin-piperaquine) dosing schedule, by patient age, for treatment of malaria (Denis et al., 2002).

Patient age, years	Formulation	Dose				
		No of granules or tablets [a]	Total, mg [b]		Based on body weight, mean mg/kg ± SD [b]	
			DHA	piperaquine	DHA	piperaquine
2-3	Granules (sachet)	1	60	480	8.1±2.2	64.8±17.3
4-6	Granules (sachet)	1.5	90	720	7.9±3.5	63.2±28.0
7-10	Tablet	1	160	1280	10.1±1.9	81.2±15.6
11-15	Tablet	1.5	240	1920	10.1±1.8	80.6±14.3
≥16	Tablet	2	320	2560	6.6±1.1	52.9±8.5

[a] For each of 4 administrations.
[b] Total of 4 administrations.

Annex III: Classification of response to treatment (WHO, 2002).

- **Early treatment failure**
  - Development of danger signs or severe malaria within the first 3 days, in the of parasitemia.
  - Parasitemia on day 2 higher than day 0 count irrespective of axillary temperature.
  - Parasitemia on day 3 with axillary temperature => 37.5°C
  - Parasitemia on day 3 => 25% of count on day 0

- **Late clinical failure**
  - Development of danger or severe malaria after day 3 in the presence of Parasitemia, without previously meeting any of the criteria of early treatment failure.
  - Presence of parasitemia and axillary temperature=> 37.5°C (or history fever) on any day to day 28, without previously meeting of the criteria of early treatment failure.

- **Late parasitological failure**
  - Presence of parasitemia on any day from day 7 to day 28 and axillary temperature < 37.5°C, without meeting of any criteria of early treatment failure or late clinical failure.

- **Adequate clinical and parasitological response**
  - Absence of parasitemia on day 28 irrespective of axillary temperature without previously meeting of any criteria of early treatment failure or late clinical failure or late parasitological failure

Annex IV: Definition of severe malaria and complications (WHO, 1996)

One ore more of the following criteria in the presence of asexual parasitemia define severe *falciparum* malaria:

1. Cerebral malaria – unrousable coma (after generalised convulsion, coma should persist for at least 30 minutes to make the distinction from post-ictal coma).
2. Severe normocytic anemia (Hb <3.0 mg/dL)
3. Renal failure (serum creatinine >3.0 mg/dL)
4. Pulmonary oedema
5. Hypoglycemia (<40 mg/dL)
6. Circulatory collapse/shock (systolic BP< 70 mm Hg in adults; or <50 mm Hg in children <5 years
7. Spontaneous bleeding/ disseminated intravascular coagulopathy
8. Repeated generalized convulsions
9. Acidemia/acidosis
10. Macroscopic hemoglobinuria

Other manifestations

1. Impaired consciousness but rousable
2. Prostration, extreme weakness (inability to stand or walk)
3. Hyperparasitemia (>5% RBC infected)
4. Jaundice (total serum bilirubin >3 mg/dL)
5. Hyperpyrexia (axillary temperature>39.5°C)

General ganger signs

1. Not able to drink or breastfeed
2. Vomiting everything
3. Recent history of convulsions
4. Lethargic or unconscious state unable to sit or stand up

Rapid selection of *Plasmodium falciparum* dihydrofolate reductase mutants by limited use of sulfadoxine-pyrimethamine

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Working manuscript

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ABSTRACT

BACKGROUND The development of *Plasmodium falciparum* resistance to sulfadoxine-pyrimethamine (SP) is a major problem for the effective treatment of malaria. The level of drug usage in the population was suggested to be a fundamental factor in the emergence and spread of drug resistance.

METHODS The aim of the study was to asses the impact of three doses of SP use on SP resistance markers in an area where SP had rarely been used. We used samples collected before, during and after a vaccine trial in Idete, Tanzania, in which the enrolled children received a dose of SP before each immunisation to clear parasitaemia. Molecular markers were analysed using a DNA-based microarray technique.

RESULTS The *pfdhfr* 108 mutation was found at baseline with a frequency of 0.49, and changed significantly between the three time points (likelihood ratio chi square test (LRX^2)= 10.16, 2 d.f, p=0.0062). There was a statistically significant increase in mutation frequencies of *pfdhfr* 59 (LRX^2=30.79, 2 d.f, p<0.0001), *pfdhfr* 51 (LRX^2=29.17, 2 d.f, p<0.0001), and *pfdhps* 436 (LRX^2=11.96, 2 d.f, p=0.0025). The *pfdhps* mutation 437 was not present at baseline, but was detected later. The increase was statistically significant (LRX^2=27.42, 2 d.f, p<0.0001). The changes in haplotype frequencies between the three time points were also statistically significant for *pfdhfr* locus (Likelihood ratio statistic (LRS) = 66.7, 14 d.f., p<0.0001) and *pfdhps* locus (LRS = 22.0, 6 d.f., p=0.0012).

CONCLUSION These data show that the three doses of SP have selected for *pfdhfr* and *pfdhps* mutations, which arise extremely rapidly under very low drug pressure. These findings have implications for intermittent preventive treatment (IPT); in the view of rapid selection of *Pfdhfr* mutations with low drug usage, other antimalarial regimens should be deployed in the areas where SP is used for IPT.

INTRODUCTION

The combination of pyrimethamine and sulfadoxine (SP or Fansidar®) is a cheap agent against chloroquine resistant *falciparum* malaria that is widely used in Africa. SP replaced chloroquine as the first-line curative antimalarial throughout Sub-Saharan Africa, because of its affordability, its ease of administration, and until recently, its effectiveness (Pearce et al., 2003). Unfortunately, its clinical efficacy is diminishing as a consequence of the development of resistance (Alifrangis et al., 2003; Mugittu at al., 2005).

In vitro resistance to pyrimethamine resistance is linked to mutations in *pfdhfr* gene. A point mutation causing a serine to asparagine change at position 108 is the first to occur, followed by mutations at positions 51, 59 and 164 (Cowman et al., 1988; Peterson et al., 1990; Foote et al., 1990). Point mutations at codons 437 and 540 of *pfdhps* gene are considered responsible for sulfadoxine resistance. Additional mutations at positions 436, 540, 581 and 613 mediate resistance to higher dosages of the drug *in vitro* (Plowe et al., 1997; Nzila et al., 2000).

Parasites with fewer than 3 *pfdhfr* mutations at position 108, 51 and 59 may be cleared by SP, regardless of *pfdhps* genotype (Wang et al., 1999; Mugittu et al., 2004). In the presence of this *pfdhfr* triple mutation form, the *pfdhps* mutant genotype increases the likelihood of treatment failure. It seems that patients infected with parasites carrying the *pfdhps* double mutant 437/540 and the *pfdhfr* triple mutant had a higher relative risk of treatment failure than did those infected with parasites carrying the *pfdhfr* triple mutant alone (Nzila et al., 2000; Gatton et al., 2006).

Although the molecular basis for SP resistance is understood, the factors promoting the development and transmission of these mutants are less clear. Drug use is considered to be the key factor promoting the emergence of SP resistance, but other parameters, such as pharmacokinetics, pharmacogenetics, level of immunity, treatment compliance, dosing, etc., play a role in clearing symptoms and parasites (White, 2004; Hastings et al., 2005). The long half life of SP has been proposed to exert a selection pressure favouring mutated parasites (Watkins & Mosobo, 1993). It was hypothesized that the level of drug usage in the population was a fundamental factor during the first phase of drug resistance, when parasites are not yet completely resistant, but are getting more and more tolerant to the drug (Hastings & Watkins, 2006).

To elucidate the effect of drug pressure on the development of SP resistance, we assessed the impact of three doses of SP used in 600 children under 5 years in an area where SP has been rarely used. The samples which were used were collected longitudinally during a vaccine trial in Idete (Tanzania) between March 1993 and July 1994. It was hypothesized that the

frequencies of *pfdhfr* and *pfdhps* mutations have been low at the baseline because SP was only used as second-line treatment at that time in Tanzania, but that three doses of SP given to a larger number of children would already select for *pfdhfr* and *pfdhps* mutations.

MATERIAL AND METHODS

Study area

The village of Idete is located about 20km west of Ifakara, southern Tanzania. It lies south of the Udzungwa Mountains, at the northern edge of the alluvial plain of the Kilombero River at an altitude of 270m. The Kilombero valley is an area of intense and perennial malaria transmission. The main rains start in March and extend through to May, and short rains occur in December and January. The prevalence of asymptomatic *P. falciparum* parasitaemia was high and showed no marked seasonality at the time of the vaccine trial (Alonso et al., 1994). 80% of infants were infected at the age of 6 months and data from an adjacent village indicated that, on average, everyone received more than 300 infective bites per year (Smith et al., 1993). Transmission of the other three species of human malarias was low and unstable.

Blood samples and drug use

The samples were collected during a vaccine (SPf66) efficacy trial in children under 5 years between March 1993 and July 1994 (Alonso et al., 1994).

Briefly, the study was a randomised double blind, placebo controlled trial, and the objective was to determine whether three doses of SPf66 reduce the incidence of clinical episodes attributable to malaria or the prevalence and intensity of parasitaemia in children aged 1-5 years, and to estimate the protection achieved. Six hundred children were recruited for this study. In total 586 children were recruited; 274 received the vaccine and 312, the placebo. At that time, the first line antimalarial treatment was chloroquine, but in order to maximize the immune response to the vaccine by avoiding the transient immuno-suppression associated with malaria parasitaemia, every individual was treated with a single curative dose of sulfadoxine-pyrimethamine (Fansidar®, Roche; Basel, Switzerland), 2 weeks before the administration of each vaccine dose or placebo. The children received 3 vaccine doses; the second dose was given 4 weeks after the first one, and the last dose 22 weeks later.

Passive case detection of clinical episodes was done at the only village dispensary (Alonso et al., 1996). Each patient attending the dispensary for a perceived illness was screened by project medical personnel, who provided 24 hours coverage. Children were then cared for by

routine medical services at the dispensary. Clinically suspected malaria episodes were treated, following national guidelines, with chloroquine and with SP when chloroquine treatment failed. The prescribed antimalarials for each person were recorded and these data were used to calculate the treatment per capita per year mean.

Molecular analysis

363 samples were randomly selected in the placebo group, 200 samples from the baseline, 81 samples from the end of the study, and 82 samples from cross sectional surveys in-between.

1) Molecular markers of drug resistance

The *pfcrt*, *pfmdr*, *pfdhfr* and *pfdhps* mutations were analysed using a DNA microarray technique as described previously (Crameri et al., 2007).

Briefly, DNA was extracted from 200µl blood pellet using the QIAamp® 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. Using gene-specific primers, the five different genes were amplified by primary and nested PCR. 10µl of each nested PCR product were diluted 1:10 with sterile water and SAP-digested in duplicate. Single base primer extension reactions were carried out using 20-30bp locus-specific extension primers, which are designed that the last bases on their 3' end precedes the SNP site. The extension over the SNP site is performed using Cy3 or Cy5-labelled ddNTPs and sequenase. The extended primers (whose last base on their 3' ends are now labelled with a fluorochrome) were denatured by heating (2 minutes at 94°C) and hybridised to specific antisense probes anchored onto aldehyde-coated microscopic slide in a humid chamber at 94°C for 1 hour. Slides were washed three times with saline-sodium citrate (SSC) and sodium dodecyl sulphate (SDS), dried with compressed air and scanned with an Axon 4100a GenePix Personnal scanner, arrays were analysed by Genepix to detect SNPs.

2) Genotyping of PfMSP2 by PCR-RFLP

Determination of multiplicity of infection (MOI) was done using *pfmsp2* genotyping as described previously (Felger et al., 1993; Felger & Beck, 2002).

Briefly, DNA was extracted from 200µl blood pellet using the QIAamp® 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. DNA was amplified by PCR, using the primers S2/S3 and S1/S4 for Primary and nested PCR, respectively. The restriction digest was done overnight at 37°C, in a total volume of 20µl, containing 7µl of nested PCR product, 10.5µl of dH_2O, 2µl of 10x buffer NEB, and 0.5µl (5

units) of HinfI. Digest products were then run on 10% polyacrylamide at 200V for 2 hours. Gels were stained 15 minutes in ethidium bromide solution, and visualised under UV light and photographed. Distinction and counting of different genotypes was achieved by analysis of the restriction fragments pattern (Felger & Beck, 2002).

Data analysis

1) Drug use

Drug pressure was calculated by dividing the number of treatments assigned to the children under five years during the vaccine trial from the health centre records from February 1993 to July 1994 by the number of inhabitants. The treatment per capita was then adjusted for 12 months (1 year).

2) Allelic frequencies

To estimate the allele frequencies of resistance markers in the sample set, a non linear statistical model was used that takes into account the effects of varying multiplicity of infection and assumes that resistant and sensitive parasite clones are transmitted independently. The likelihood of a sample containing no resistant clones is $(1 - p)^n$, where p is the frequency for the mutant allele and n is the multiplicity of infection of the sample. Similarly, the likelihood for the sample to contain no wild-type allele is p^n and for a mixture of both, a wild-type and a resistant allele, is $1 – p^n – (1 - p)^n$. The likelihood over the whole data set for p is computed as the product of this likelihood over all samples, using values of n derived from *Pfmsp2* genotyping results (Schneider et al., 2002). A simple one-dimensional search routine was used to maximize this likelihood and to obtain estimates of p (Teukolsky et al., 2002). Allelic frequencies were calculated separately for each mutation for each time point of the study. Statistical significance changes in allele frequencies between time points were assessed using likelihood ratio tests with 2 degrees of freedom.

Haplotype frequency

The haplotype frequencies were analyzed using a maximum likelihood method as previously described (Hastings & Smith, submitted). Statistical significance changes in haplotype frequencies between time points were assessed using likelihood ratio tests and they were assessed for each gene not for each haplotype separately.

RESULTS

Drug use

In total, 6103 paediatric attendance were recorded in the database from February 1993 to July 1994, and the population of Idete was then 4758 inhabitants. About 60% of the children who attended the dispensary received an antimalarial, either chloroquine (CQ) or sulfadoxine-pyrimethamine (SP). Overall it was estimated that 3468 patients were treated with chloroquine, 146 with SP and 469 with cotrimoxazole. The treatment per capita was 0.49 CQ, 0.02 SP and 0.07 cotrimoxazole treatments per child and per year.

Allele frequencies

Full genotypes could be established in 97% samples (N=352), 189 from the baseline, 82 in-between and 81 at the end of the vaccine trial. Mutations were found in *pfdhfr* codons 51, 59 and 108 and *pfdhps* codons 436, 437 and 540 (Table 1). The *pfdhfr* 59, *pfdhps* 437 and 540 frequencies increased at intermediate stage and decreased slightly at the end (Figure 2). The *pfdhfr* 51 frequency was 4.5 times higher at the end. Those changes were statistically significant for all SNPs except for *pfdhps* 540 (Table 1).

There was no significant change in frequencies of SNPs associated with CQ resistance (Appendix B, figure 2).

Table1: Mutant allele frequency in *Pfdhfr* and *Pfdhps* genes

Mutation	Frequency with 95% CI					
	Baseline	Intermediate	End	LRX^2	df	p values
Pfdhfr						
S108N	0.49 0.40-0.58	0.44 0.31-0.58	0.51 0.38-0.65	10.16	2	**0.0062***
C59R	0.05 0.02-0.08	0.15 0.10-0.24	0.10 0.04-0.18	30.79	2	**<0.0001***
N51I	0.02 0.01-0.03	0.09 0.04-0.15	0.09 0.04-0.17	29.17	2	**<0.0001***
Pfdhps						
S436A	0.08 0.03-0.13	0.05 0.02-0.09	0.11 0.05-0.18	11.96	2	**0.0025***
A437G	0.00 0.00-0.00	0.05 0.01-0.11	0.04 0.01-0.08	27.43	2	**<0.0001***
K540E	0.03 0.02-0.05	0.09 0.03-0.17	0.05 0.02-0.09	11.14	2	0.5656

*Statistically significant at 95% level

Haplotype Frequency

The frequency in different haplotypes is summarized in Table 2. The frequency of *Pfdhfr*-double mutant (S108N + C59R) and triple mutant increased at intermediate stage and decreased slightly at the end (Figure 3). The same trend was observed for *Pfdhps* double mutant, whereas the frequency of *Pfdhfr*-single mutant decreased at intermediate stage and increased slightly at the end (Figure 3).

The changes in haplotype frequencies between the three time points were statistically significant for *dhfr* gene (Likelihood ratio statistic (LRS) = 66.7, 14 degree of freedom (d.f.), p<0.0001) and for *dhps* gene (LRS = 22.0, 6 d.f., p=0.0012).

Table 2: Haplotype frequency in *Pfdhfr* and *Pfdhps* genes

Mutation	Frequency with 95% CI		
	Baseline	**Intermediate**	**End**
	Pfdhfr		
wild type	0.55 0.50-0.59	0.58 0.51-0.64	0.50 0.44-0.56
single mutant	0.39 0.34-0.43	0.20 0.14-0.26	0.32 0.26-0.38
S108N +C59R	0.05 0.03-0.07	0.15 0.10-0.21	0.06 0.03-0.10
S108N + N51I	0.01 0.003-0.02	0.01 0.002-0.04	0.07 0.04-0.10
triple mutant	0.01 0.002-0.02	0.06 0.03-0.10	0.04 0.02-0.07
	Pfdhps		
wild type	0.99 0.96-1.00	0.93 0.89-0.95	0.95 0.92-0.96
K540E	0.01 0.002-0.03	0.01 0.002-0.03	0.01 0.002-0.02
A437G	0.00 0.00-0.01	0.00 0.00-0.02	0.00 0.00-0.006
double mutant	0.00 0.00-0.009	0.06 0.03-0.09	0.04 0.02-0.06

DISCUSSION

SP was used during the vaccine trial as an effective drug to clear parasitaemia in all individuals taking part in the study (Alonso et al., 1994). SP was the second-line drug for treatment of uncomplicated chloroquine-resistant *falciparum* malaria in Tanzania, and no resistance has been reported near the proposed area at the time of vaccine trial (Armstrong & Philips-Howard, 1992). During the vaccine trial, all the treatment given to children under five years coming to the health centre were recorded (Alonso et al., 1996), and these data show that SP use was very low at that time in Idete (Figure 1).

A high frequency of S108N mutation was found, which could be explained by the fact that pyrimethamine resistance was probably present at low frequency in the *P. falciparum* population before drug challenge. This has been suggested by the early observations of treatment failures within months of sustained prophylaxis with pyrimethamine in Tanzania (Clyde, 1954; Jones, 1954). This high frequency could also be due to the use of cotrimoxazole (CTXZ). In this area the use of CTXZ was higher than the use of SP at the health centre (Figure 1). The drug has been shown to be cross resistant with pyrimethamine *in vitro* (Iyer et al., 2001), and has already been suggested to account for a low background rate of *Pfdhfr* mutations in the population (Doumbo et al., 2000; Khalil et al., 2005; Marks et al., 2005). The high frequency of S108N could also be due to the uncontrolled use of SP in the population. But our data show that SP use was still very low at health centre to treat children under five years, who represent the most important part of the population at risk of getting clinical malaria and thus seek treatment.

The *Pfdhps* 437 mutation has been suggested to be important in SP resistance (Triglia et al., 1998; Marfurt et al., submitted). This mutation was not present at baseline, and reached 5% after SP treatment. Concerning the other *Pfdhps* mutations, we found *Pfdhps* 436 and *Pfdhps* 540, confirming earlier findings that *Pfdhps* 436 is most common where SP use and resistance is low (Plowe et al., 1997; Mawili-Mboumba et al., 2001).

Drug pressure is generally assumed to be responsible for the development and spread of antifolate-resistant malaria (Hastings &Watkins, 2005). These results show a low level of *Pfdhfr* double and triple mutant and no *Pfdhps* double mutant before the vaccine trial begins. There was a decrease in single mutation, and an increase in double and triple mutation and no change in frequency of wild type (Figure 3), meaning that parasites which had already a mutation and could tolerate longer sub-therapeutic levels of SP have been selected by the use of three doses of SP and acquired new mutations This is consistent with models which have shown SP resistance to evolve in a ordered stepwise manner (Hastings & Donnelly, 2005;

Hastings & Watkins, 2006). Several other studies have shown that *Pfdhfr* mutations are selected after a single episode of SP treatment (Wang et al., 1997; Kun et al., 1999; Doumbo et al., 2000; Tinto et al., 2007).

Spreading of *Pfdhfr* double and triple mutant has been shown to be important in SP resistance (Nair et al., 2003; Roper et al., 2003). These highly resistant parasites could also have been imported in Idete, but this is unlikely to be the case. A clinical trial conducted two years after the vaccine trial in 1996 in the same area found that SP treatment failure was two times higher in Idete (13.2%) compared to Ifakara (5%), a village situated 30km from Idete (Mshinda, 2000).

The higher treatment failure rate in Idete shows that the resistance observed in our study was not only limited to the treated people. Indeed, about 600 children were recruited for the study, this sub population acted as a reservoir for resistant parasites, which might have later spread in the population. The long elimination half-life of SP combined with the high transmission intensity in Idete have boosted the spread of resistant parasites, as a result of a large part of the population having sub-therapeutic levels of SP and thus selecting for resistant parasites. Moreover, the frequency of *Pfdhps 437, 540* and *Pfdhfr 59* increased at intermediate stage as a result of parasites selected just after the treatment. The frequency then decreased and stayed at a level which was still higher one year later than at the beginning of the vaccine trial, representing the impact on the whole population. These results confirm earlier finding, which showed *Pfdhfr* triple mutant to be suitable as molecular marker for detecting emerging SP resistance in areas where SP recently has been introduced (Alifrangis et al., 2003).

Despite its compromised efficacy, SP is still a component of combination therapies being considered or implemented in some countries (Marfurt et al., 2007; Hwang et al., 2007). It is also the only drug available for intermittent preventive treatment of pregnant women and infants (Greenwood et al., 2005). Some studies have suggested that the drug pressure exerted sporadically by IPT might lead to further substantial selection of drug resistant parasites (Marks et al., 2005; Tinto et al., 2007). It remains to be determined whether the benefits, in particular the significant reduction in the number of episodes of clinical malaria, of IPT with SP outweigh the possible increase in drug resistance. The potential impact of widespread cotrimoxazole prophylaxis on antifolate resistant malaria in areas where both malaria and HIV are prevalent is also an important issue.

These findings should be carefully assessed in light of the evidence of cross-resistance with CTXZ and rapid selection for *Pfdhfr* mutants under low drug pressure. New antimalarials

with different mechanisms of action to antifolates should be deployed in areas where either SP or cotrimoxazole are used for preventive treatment.

Based on these data, the selection of double and triple mutant in *Pfdhfr* and *Pfdhps* in Idete is probably due to the drug pressure caused by the 3 doses SP given to the 600 children during the SPf66 vaccine trial between March 1993 and July 1994, and that could also explain the highest SP treatment failure found in Idete compared to Ifakara two years later.

The high frequency of *Pfcrt* 76 and *Pfmdr* 86 mutations (data not shown) suggests also that chloroquine resistance was very high. A clinical failure rate of 33% was found in a study conducted 2 years later (Mshinda, 2000). This high failure rate of CQ suggests that SP was more and more used as second line treatment, thus increasing the SP pressure on parasites population.

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FIGURES

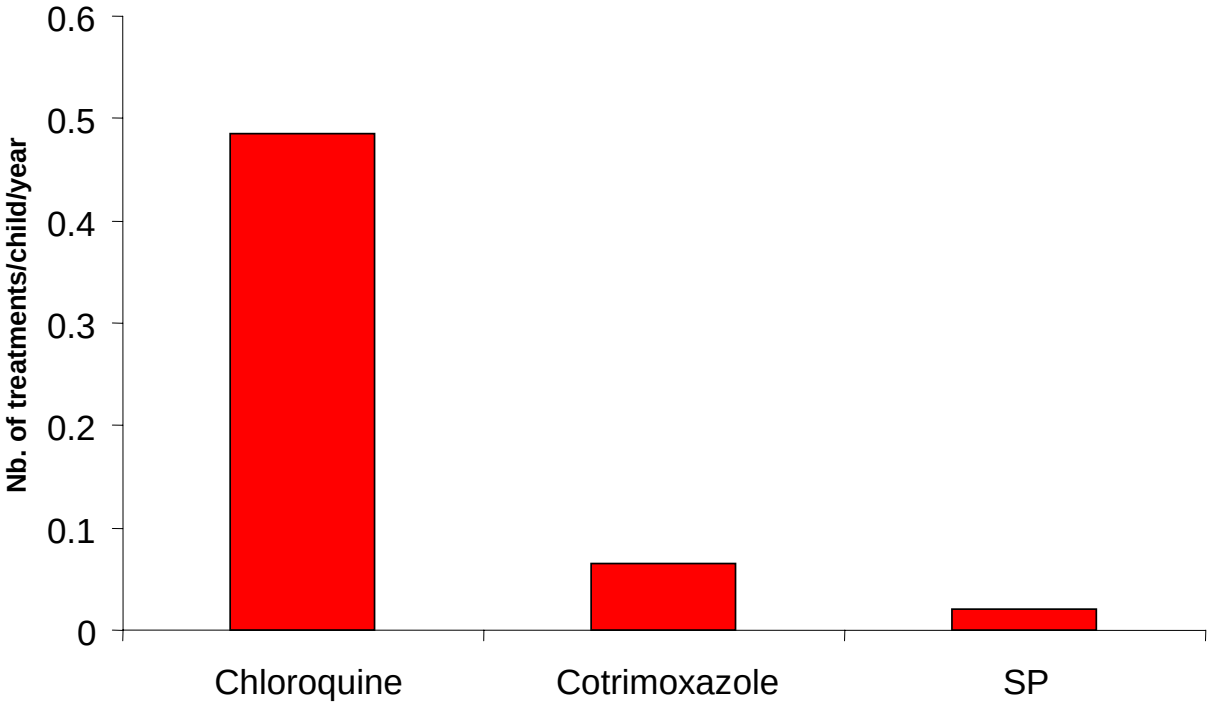

Figure 1: Drug use

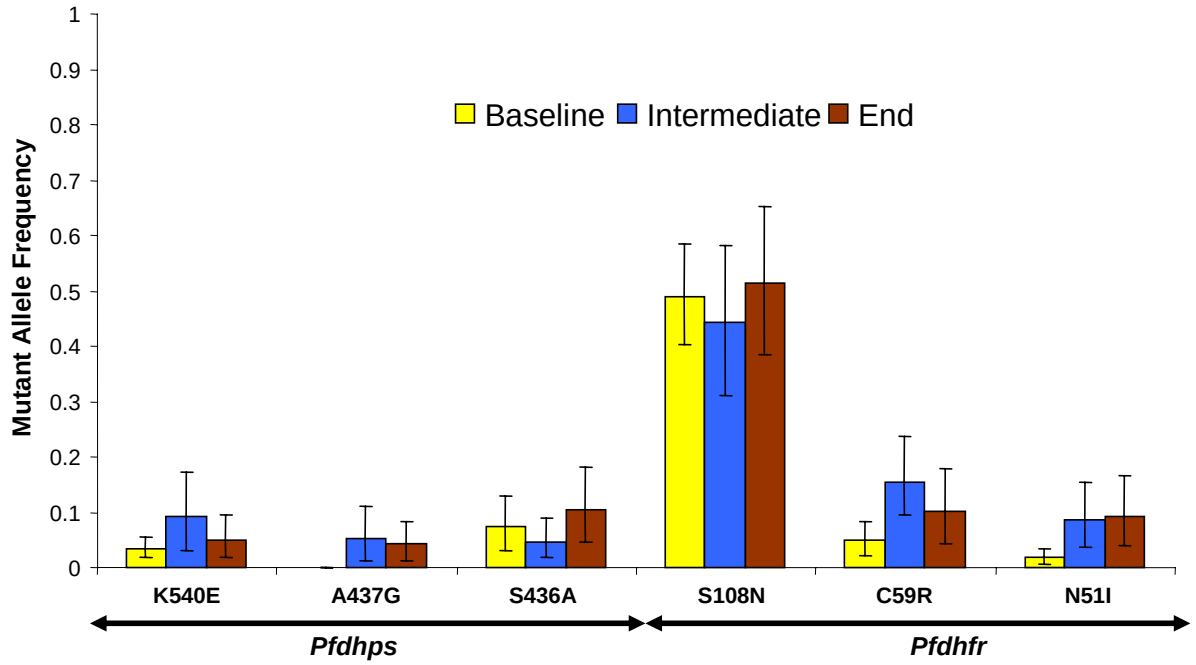

Fig.2: Mutation frequencies in *Pfdhfr* and *Pfdhps* genes

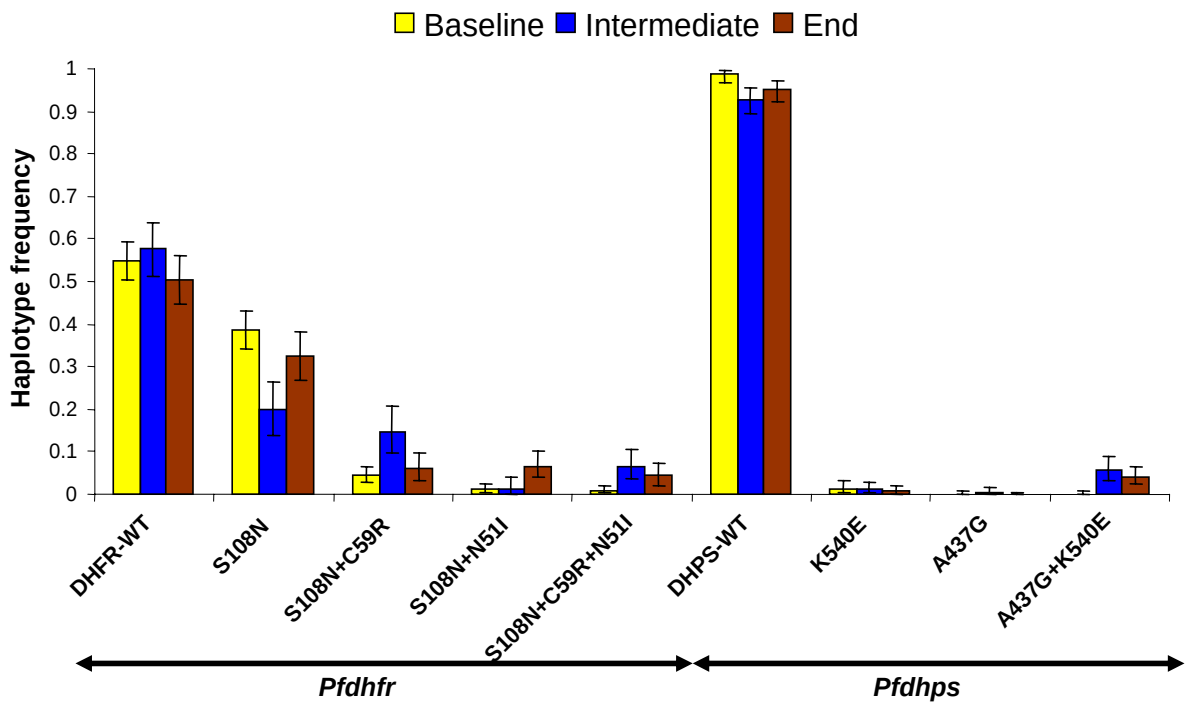

Fig. 3: Haplotype frequency in *Pfdhfr* and *Pfdhps* gene

Dynamics of amodiaquine, chloroquine and sulfadoxine-pyrimethamine clinical effectiveness in relation to drug pressure and frequency of parasite molecular markers of resistance

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Working manuscript

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ABSTRACT

BACKGROUND Antimalarials use has always been thought to be the key factor leading to *Plasmodium falciparum* resistance. The morbidity surveillance set up in Wosera [Papua New Guinea (PNG)] in 1990 offers a unique opportunity to investigate the dynamics of treatment effectiveness as a function of drug pressure and increasing frequency of molecular markers involved in chloroquine (CQ) and sulfadoxine-pyrimethamine (SP).

METHODS We conducted on a yearly basis a retrospective survey to assess the dynamics of treatment effectiveness between 1991 and 2002 using records on antimalarial use from Health centres. The covered period included two successive treatment policies, namely amodiaquine (AQ) or CQ from 1991 to 2000 and SP + AQ or CQ onwards. DNA-based microarray technique was used to analyze mutations in genes related to CQ and SP resistance.

RESULTS The treatment failure rate increased 3.5 times from 1996 to 2000 and then decreased dramatically onwards as a consequence of treatment policy change. The increasing *in vivo* resistance to 4-aminoquinolines was supported by an increasing frequency in *Pfcrt* and *Pfmdr* mutations during the 12 year-period of follow-up with a mean of 0.40 CQ and 0.55 AQ treatments per person per year in the population. The frequency of *Pfdhfr* 108 and 59 mutations increased also during the same period with a mean of 0.22 treatments per person/year. Significant correlation coefficients were found between the selection coefficients of *Pfdhf*r mutations and SP use, but not between *Pfcrt/Pfmdr* mutations and CQ/AQ use.

CONCLUSION The study provides evidence that drug pressure is a key element in the emergence of parasite resistance and indicates that the longitudinal analysis of the frequency of molecular markers of parasite resistance to several drugs could give a more comprehensive appraisal of the status and longitudinal dynamics of resistance.

INTRODUCTION

The effectiveness of the most widely used first-line antimalarials chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) has been heavily compromised by the emergence and spread of *P. falciparum* resistance to these drugs (Trape et al., 1998; Sibley et al., 2001).
In vivo studies remain the gold standard for monitoring antimalarial drug efficacy and are the primary source of information for establishing recommendations for malaria chemotherapy and prophylaxis. However, *in vivo* measures such as IC50s do not translate directly into likely clinical efficacy, and *in vivo* testing is only one of several sources of data currently available to guide policy makers (Hastings et al., 2007).

Resistance to CQ and SP has been associated with the accumulation of multiple mutations in *Pfcrt/Pfmdr* and *Pfdhfr/Pfdhps* genes respectively (Peterson et al., 1988; Triglia et al., 1997; Wang et al., 1997; Fidock et al., 2000; Reed et al., 2000), which once established, spread across vast continent-wide areas (Cortese et al., 2002; Wootton et al., 2002; Roper et al., 2004). This has led to the promotion of molecular monitoring of parasite resistance as a complementary tool to assess treatment effectiveness over the years and regions (Quaye and Sibley, 2002; Plowe, 2003; Plowe et al., 2007). However molecular markers are not considered by policymakers as sufficient criteria to decide on policy change (Hastings et al., 2007; Mbacham & Njikam, 2007). Other parameters, such as pharmacokinetics, pharmacogenetics, level of immunity, treatment compliance, dosing, etc., play a role in clearing symptoms and parasites (White, 2004; Hastings and Watkins, 2005).

Few studies have assessed the relationships between different measures of resistance over extended periods. Most such studies have not estimated drug pressure (Kublin et al., 2003; Plowe et al., 2004; Babiker et al., 2005). A 10-year study in Senegal quantified drug pressure, and found that clinical resistance to both SP and CQ, as well as the frequency of resistance markers, disseminated very rapidly in an area where antimalarial use was strictly controlled and used only for parasitologically diagnosed malaria (Noranate et al., 2007).

The conditions involved in the positive selection of resistant parasites, and the selective pressure contributing to their spread are largely unknown. Drug usage is thought to be one of the important factors leading to drug resistance, but its impact on resistance spread is still not well known.

This study, which is based on longitudinal morbidity surveillance, provides a unique data set to investigate the dynamics of treatment effectiveness as a function of drug pressure and

increasing frequency of molecular markers involved in CQ and SP in an area with high diversity and complex patterns of malaria epidemiology.

MATERIAL AND METHODS

Study area

The Wosera area [Eastern Sepik Province, Papua New Guinea (PNG)] is characterized by a tropical wet climate. Rainfall is the main climatic feature which shows some seasonality, with a wet season from October to April and a dry season from May to September. The area is highly endemic for malaria, with a mean parasite prevalence of 52% *P. falciparum* is the predominant specie, but *P. vivax* and *P. malariae* are also common (Genton et al., 1995; Mehlotra et al., 2002). Transmission is perennial, and the number of *P. falciparum* infective bites per year is around 50 (Benet et al., 2004).

Malaria is the commonest cause of outpatient presentation and accounts for 27% of all attendances at health facilities (Müller et al., 2003). Awareness of the cause of malaria and methods of treatment is high due to the relatively good access to the health services and to regular information sessions delivered in the area. No drug circulates in the community, as antimalarials are received at health centre on a daily basis and no commercial outlet is present in the area (Genton et al., 1995).

Patients and follow up

Demography and morbidity surveillance systems were set up in the Wosera in 1990 as a part of the Malaria Vaccine Epidemiology and Evaluation Project (Alpers et al., 1992; Genton et al., 1995). All patients seen by the health centre staff are recorded with details of age, sex, place of residence, identity (ID) number, presumptive diagnosis and treatment. These data are available in a digital form for the period of 1990-2002. In addition, when a patient presents with symptoms of presumptive malaria, he/she undergoes physical examination by an IMR surveillance nurse, blood collection for parasitological and haematological assessment (for details see Genton et al., 1994). Treatment procedures at the health centres follow the guidelines of the Papua New Guinea Department of Health, which recommend malaria treatment for all patients with fever (PNG, department of Health, 1993). From 1990 to 2000, the first-line treatment for uncomplicated malaria was amodiaquine (AQ) for children <20kg, chloroquine (CQ) for others; primaquine one dose was supposed to be given from 1997 with AQ or CQ in order to reduce the transmission intensity. Second-line was the combination of

quinine + sulfadoxine/pyrimethamine (SP). In 2001, the first-line treatment changed to the combination of AQ+SP or CQ+SP, depending on the weight. Individuals recruited in the morbidity surveillance can be linked to the demography surveillance system through their ID number, and thus followed for reattendance to the health facility within any given period of time.

Molecular analysis

About fifty samples were randomly selected from patients presenting at Kunjingini Health Subcentre each year, from 1991 to 2002. Only samples from patients infected with >1000 *P. falciparum* parasites /µl [>25 asexual parasites /200 white blood cells (WBCs)] were analyzed.

1) Molecular markers of drug resistance

The *Pfcrt*, *Pfmdr*, *Pfdhfr* and *Pfdhps* mutations (total of 28 SNPs) were analysed using a DNA microarray technique as described previously (Crameri et al., 2007).

2) Genotyping of MSP2 by Genescan

Determination of multiplicity of infection (MOI) was done using Genescan-based MSP2 genotyping as described previously (Falk et al., 2006).

Data analysis

Treatment effectiveness

Treatment failure rate was estimated for each year as the proportion of confirmed malaria cases that were administered an antimalarial treatment course and who reattended for a subsequent confirmed episode of malaria within a given period of time, i.e. 8-28 or 29-42 days, over the total number of confirmed malaria cases (as in Noranate et al. 2007). This was done using the ID number assigned to each individual for the follow up.

Drug use

For inhabitants of the immediate catchment of Kunjingini (corresponding to the area analyzed by Müller et al. 1998), the health centre represents the only source of primary care. Thus the drug use was estimated only for the inhabitants of the core villages (Kunjingini 1 and 2, Apusit, Nale and Mul). For the years 1991-93, drug pressure could thus be calculated by dividing the number of treatments assigned to people from these villages from the health

centre records by the total population determined in the demography database. From 1994 onwards the detailed health centre records were not available. No reliable data on drug usage were found for years 1994 to 1996. From 1997 onwards, treatment data were available for the presumptive cases that were investigated, and drug pressure was estimated by dividing the recorded treatments per capita by the proportion of possible days during which the research team was recording these data. Because of the inconsistency of the records, the way the drug use was assembled is not prompt to confidentially estimate separately the treatment failure rate for each drug.

Allelic frequencies

To estimate the allele frequencies of resistance markers in our sample set, we used a non linear statistical model that takes into account the effects of varying multiplicity of infection, and assumes that resistant and sensitive parasite clones are transmitted independently. The likelihood of a sample containing no resistant clones is $(1 - p)^n$, where p is the frequency for the mutant allele and n is the multiplicity of infection of the sample. Similarly, the likelihood for the sample to contain no wild-type allele is p^n and for a mixture of both, a wild-type and a resistant allele, is $1 – p^n – (1 - p)^n$. The likelihood over the whole data set for p is computed as the product of this likelihood over all samples, using values of n derived from *msp2* genotyping results (Schneider et al., 2002). We used a simple one-dimensional search routine to maximize this likelihood, and thus obtained estimates of p (Teukolsky et al., 2002). Allelic frequencies were calculated separately for each mutation for each year of the study.

Haplotype frequency

The haplotype frequencies were analyzed using a maximum likelihood method as previously described (Hastings & Smith, submitted).

Selection coefficient

The selection coefficient was calculated for each SNP as described by (Anderson and Roper, 2005), from the slope plotting Ln (R/S), with R and S being the frequency of resistant and sensitive allele, respectively, against the number of estimated generations. The selection coefficient for each SNP was the slope obtained by regression analysis for the 12-year period.

S, which indicates the relative survival of resistant and sensitive parasites at each generation, was calculated using an estimate of 6 generations per year for *P. falciparum*.

The investigations on relationships between selection coefficients and treatment effectiveness or drug use were done using regression analysis. The selection coefficients used here were yearly selection coefficients.

RESULTS

Treatment effectiveness

A total of 25021 patients were investigated by IMR team from 1991 to 2002 (Appendix A, figure 2).

Overall 148 of 6678 patients (2.2%) with a confirmed malaria diagnosis and treated with an antimalarial failed during the 8-28 days period and 122 of them (1.8%) during the 29-42 days period of follow up. There was no trend for treatment failure rate from day 8-28 between 1991 and 1995 (Figure 1). There was no record of treatment failure in 1995, probably because the follow-up that year was not done for those re-attending. It then progressively increased from 1996 till 1999; there was 3.8 more failure cases in 1999 compared to 1996 (Figure 1). From 1999, the treatment failure rate decreased dramatically, resulting in a 3.5 times decrease in 2002 (Figure 1). There was no trend for treatment failure rate from day 29-42. Nevertheless, the administration of a second treatment from day 29-42 was 2.5 times more likely in 1999 than in 1991, and 3.8 times less in 2002 (Figure 1).

Drug use

Overall we estimated that 18’365 amodiaquine treatments, 13’279 chloroquine treatments, 863 quinine treatments, 6’592 SP treatments and 11’360 primaquine treatments were delivered by the Kunjingini Health Centre between 1991 and 2002. The population of the core villages averaged 2’022 people and accounted for 39 % of the treatments, meaning that the effective population served by the health centre was 5185 people who each received an average of 0.81 treatments per annum during the follow-up. From 1991 to 1993, the three drugs prescribed and given to the patients in the Health centres were amodiaquine (AQ), chloroquine (CQ) and quinine (Q) (Figure 2). The treatment per capita decreased progressively for AQ and CQ from 1991 to 1999, resulting in a 2.25 and 1.93 fold decrease respectively for AQ and CQ between 1992 and 1999. It then stayed relatively stable (Figure

2). The treatment per capita was always higher for AQ than for CQ. The use of quinine was low from the beginning with 0.05 treatments per person/year, and decreased even to 0.01 treatments per person/year in 2002. Primaquine (PQ) started to be used in 1997 with 0.58 treatment per capita; it increased rapidly to 0.95 in 2000 and then decreased dramatically to almost 0 in 2002 (Figure 2). Sulfadoxine-pyrimethamine (SP) started to be used in 1997 with 0.01 treatments/person/year; the treatment per capita stayed at low levels till 1999, and then increased rapidly with policy change to reach 0.86 and 0.74 treatments per person per year in 2001 and 2002 respectively (Figure 2).

Multiplicity of infection (MOI)

The multiplicity of infection did not show a significant trend over the twelve years. The mean of MOI over this period was 1.4 ± 0.15 clones of *P. falciparum* per sample, with the lowest MOI in 1991 (1.21) and the highest in 2000 (1.78) (Appendix A, figure 5).

Allele frequencies

Mutation analyses were successfully accomplished in 98% (N=572) of the samples randomly selected each year. Polymorphisms were found in *Pfmdr1* codons N86Y, Y184F, and N1042D, *Pfcrt* codons C72S, K76T, H97Q, A220S, N326D, I356L and I356T, *Pfdhfr* codons C59R and S108N, and *Pfdhps* codon A437G (Figure 3). Most frequencies of the mutant alleles increased over the 12-year period, except for Y184F, N1042D, I356T, H97Q and A437G; these latter mutations were at very low frequencies and showed no trend. For CQ resistance related polymorphisms, there was already a high frequency in 1991. For most of them, the frequency was higher than 0.5 (Figure 3). I356L and C72S increased most over the 12-year period (1.67 and 1.63 times respectively) (Figure 3). The *Pfcrt* K76T and *Pfmdr1* N86Y mutation frequencies were already high in 1991 with 0.66 and 0.57 respectively, and increased 1.34 and 1.18 times respectively from 1991 to 2002 (Figure 3). A second mutation (the I356T form) was found at low frequencies and not in all years (0-0.16 in 1992) at codon CRT 356. Regarding SP resistance, only the two mutations C59R and S108N were found in *Pfdhfr*. The frequencies of these mutant alleles decreased between 1991 and 1995, from 0.47 to 0.24 for S108N, and 0.26 to 0.07 for C59R. From 1995 onwards, the frequency of mutant N108N and C59R increased 3 and 6.4 times respectively (Figure 3). For *Pfdhps*, only the A437G mutation was found in the period of 1992 to 2002, with a frequency of 0.02.

Haplotype frequencies

The frequency of *Pfdhfr* wild type did not vary significantly between 1991 and 1998, it then dramatically decreased till 2002 (Figure 4). There was no significant trend for *Pfdhfr* double mutant between 1991 and 1997, it then increased from 1998; the frequency was 2.5 times higher in 2001 (Figure 4).

Selection coefficient

The selection coefficients are significant for two *Pfcrt* codons, i.e. I356L [2.53% (1.31-3.75%] and C72S [2.60% (1.47-3.73%)], and two *Pfdhfr* codons, i.e. C59R [2.25% (0.09-4.41%)] and S108N [1.95% (0.34-3.57%)] (Table 1). The selection coefficient were also significant for two *Pfdhfr* haplotypes, i.e. *Pfdhfr* wild type [-1.76% (-3.27- -0.23%] and *Pfdhfr* double mutant [2.15% (0.03-4.27%)].

There were positive trends but no significant correlations between the failure rate on day 8-28 and the selection coefficients of *Pfcrt* and *Pfmdr* mutations (Table 2). The association was also positive, but not significant between selections coefficients of *Pfdhfr* mutations and treatment effectiveness (Table 2).

The same analysis was done between selection coefficients and drug use patterns. There were always negative correlations between selection coefficients of *Pfcrt* and *Pfmdr* mutations and quinine, amodiaquine and chloroquine use, but the correlations were not significant (Table 3), except for quinine use and *Pfcrt* 72 selection coefficient ($r = -0.68$, $p = 0.046$). However the correlations were positive and significant between selection coefficients of *Pfdhfr* polymorphisms and the treatment per capita for SP with $r = 0.92$, $p=0.001$ for *Pfdhfr* 108, and $r = 0.83$, $p=0.006$ for *Pfdhfr* 59.

DISCUSSION

In this study, the dynamics of clinical effectiveness of antimalarials were investigated in relation to drug pressure and molecular marker frequencies over a period of twelve years in Wosera, an area of high transmission intensity in Papua New Guinea.

The increasing treatment failure rate observed between 8-28 days from 1996 to 1999 is a consequence of the increasing resistance to the standard treatment with 4-aminoquinolines (CQ and AQ). The increasing levels of *in vivo* resistance to 4-aminoquinolines observed in PNG since the 1980s (Müller et al., 2003; Genton et al., 2005) are supported by the increasing

frequency of mutant alleles in CQ resistance (CQR) related *Pfcrt* and *Pfmdr* genes that were observed from 1991 to 2002. The treatment failure rate started to decrease in 2000, the year of the introduction of the new first line treatment policy. The addition of SP to either AQ or CQ has definitely improved the effectiveness of the standard treatment. However in Karimui (Simbu Province), treatment failure with the above combinations was at day 28 already 28% in 2003 (Marfurt et al., 2007). Molecular data confirmed that resistance to SP has continued to increase after policy change (Mita et al., 2006; Marfurt et al., submitted). Molecular data show a steady increase of *Pfdhfr* 108 and *Pfdhfr* 59 mutation frequency from 1999 onwards, which fits almost perfectly the increase of SP use that we estimated from the health facility records. The increase of SP use and related mutation frequencies one year prior to policy change is likely to be due to a higher recourse to second line treatment because of failure with AQ or CQ alone.

No significant correlation was found between the dynamics of molecular markers of CQ and AQ or SP, reflected by their selection coefficients, and treatment effectiveness, (although all correlation coefficients were positive). One reason for the lack of significance might be that most polymorphisms in *Pfcrt* and *Pfmdr* had reached fixed values during the 12-year period. Also, the addition of SP in the middle of the observation period (2000) has improved the effectiveness of the treatment, thus modifying the dynamics of the treatment failure rate, but did not stop the increase in *Pfcrt*, *Pfmdr, Pfdhfr* and *Pfdhps* mutations.

One can argue that our definition of treatment failure was not the one usually used in proper *in vivo* studies. It is believed nevertheless that our method is reliable to identify repeated episodes of malaria that are likely to be due to recrudescence of parasites, even if new infections may also play a role. The increasing trend of treatment failure rate up to 2000 and the sharp decrease in the following years after the introduction of the new first line treatment validates the approach. The same methodology was used in the only other longitudinal assessment of clinical resistance (Noranate et al., 2007).

Drug use has been thought to be one of the key factor leading to parasite resistance (D'Alessandro and Buttiens, 2001), but its impact on resistance spread is still not well known. The impact of the drug pressure, defined as the treatment per capita, was evaluated on the selection coefficients of *Pfcrt*, *Pfmdr*, *Pfdhfr* and *Pfdhps* mutations. Surprisingly, negative correlations were found between pressure of AQ, CQ and Q pressure and the selection coefficients of some *Pfcrt* and *Pfmdr* mutations. AQ and CQ pressure decreased almost all over the studied period and yet the mutation frequencies of *Pfcrt* and *Pfmdr* were increasing. It has already been suggested that parasite resistance will undoubtedly increase to the point of

drug treatment failure, as long as there is any significant drug use (Mackinnon and Hastings, 1998). It is likely that the drug has to be almost completely removed from the population to stop or reverse the drug resistance (Kublin et al., 2003; Laufer and Plowe, 2004).

That drug pressure had a positive and significant correlation with selection coefficients of two *Pfdhfr* mutations was also observed (Table 3). Although SP use was not so common, it is possible that cotrimoxazole given to treat other causes of fever, might have played a role. It is also possible that SP was not systematically recorded in the health facility registry when it was given with quinine as second line. Lastly the pressure exerted by antifolates used during mass drug administration with pyrimethamine in the 60s and 70s may have contributed to the emergence and development of *Pfdhfr* mutations (Marfurt et al., submitted).

Drug use in population is difficult to quantify, particularly in malaria endemic areas, where most of the time public health services do not have adequate infrastructure to get that kind of data. In Wosera, most of people have access to drugs through the health centres only (Genton et al., 1995), thus making it possible to make confident estimation of drug use in the population, even if it was not possible to get reliable data for some years.

The selection coefficient indicates the proportional difference in survival of parasites bearing different alleles at each generation (Anderson and Roper, 2005). In this study, AQ and CQ pressure were quite high with a mean of 0.55 AQ and 0.40 CQ treatments per year per person. Taking into account that 56% of patients were treated without having *P. falciparum* (data not shown) and the long elimination half life of the drugs, a great proportion of the population had sub-therapeutic levels of the drug at any time, thus being able to select resistant parasites. The lack of significance between *Pfcrt/Pfmdr* selection coefficients and AQ/CQ pressure is easily explainable by the fact that mutation frequency had reached fixed levels during the period of follow-up.

It has been proposed that the first phase in which the parasite acquire mutations and get more and more tolerant is longer that the second phase of acquiring resistance, which is very fast (Watkins et al., 2005). This is shown here by positive and significant correlation between SP pressure and *Pfdhfr* selections coefficients; even if the drug pressure was lower for SP with a mean of 0.22 treatments per person per year. These results indicate that drug pressure is a key element in the emergence of resistance; once mutations are well established in the population, even a strict use of drugs can not stop the resistance increase (Noranate et al., 2007).

Longitudinal studies of treatment effectiveness provide the best information on which to base treatment policy changes. Simpler standard *in vivo* efficacy studies are better suited for ongoing efficacy monitoring. These surveys are expensive to perform making their sample

sizes relatively small. Conversely, the longitudinal analysis of the frequency of molecular markers of parasite resistance to several drugs is relatively simple. Sample sizes can be much larger and the analysis can be conducted on parasites collected in malaria patients but also on those collected from community surveys. Consequently they give a more comprehensive appraisal of the status and longitudinal dynamics of resistance (Talisuna et al., 2003; Hastings et al., 2007; Marfurt et al., submitted; Mugittu et al., submitted).

Predicting future levels of resistance is necessary for planning and instituting strategies to delay its emergence and spread (Hastings and D'Alessandro, 2000). Amodiaquine in combination with SP has been proven to be efficacious in areas where both drugs have been used previously as monotherapy (Dorsey et al., 2002; Rwagacondo et al., 2003), but this combination is already failing in PNG (Mita et al., 2006; Marfurt et al., 2007). This shows the utility of longitudinal studies using molecular markers. Indeed, our data show that resistance to SP was increasing in PNG before the large scale use of the drug, predicting a short useful therapeutic life (UTL) of the combination. Several theoretical models have been proposed to explain the dynamics of emergence and spread of drug resistance (Hastings, 1997; White, 1999; Hastings et al., 2002; Watkins et al., 2005; Hastings and Watkins, 2006), but these models need to be integrated with longitudinal field data to enable qualitative inferences to be made. More longitudinal studies are needed to asses the exact relationship between treatment effectiveness, molecular markers and drug use in different settings with different malaria epidemiology patterns. They should try to have more accurate estimate of drug use in the population, as this measure is the most difficult to quantify.

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TABLES AND FIGURES

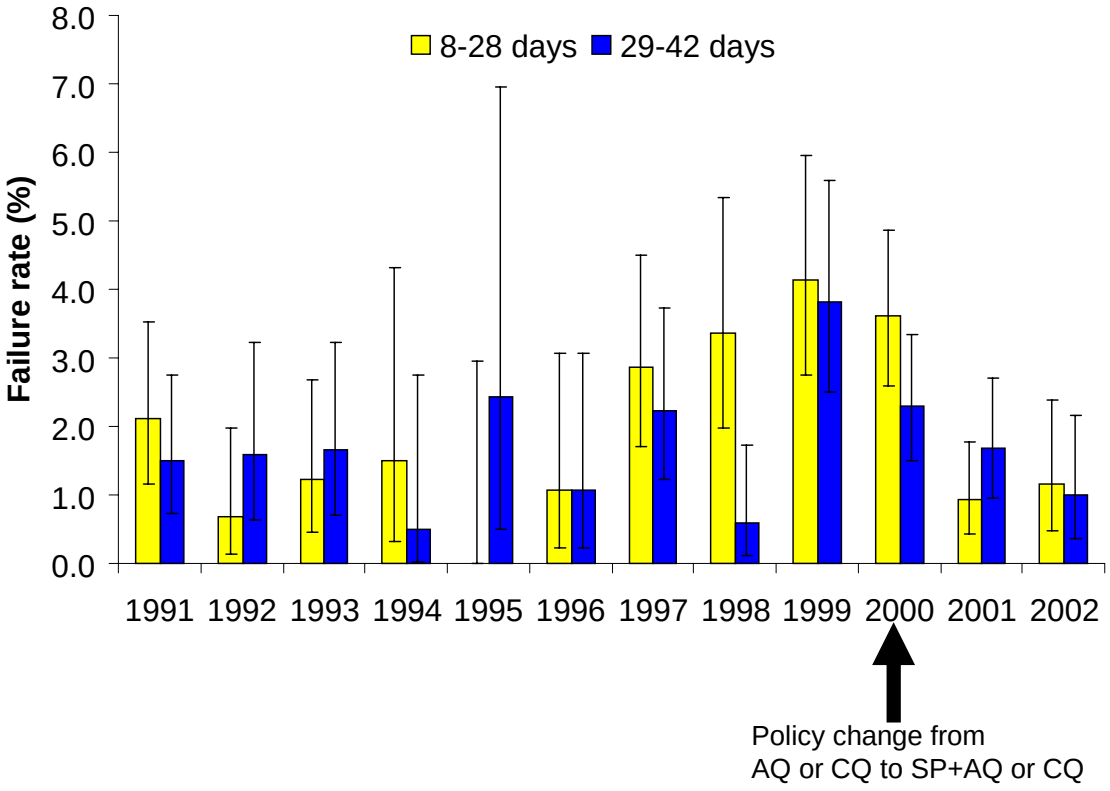

Figure 1: Treatment failure rate

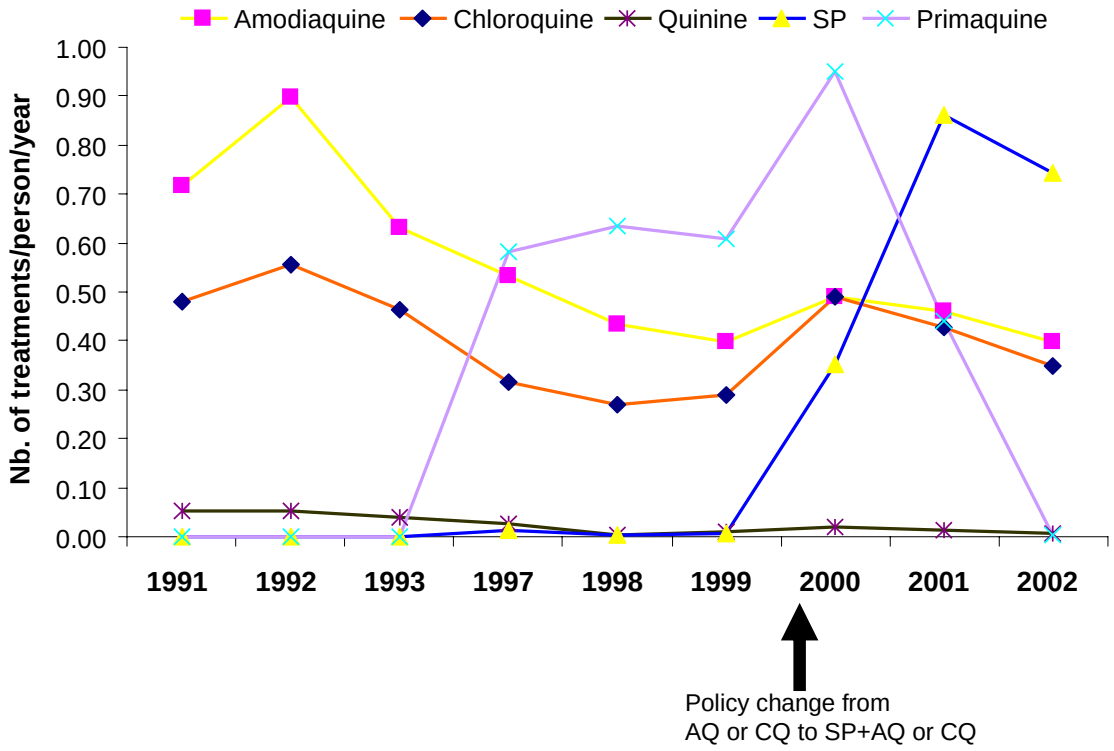

Figure 2: Drug use in the Wosera district

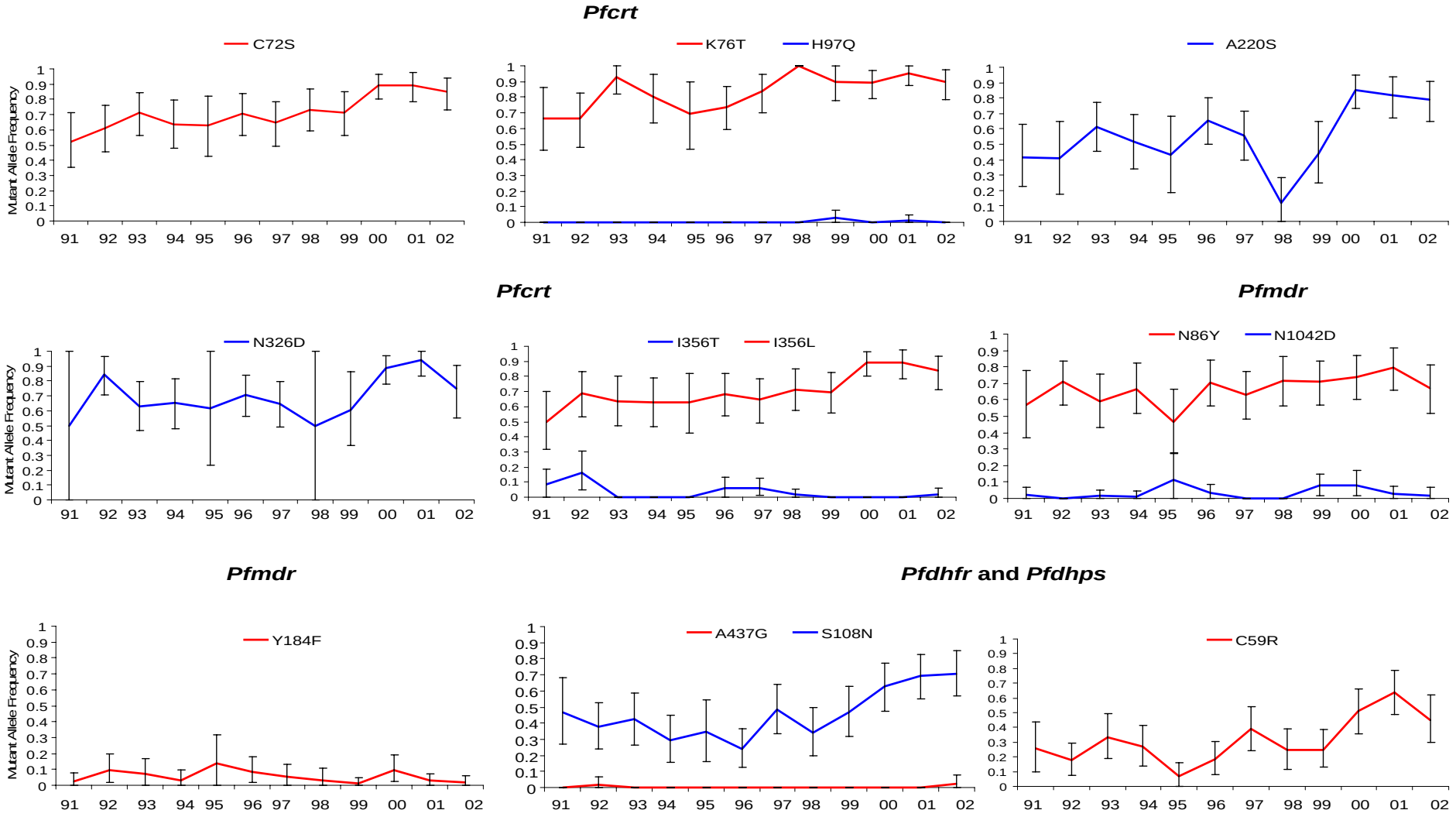

Figure 3: Allelic frequencies of *Pfcrt, Pfmdr, Pfdhfr* and *Pfdhps* mutations by year

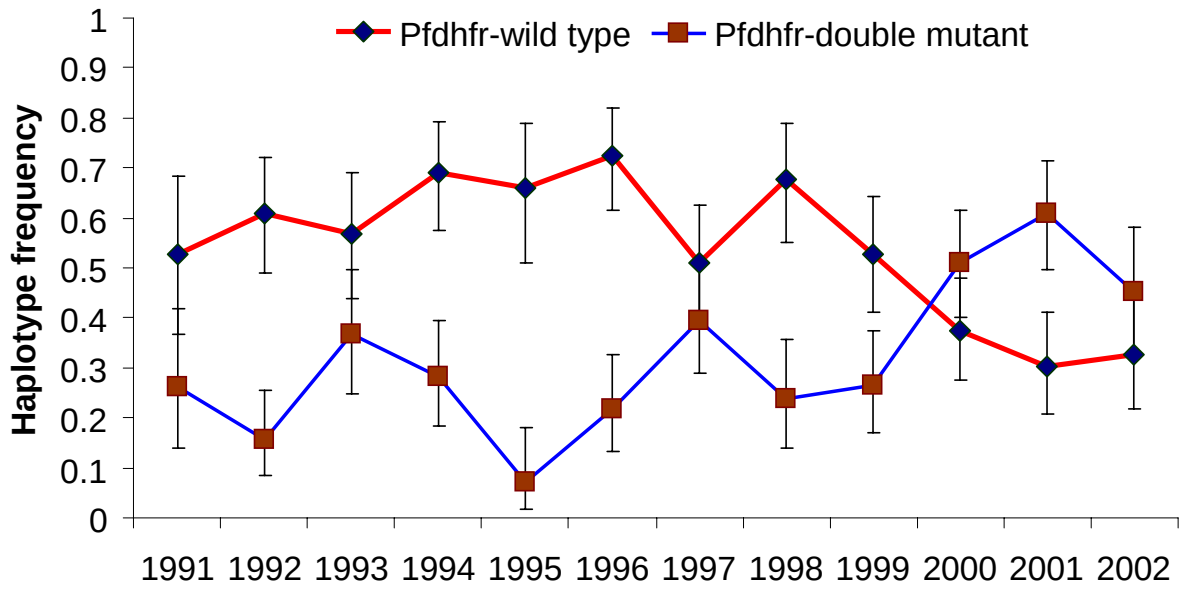

Figure 4: Haplotype frequency of *Pfdhfr* wild type and double mutants

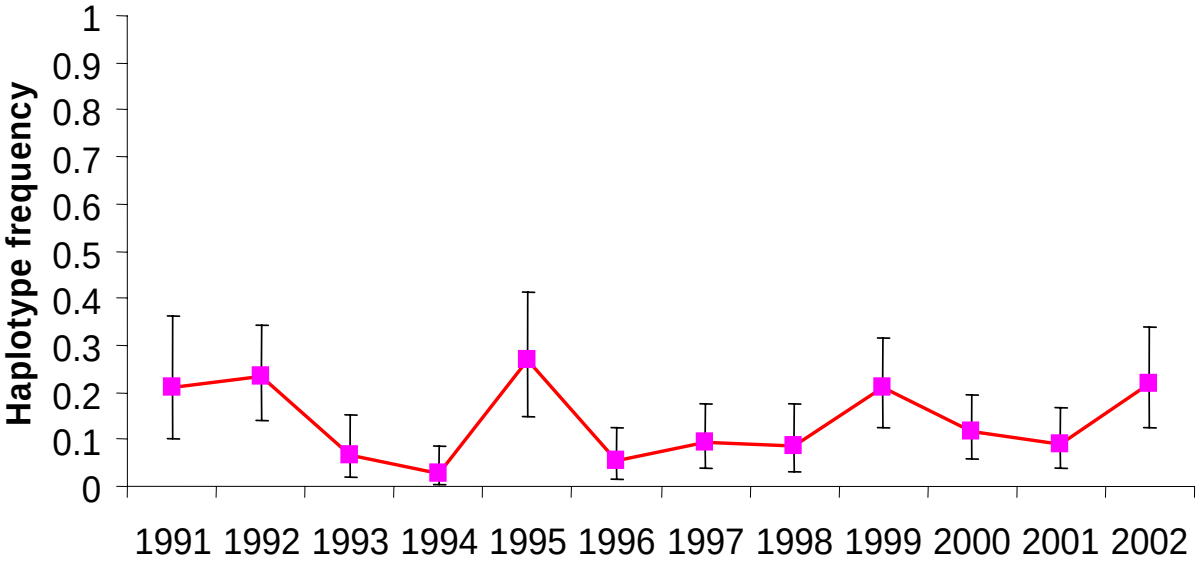

Figure 5: Haplotype frequency of *Pfdhfr* single mutant

Table 1: Selection coefficients*

Genes	SNPs	Selection coefficient (S)	95% Conf. Interval
Pfcrt	**I356L**	**2.53%**	**1.31% - 3.75%**
Pfcrt	N326D	1.70%	-073% - 4.13%
Pfcrt	I356T	-4.94%	-19.26% - 09.38%
Pfcrt	A220S	2.15%	-0.82% - 5.12%
Pfcrt	H97Q	6.13%	-2.54% - 14.80%
Pfcrt	K76T	4.08%	-4.90% - 13.05%
Pfcrt	**C72S**	**2.60%**	**1.47% - 3.73%**
Pfmdr	N1042D	3.27%	-8.76% - 15.31%
Pfmdr	Y184F	-1.27%	-3.73% - 1.20%
Pfmdr	N86Y	1.03%	-0.04% - 2.10%
Pfdhps	A437G	0.95%	-8.77% - 10.67%
Pfdhfr	**S108N**	**1.95%**	**0.34% - 3.57%**
Pfdhfr	**C59R**	**2.25%**	**0.09% - 4.41%**
Genes	**Haplotype**		
Pfdhfr	**Wild type**	**-1.76%**	**-3.27% - -0.23%**
Pfdhfr	Single mutant	0.11%	-2.45% - 2.69%
Pfdhfr	**Double mutant**	**2.15%**	**0.03% - 4.27%**

*assuming 6 generations of *P. falciparum* per year

Table 2: Correlations between *pfcrt*, *pfmdr* and *Pfdhfr* mutations selection coefficients and drug use

		correlation coefficient (r)	P value (regression analysis)
Selection coefficient			
Genes	**SNPs**		
		Quinine use	
Pfcrt	**I356L**	-0.61	0.081
Pfcrt	**K76T**	-0.55	0.318
Pfcrt	**C72S**	**-0.68**	**0.046***
Pfmdr	**N86Y**	-0.58	0.100
		Amodiaquine use	
Pfcrt	**I356L**	-0.50	0.169
Pfcrt	**K76T**	-0.43	0.252
Pfcrt	**C72S**	-0.62	0.075
Pfmdr	**N86Y**	-0.37	0.252
		Chloroquine use	
Pfcrt	**I356L**	0.04	0.920
Pfcrt	**K76T**	-0.56	0.113
Pfcrt	**C72S**	-0.05	0.900
Pfmdr	**N86Y**	-0.06	0.878
		Primaquine use	
Pfcrt	**I356L**	0.45	0.219
Pfcrt	**K76T**	0.36	0.348
Pfcrt	**C72S**	0.45	0.228
Pfmdr	**N86Y**	0.53	0.139
		Sulfadoxine-pyrimethamine (SP) use	
Pfdhfr	**S108N**	**0.92**	**0.001***
Pfdhfr	**C59R**	**0.83**	**0.006***
Genes	**haplotype**		
		Sulfadoxine-pyrimethamine (SP) use	
Pfdhfr	**Wild type**	**-0.90**	**0.001***
Pfdhfr	**single mutant**	0.02	0.963
Pfdhfr	**double mutant**	**0.79**	**0.012***

* Statistically significant at 95% level

General Discussions and Conclusion

General Discussions and conclusions

In this study, the dynamics of drug resistance associated molecular markers of malaria parasite were investigated in two areas of different transmission intensity with two different drug pressures. In Idete, Tanzania, the goal was to evaluate the impact of a low drug pressure limited in time on the development of resistance, when Sulfadoxine-pyrimethamine (SP) was introduced in an area of very high transmission intensity. The dynamics of treatment effectiveness as well as the dynamics of molecular markers under a sustained drug pressure in Wosera, Papua New Guinea (PNG), an area of moderate transmission intensity were also investigated longitudinally.

The difference in transmission intensity between these study sites was reflected in our data in the average number of different *Plasmodium falciparum* strains, called the multiplicity of infections (MOI). The average MOI was 4.08 in Idete and 1.39 in Wosera.

Chloroquine (CQ), amodiaquine (AQ) and SP use was estimated in Wosera from health centres data, and for Idete, CQ/SP treatments were estimated from health centre data during the SPf66 vaccine trial (Alonso et al., 1996). Only treatments given to children under five years were recorded during the vaccine trial (Alonso et al., 1996), but we can assume that they represent the majority of malaria cases, thus representing the drug use in this age group. In both places presumptive treatment is common, indeed, it is the official politic in Wosera to treat all malaria presumptive cases (Genton et al., 1995). In Tanzania most people have access to antimalarials through the private market (Kindermans et al., 2007), thus the parasite populations in both places are under high drug pressure.

V.1. Drug resistance DNA-microarray

The microarray chip is an extraordinary tool to analyze point mutations that are linked to drug resistance on a large scale in epidemiological studies. This semi-high throughput method has been shown to be cheaper than other methods that are currently used to analyse single nucleotide polymorphisms (SNPs) involved in malaria drug resistance (Crameri et al., 2007). The advantage of the DNA-microarray is not only the cost and the analysis of all known SNPs simultaneously, but it is also easy to add new mutations when they are found. This method has also been shown to detect minority mutations in field samples coming from high transmission areas, which will be an important factor in the surveillance of molecular markers prior to the reintroduction of a drug (Juliano et al., 2007). Although this technique has been developed for malaria drug resistance, other applications have already been considered. A

HIV-chip is currently developed and the first results are encouraging. Another system is also developed to analyse point mutations in human genes coding for enzymes that are linked to drug metabolism, which could add valuable information when drug policy has to be changed, because treatment effectiveness does not only depend on the drug efficacy and resistance, but also on other factors as immunity, compliance, pharmacokinetics, pharmacogenetics, etc…
However, the currently used technique to identify SNPs could be improved e.g. by using multiplex-PCR to amplify the different genes. Currently twenty PCR reactions (primary and nested) are needed to amplify target sequences of 5 genes, and this is time and material consuming. The development of multiplex PCR would significantly reduce cost and hand-on times, and thus could be easily used in dedicated laboratories in endemic areas

V.2. Usefulness of molecular markers

Molecular markers for drug resistant malaria are based on genetic changes that confer parasite resistance to drugs used to treat and prevent malaria. These molecular markers have been used as tools for surveillance of resistance (Djimde et al., 2001a; Djimde et al., 2001b; Kublin et al., 2002), and their potential value to policy makers has been demonstrated by their use to help control a malaria epidemic (Djimde et al., 2004), to guide national malaria treatment policies (Mugittu et al., 2004) and to monitor changes in parasite drug susceptibility following changes in malaria drug treatment policy (Kublin et al., 2003). However, their application as tools for surveillance has been challenged?, because their relation with *in vivo* outcomes is not so straightforward (Plowe, 2003), and they are not considered by policy makers as sufficient criteria to decide change in treatment policy (Hastings, 2007; Mbacham and Njikam, 2007). Validated genetic determinants of resistance would vastly improve surveillance and, thus, the effectiveness of resources for the treatment and control of malaria. This information can provide an efficient measure of changes in drug resistance long before they are reflected in clinical drug failure. Thus molecular markers represent a public health tool of great potential value ((Plowe et al., 2007).
In our study, we found that the decrease in AQ/CQ effectiveness was paralleling an increase mutation frequency in *pfcrt* and *pfmdr*, making molecular markers valuable tools to assess the effectiveness of antimalarials on longitudinal studies. These molecular markers were also useful to detect emergence of SP resistance in Idete under a defined and very low drug pressure. Both studies showed also that SPR was increasing in the absence (Wosera) or very low drug pressure (Idete), suggesting that other factors such as cross resistance with other antifolate drugs such as cotrimoxazole could account for the development and spread of SPR.

The addition of SP to AQ or CQ was thought to delay the development of resistance to the new combination treatment in PNG, but it was not known that SP resistance was developing that fast, even without low drug pressure. On the other hand, a long history of AQ/CQ use in PNG has severely compromised their efficacy (Marfurt et al., 2007). This shows the need of a system of surveillance using longitudinal treatment effectiveness data, and molecular markers to help in changing treatment policy.

V.3. Development of SP resistance in the absence of high drug pressure

There are two properties of SP that may have contributed to the remarkable fast spread of resistance: the apparent stimulation of gametocytogenesis associated with poor therapeutic responses to SP and the widespread exposure of *P. falciparum* parasites to sub-therapeutic SP concentrations either during its long elimination phase from the body or following sub-therapeutic dosing (Terlouw et al., 2003; Watkins and Mosobo, 1993). Moreover, the ability of malarial parasites to salvage intact folate or its metabolite to supplement *de novo* synthesis may contribute to the observed *in vivo* parasite resistance to antifolate drugs, particularly sulfa drugs (Kinyanjui et al., 1999; Wang et al., 2004). Recently, a high blood folate concentration was associated with an increased risk of treatment failure, even between patients with the same age, blood drug concentrations, parasite density and parasite *Pfdhfr/Pfdhps* genotype (Dzinjalamala et al., 2005).

We can conclude from both studies in Wosera and in Idete that SP resistance (SPR) develops very fast, even under low drug pressure. The use of three doses of SP during a vaccine trial in Idete has selected for double and triple mutants in *Pfdhfr* and double mutant in *Pfdhps*, and these haplotypes have been associated with resistance to SP (Mugittu et al., 2004; Nzila et al., 2000). In Wosera, there was an increasing frequency of *Pfdhfr* double mutant from 1991 to 1999 in the absence of a high drug pressure. The presence of this double mutant is consistent with other studies which have found *Pfdhfr* double mutant to be more common than triple mutant in Melanesia (Mita et al., 2007), and this double mutant when combined with *Pfdhps* 437, has also been associated with treatment failure in Papua New Guinea (Marfurt et al., submitted).

It is interesting to observe that the low usage of SP in Wosera, and more surprisingly the use of 3 doses of SP in Idete have led to increased resistance. This question is of high significance in the light that many people are calling for intermittent preventive treatment (IPT) to be used more widely, and there is even discussion of expanding preventive treatment beyond pregnant women and infants, but also to children (Greenwood, 2007).

Intermittent presumptive treatment in pregnancy (IPTp) has been shown to reduce the incidence of severe maternal anaemia and to improve birth-weight (Vallely et al., 2007). Its use in areas of medium to high transmission is recommended by World Health Organization (WHO, 2007), even if its efficacy is reduced in HIV-positive women (Van Eijk et al., 2004). Intermittent presumptive treatment in infants (IPTi) lowered mortality, morbidity and severe anaemia from malaria substantially in the first year of life (Schellenberg et al., 2001), and the protection was sustained during the following year in the absence of any further drug administration (Massaga et al., 2003; Schellenberg et al., 2005). However, this latter effect has not been seen in other studies (Chandramohan et al., 2005; Macete et al., 2006). On the other hand, IPTi is difficult to sustain over long periods, could select for drug resistance, and could impede the development of natural immunity (Greenwood, 2006).

Actually, the recommended regimen for IPTp is at least 2 doses of SP given in the second and third trimesters as part of the routine scheduled antenatal clinic visits (WHO, 2007). But it has been suggested that HIV-infected women may require at least 3 courses to achieve the same benefits seen in HIV-negative women with 2 courses (Parise et al., 1998, Filler et al., 2006; Hamer et al., 2007). There is no official regimen for IPTi, but generally 3 doses of SP are given at the time of routine vaccinations delivered through WHO's Expanded Program on Immunisations (EPI) (Schellenberg et al., 2001; Macete et al., 2006). However other drugs are currently tested through the IPTi consortium (IPTi consortium, 2008), and IPTp clinical trials are currently underway in several places in Africa to evaluate other drugs such as mefloquine, SP plus artesunate or amodiaquine (Vallely et al., 2007).

Widespread implementation of IPT will undoubtedly increase drug pressure and favours drug resistant parasites, but the effect will depend probably on the background pattern of resistance and the overall pattern of drug usage in the population. The fact that widespread use of IPT will encourage the spread of drug resistant parasites is still a matter of controversy (Greenwood, 2006). Using molecular markers, some studies have suggested that the use of IPTi increased the prevalence of resistant parasites (Marks et al., 2005; Cisse et al., 2006), but others did not observe this (Mayor et al., 2005; Sokhna et al., 2008). In this study it was shown that in Idete the use of three doses of SP in children has selected for resistant parasites. The increasing frequency of *Pfdhfr* mutations in Wosera could also be due to the very low usage of SP in combination with quinine as second line treatment. In both places SP resistance was low, thus making it possible to see the impact of low usage of SP on the development of resistance using molecular markers.

The reason why some studies did not find an effect of IPT on drug resistant parasites, could be due to the fact, that in most places SP resistance was already high and its use more common in the community, thus being difficult to measure the exact effect of a low and defined pressure on the development of resistance. The study conducted by Marks et al. in Ghana was also in an area where SP resistance and SP use were very low (Marks et al., 2005), confirming that the results in other studies were confounded by the high level of SP resistance in the population.

It has been postulated that IPTi would have only a marginal effect on resistance as intermittent treatment would constitute only a small proportion of a country's total malarial treatment doses (Schellenberg et al., 2001). It has also been suggested that by reducing the incidence of clinical malaria fewer treatment doses would need to be given. However, resistance is more likely to develop in infants whose immune system is immature. Moreover, in areas of high transmission, infants make up a high proportion of the infected population at any time. They also carry *Plasmodium* blood stage densities that are typically orders of magnitude higher than adolescents or adults (Bloland et al., 1999), and are therefore an important reservoir for infection, including infection with resistant parasites as it has been shown in our study in Idete.

For these reasons, IPTi is more likely to promote the development of SP resistance than IPTp in areas with low SP resistance. Indeed, as it was shown in Idete, children under 5 years harbour a large part of the parasite population (Akim et al., 2000; Drakeley et al., 2000) which is exposed to sub-therapeutic levels of the drug, thus making it possible for partially resistant (or tolerant) parasites to establish and to spread (Hastings & Watkins, 2006). In addition, in many endemic areas, most of people take antimalarials presumptively, making an even higher proportion of the population parasite being exposed to drug sub-therapeutic levels. This argues against the introduction of new drugs in IPT programs, unless SP efficacy has been shown to decline. This will lead to the fast development of resistance to the new drug. The solution would probably be to use other antimalarials which have different mode of action than SP in areas where this latter is used for IPT, thus the SP resistant parasites escaping from IPT would be cleared by the other antimalarial which would be used in the population to treat clinical malaria. This will avoid the spread of SP resistant parasites in the population, allowing a longer use of SP in IPT.

V.4. Cross-resistance between cotrimoxazole and sulfadoxine-pyrimethamine

Cotrimoxazole is an antibacterial antifolate, which is a combination of trimethoprim and sulfamethoxazole. Cotrimoxazole has been shown also to be active against malaria in humans (Bloland et al., 1991; Hutchinson et al., 1982). The components of cotrimoxazole target selectively dihydrofolate reductase (DHFR) and dihydropteorate synthetase (DHPS), the same folate pathway enzymes targeted by sulfadoxine-pyrimethamine (Sibley et al., 2001). Both the single and double mutant mutants (108N and 108N +51I or 108N +59R) confer trimethoprim resistance on *P. falciparum Pfdhfr in vitro* (Sibley et al., 2001).

This drug has been used for decades in Africa and in Papua New Guinea for treatment of lower respiratory infections (Jelinek et al., 1999; Marfurt et al., submitted). The use of cotrimoxazole (CTXZ) could also explain the high prevalence of *Pfdhfr* 108N in Idete. From our study, it is likely that the use of CTXZ for bacterial infections treatment may have already selected for *Pfdhfr* 108 mutant, and the data from the health centre show that CTXZ was more used than SP in the treatment of children under five years. The increasing frequency of *Pfdhfr* 108N and 59R in Wosera in the absence of high SP pressure could also be explained by the use of cotrimoxazole together with the low SP use.

The potential impact of widespread CTXZ prophylaxis on antifolate resistant malaria in areas where both malaria and HIV are prevalent is still controversial (Laufer and Plowe, 2006). Despite the cross resistance observed *in vitro* between pyrimethamine and trimethoprim (Iyer et al., 2001) and between sulfamethoxazole and sulfadoxine (Triglia et al., 1997), the intermittent use of cotrimoxazole has not yet been associated with selection of *Pfdhfr* and *Pfdhps* mutants *in vivo* (Thera et al., 2005; Malamba et al., 2006). But all these studies were conducted over a short period, generally 3 months.

From the data in Wosera, it can be seen that SP resistance was increasing over many years without reported drug pressure, but with a continued use of cotrimoxazole (see appendix A, figure 6). Moreover, our data were population-based; they were not restricted on a small group as it was the case in the other studies. Combined with very low SP use, the use of cotrimoxazole has probably contributed to the increase of SP resistance in Wosera as it has been described previously (Doumbo et al., 2000; Marks et al., 2005).

Until recently, CTXZ was only used to treat bacterial infections, but it has now been recommended by WHO for prophylaxis of opportunistic infections for people living with HIV/AIDS in a variety of African settings (UNAIDS/WHO, 2000). Prolonged daily prophylaxis with cotrimoxazole would be more conducive to selection for resistant parasites. People taking cotrimoxazole prophylaxis while concurrently infected with HIV and *P.*

falciparum may not respond to SP, incurring an increased risk of severe disease and death. The widespread use for prophylaxis in HIV-infected individuals could promote SP resistance in areas where this latter is used for intermittent preventive treatment.

V.5. Conclusion

The conclusion which can be drawn from the two studies is that SP resistance (SPR) can develop very fast even under very low drug pressure. The development of SPR seems also to be induced by cross resistance to cotrimoxazole (CTXZ). This has implications for ongoing prevention programs such as intermittent preventive treatment (IPT), as SP is the only drug available for IPT and the use of CTXZ in the treatment of bacterial infections in HIV-positive people in places where both diseases are highly prevalent. However these factors are likely to favour SP resistance in areas where SP resistance and use are low.

References: Introduction & General Discussions

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Appendix

Additional data from Wosera

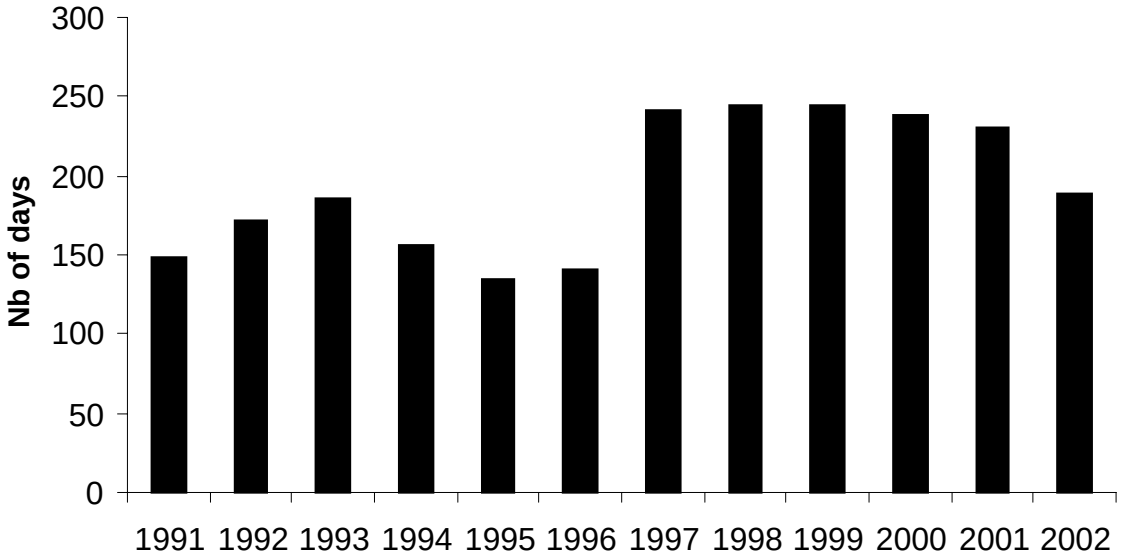

Figure 1: Number of days of PNG Institute of Medical Research (PNGIMR) surveillance. The graph represents the number of days per year on which patients from Kunjingini Health Centre were investigated by IMR team.

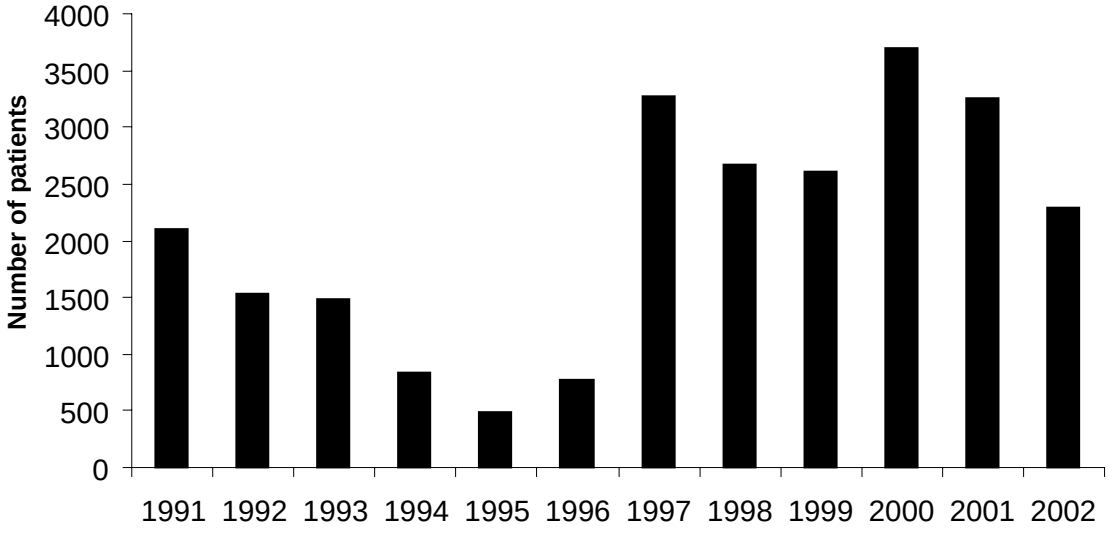

Figure 2: Number of patients investigated by IMR team. Estimated using the ID numbers assigned to each individual in the demographic surveillance and the records from IMR surveillance.

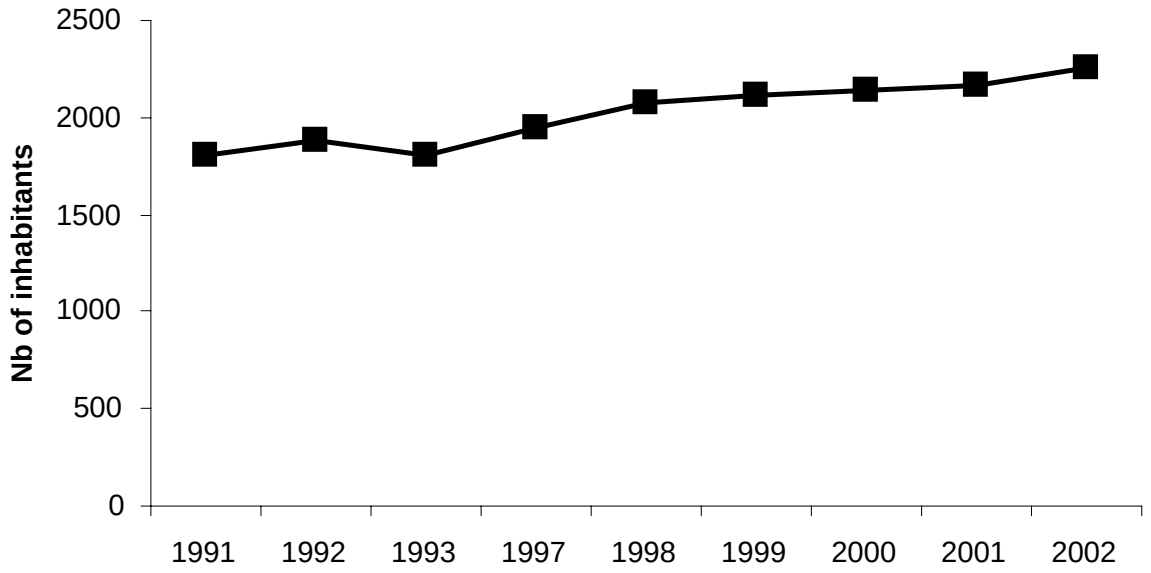

Figure 3: Population from the core villages (Apusit, Mul, Nale, Kunjingini 1, Kunjingini 2). Data obtained from the demographic surveillance. These villages are the closest to Kunjingini Health Centre, and their populations have access to healthcare through the Kunjingini Health Centre.

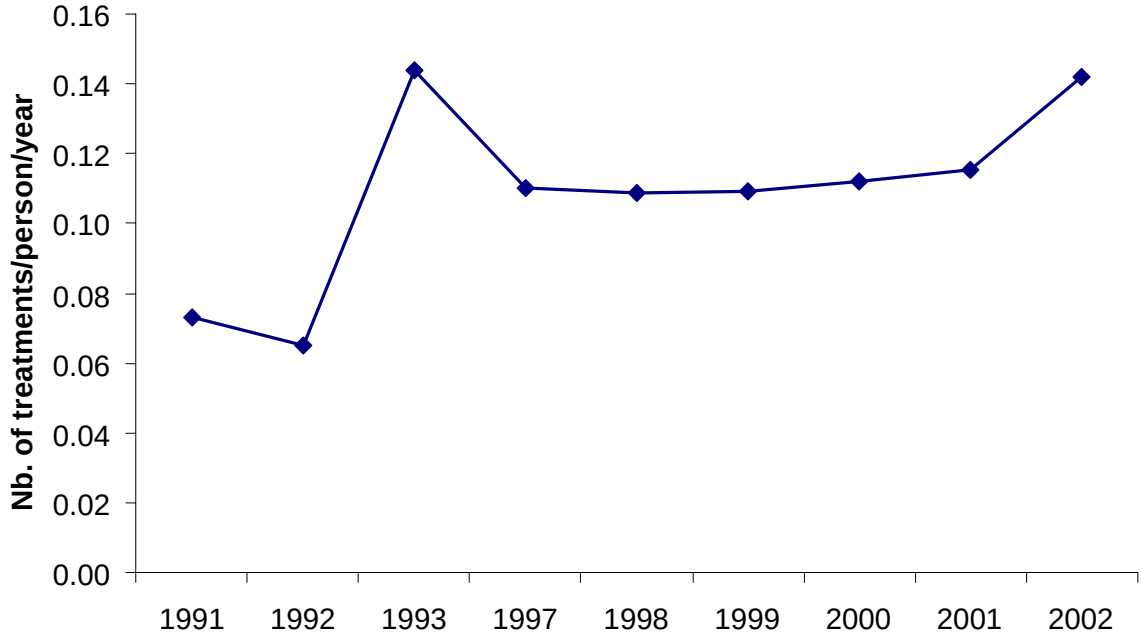

Figure 4: Cotrimoxazole use. Treatment per capita calculated from IMR surveillance records.

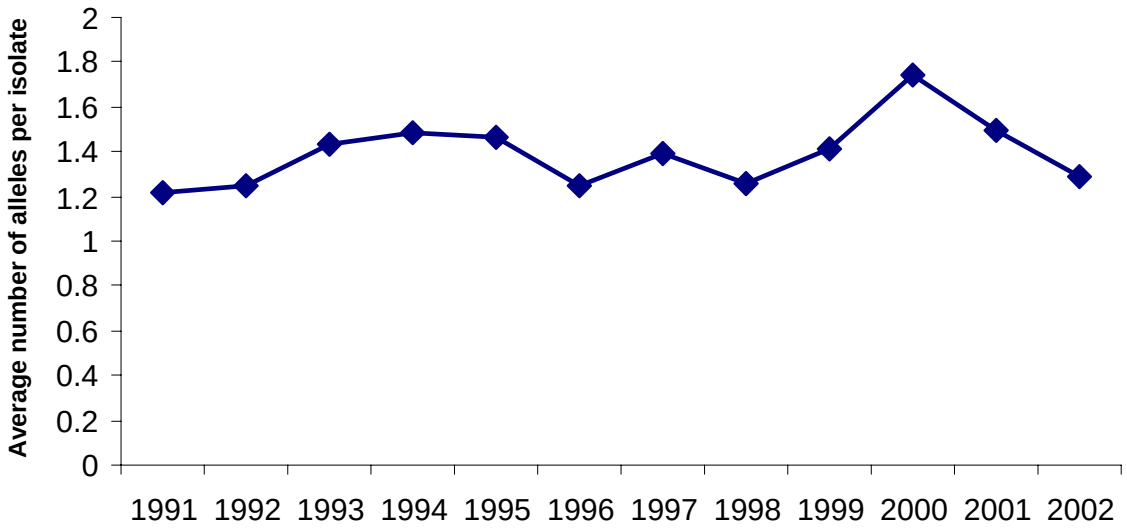

Figure 5: Multiplicity of infection (MOI)

Additional data from Idete

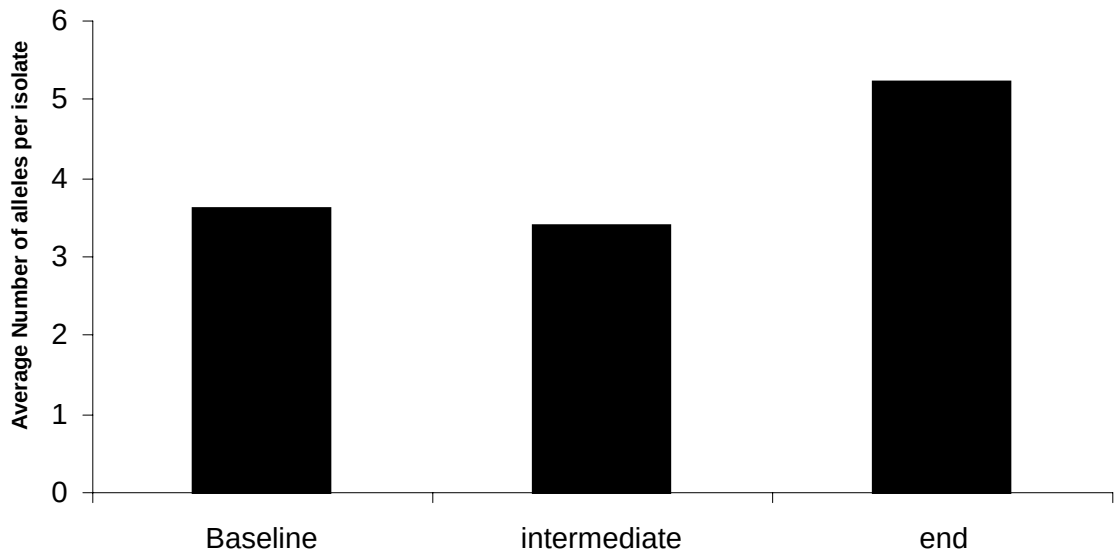

Figure 1: Multiplicity of infection (MOI)

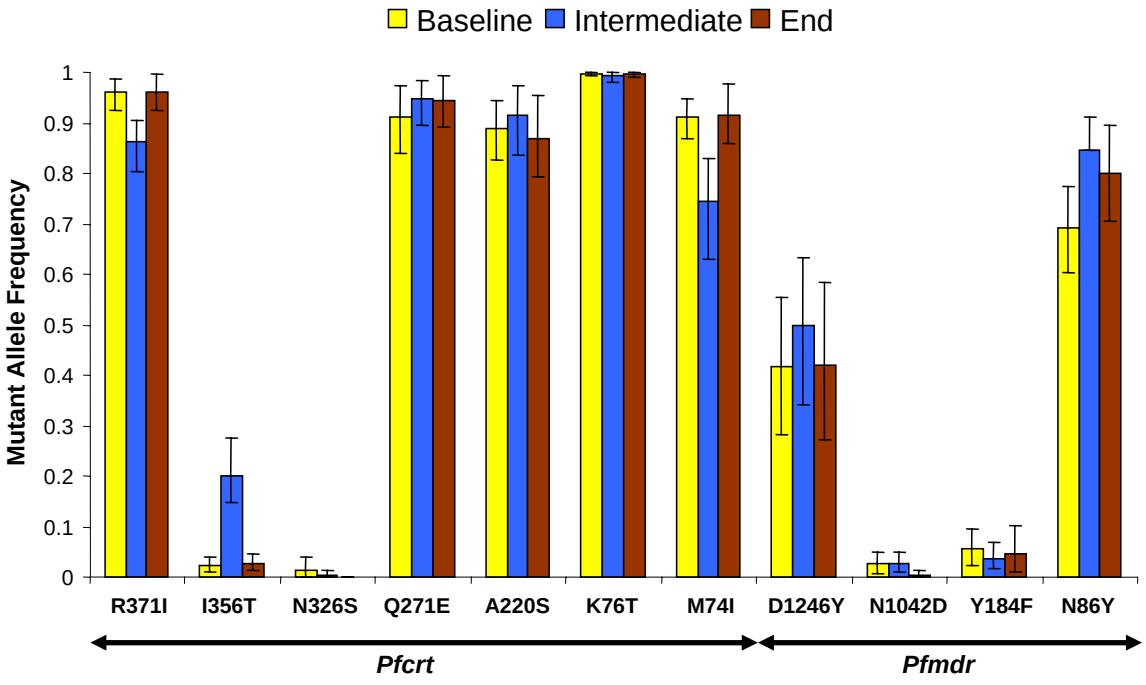

Figure 2: Mutation frequencies in *Pfcrt* and *Pfmdr* genes

Table 1: Selection coefficients assuming 6 generations of *P. falciparum* per year

Genes	SNPs	Selection coefficient (S)	95% Conf. Interval
Pfdhps	S436F	0.90%	-130.50% - 132.31%
Pfdhps	A437G	113.17%	-181.13% - 407.44%
Pfdhps	K540E	8.29%	-107.35% - 123.92%
Pfdhfr	S108S	-0.05%	-42.23% - 42.13%
Pfdhfr	N51I	21.73%	-139.24% - 57.39%
Pfdhfr	C59R	12.90%	-88.81% - 114.61%

Standard Operating Procedure

Analysis of malaria drug resistance markers using DNA microarray (chip) technology

Version 1.9 (Revision Jutta_September, 2006)

Table of Content

1. DNA preparation

DNA is isolated from whole blood (anti-coagulated with EDTA) or red blood cell pellets using QIAamp® 96 DNA Blood Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions. [http://www1.qiagen.com/literature/protocols/QIAamp96DNABlood.aspx]

2. Amplification of target sequences

2.1 Primary PCR

2.1.1 Primary PCR mix

Reagents	**1 reaction**	**104 reactions (96 patients)**	**final conc.**
H_2O	30.0 µl	3120 µl	
10 x PCR buffer (without $MgCl_2$)	5.0 µl	520 µl	1 x
dNTP mix (2mM)	5.0 µl	520 µl	200 µM
$MgCl_2$ (25mM)	6.0 µl	624 µl	3 mM
Primary PCR primer mix (10 µM each) **#**	1.0 µl	104 µl	200 nM each
Taq polymerase 5U/µl	0.50 µl	52 µl	0.05 U/µl
Final volume	**47.5 µl**	**4940 µl**	
DNA	2.5 µl		
Final volume	**50 µl**		

Primary PCR primer mixes (sequence information: see Appendix):

1. P 1-1 (*pfmdr1* PCR I: P 1-1 for / P1-1 rev, 10 µM each in TE buffer)
2. P 3-1 (*pfmdr1* PCR II: P 3-1 for / P3-1 rev, 10 µM each in TE buffer)
3. P 5-1 (*pfdhfr* PCR: P 5-1 for / P5-1 rev, 10 µM each in TE buffer)
4. P 8-1 (*pfdhps* PCR: P 8-1 for / P8-1 rev, 10 µM each in TE buffer)
5. P 10-1 (*pfcrt* PCR I: P 10-1 for / P10-1 rev, 10 µM each in TE buffer)
6. P 11-1 (*pfcrt* PCR II: P 11-1 for / P11-1 rev, 10 µM each in TE buffer)
7. P 12-1 (*pfcrt* PCR III: P 12-1 for / P12-1 rev, 10 µM each in TE buffer)
8. P 16-1 (*pfcrt* PCR IV: P 16-1 for / P16-1 rev, 10 µM each in TE buffer)
9. P 17-1 (*pfATPase6* PCR: P 17-1 for / P17-1 rev, 10 µM each in TE buffer)
10. P 18-1 (*pfcrt* PCR V: P 18-1 for / P18-1 rev, 10 µM each in TE buffer)

2.1.2 Primary PCR program

96 °C 180 sec

96 °C 30 sec
52 °C 90 sec **20 cycles for clinical (symptomatic) samples**
72 °C 90 sec **25 cycles for community (asymptomatic) samples**

Hold at 4 °C

2.2 Nested PCR

2.2.1 Nested PCR mix

Reagents	1 reaction	104 reactions (96 patients)	final conc.
H_2O	60.0 µl	6000 µl	
10 x PCR buffer (without $MgCl_2$)	10.0 µl	1000 µl	1 x
dNTP mix (2mM)	10.0 µl	1000 µl	200 µM
$MgCl_2$ (25mM)	12.0 µl	1200 µl	3 mM
Nested primer mix (10µM each)§	2.0 µl	200 µl	200 nM each
Taq polymerase 5U/µl	1.0 µl	100 µl	0.05 U/µl
Final volume	**95 µl**	**9500 µl**	
Primary PCR product	5.0 µl		
Final volume	**100 µl**		

§ Nested PCR primer mixes (sequence information: see Appendix):

11. P 1 (*pfmdr1* PCR I: P 1 for / P1 rev, 10 µM each in TE buffer)
12. P 3 (*pfmdr1* PCR II: P 3 for / P3 rev, 10 µM each in TE buffer)
13. P 5 (*pfdhfr* PCR: P 5 for / P5 rev, 10 µM each in TE buffer)
14. P 8 (*pfdhps* PCR: P 8 for / P8 rev, 10 µM each in TE buffer)
15. P 10 (*pfcrt* PCR I: P 10 for / P10 rev, 10 µM each in TE buffer)
16. P 11 (*pfcrt* PCR II: P 11 for / P11 rev, 10 µM each in TE buffer)
17. P 12 (*pfcrt* PCR III: P 12 for / P12 rev, 10 µM each in TE buffer)
18. P 16 (*pfcrt* PCR IV: P 16 for / P16 rev, 10 µM each in TE buffer)
19. P 17 (*pfATPase6* PCR: P 17 for / P17 rev, 10 µM each in TE buffer)
20. P 18 (*pfcrt* PCR V: P 18 for / P18 rev, 10 µM each in TE buffer)

2.2.2 Nested PCR program

96 °C 180 sec

96 °C 30 sec
52 °C 90 sec **<u>20 cycles</u> for clinical (symptomatic) samples**
72 °C 90 sec **<u>25 cycles</u> for community (asymptomatic) samples**

Hold at 4 °C

3. <u>SAP (Shrimp Alkaline Phosphatase) digest of PCR products</u>

3.1 Preparation of PCR products

By using a multichannel pipette, pool 10 µl of all the 10 nested PCR reactions from each patient into a new 96 well plate. Mix and centrifuge briefly. **= PCR pool plate**

Transfer 10 µl of each well into a new 96 well plate and add 90 µl of H_20 to each well.
Mix and centrifuge briefly. **= PCR pool plate 1:10**

3.2 SAP digest

NOTE Each PCR pool from **PCR pool plate 1:10** has to be SAP digested **in duplicate** because we have to perform **2 extension reactions** per patient!

3.2.1 SAP master mix

Reagents	**1 reaction**	**104 reactions (48 patients)**
H_2O	4.0 µl	416 µl
10 x SAP buffer	1.0 µl	104 µl
Shrimp Alkaline Phosphatase (SAP) 1U/µl	2.0 µl	208 µl
Final volume	**7.0 µl**	**728 µl**
PCR pool 1:10	5.0 µl	
Final volume	**12.0 µl**	

- Using a multichannel pipette, transfer 5 µl of the PCR pool 1:10 to the **SAP plate**. Remember: you need **two wells per patient** (i.e., 1 SAP plate contains 48 patients).
- Add 7 µl of the SAP master mix to each well.
- Mix and centrifuge briefly, start SAP program.

SAP plate

SAP plate	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A) SAP 1	Patient1	Patient2	Patient3	Patient4	Patient5	Patient6	Patient7	Patient8	Patient9	Patient10	Patient11	Patient12
B) SAP 2	Patient1	Patient2	Patient3	Patient4	Patient5	Patient6	Patient7	Patient8	Patient9	Patient10	Patient11	Patient12
C) SAP 1	Patient13	Patient14	Patient15	Patient16	Patient17	etc.						
D) SAP 2	Patient13	Patient14	Patient15	Patient16	Patient17	etc.						
E) SAP 1												
F) SAP 2												
G) SAP 1											Patient47	Patient48
H) SAP 2											Patient47	Patient48

3.2.2 SAP program

SAP digest: 1 hour at 37 °C
Inactivation of SAP digest: 15 min at 90 °C

NOTE This reaction is performed in a PCR machine.

4. Primer extension

4.1 Preparation of ddNTP mixes

Combination 1	Combination 2
ddATP Cy3	ddUTP Cy3
ddCTP Cy3	ddCTP Cy3
ddGTP Cy5	ddATP Cy5
ddUTP Cy5	ddGTP Cy5

- To get a **2.5 µM** final concentration of ddNTP mixes (**Combination 1**, **Combination 2**), dilute Cy3- and Cy5-labelled ddNTP's stock solutions (100 µM at -80°C) 1:40 in TE buffer:

  1. 4 x 25 µl 100 µM ddNTP stock = 100 µl. Add 900 µl TE buffer = 1 ml 2.5 µM ddNTP mix.
  2. Make aliquots and store 2.5 µM ddNTP mix at **-20°C**!

4.2 Preparation of extension primer mixes

Gene	Combination 1 (25 oligos)	Combination 2 (15 oligos)
Pfdhps	437, 540, 581, 613, 640	436, 613B, 645
Pfdhfr	16, 51, 59, 108, 164	108B, 164B
Pfmd1r	86, 184, 1034, 1042	1246
Pfcrt	72, 75B1, 152, 271, 326, 326B, 356, 356B	74, 76, 97, 163, 220, 371
PfATPase6	538, 769, 769B	574, 623, 683

- To get a **62.5 nM** final concentration of extension primer mixes (**Combination 1**, **Combination 2**), dilute extension primer stock solutions (10 µM in TE buffer) 1:160 in TE buffer:

  1. **Combination 1**: 25 x 2 µl = 50 µl plus 270 µl TE buffer
  2. **Combination 2**: 15 x 2 µl = 30 µl plus 290 µl TE buffer

  Store extension primer mixes at **+4°C**!

4.3 Reaction mix combination 1

Combination 1	1 reaction	52 reactions (48 patients)	final conc.
H_2O	1.6 µl	83.2 µl	
10 x Sequenase buffer	2 ul	104 ul	1 x
Extension primer mix Combination 1 (62.5 nM)	2 ul	104 ul	6.25 nM
ddNTP mix Combination 1 (2.5 µM)	2 ul	104 ul	0.25 µM
Thermo Sequenase (5U/µl)	0.4 µl	20.8 µl	0.1 U/µl
Final volume	**8 ul**	**416 ul**	

4.4 Reaction mix **combination 2**

Combination 2	1 reaction	52 reactions (48 patients)	final conc.
H_2O	1.6 µl	83.2 µl	
10 x Sequenase buffer	2 ul	104 ul	1 x
Extension primer mix Combination 2 (62.5 nM)	2 ul	104 ul	6.25 nM
ddNTP mix Combination 2 (2.5 µM)	2 ul	104 ul	0.25 µM
Thermo Sequenase (5U/µl)	0.4 µl	20.8 µl	0.1 U/µl
Final volume	**8 ul**	**416 ul**	

- Add 8 µl of the extension reaction mixes **Combination 1** and **Combination 2** to the SAP digested PCR products in the SAP plate = **EXTENSION plate** (Final volume = **20 µl**)
- Start extension program

EXTENSION plate

SAP→EXTENSION	1	2	3	4	5	6	7	8	9	10	11	12
A) COMB 1	Patient1	Patient2	Patient3	Patient4	Patient5	Patient6	Patient7	Patient8	Patient9	Patient10	Patient11	Patient12
B) COMB 2	Patient1	Patient2	Patient3	Patient4	Patient5	Patient6	Patient7	Patient8	Patient9	Patient10	Patient11	Patient12
C) COMB 1	Patient13	Patient14	Patient15	Patient16	Patient17	etc.						
D) COMB 2	Patient13	Patient14	Patient15	Patient16	Patient17	etc.						
E) COMB 1												
F) COMB 2												
G) COMB 1											Patient47	Patient48
H) COMB 2											Patient47	Patient48

4.5 Primer extension program

94 °C 60 sec

94 °C 10 sec **35 cycles**
50 °C 40 sec

Hold at 4 °C

4.6 Denaturation

- Pool extension reaction mixes **Combination 1** and **Combination 2** with a multichannel pipette (Final volume = **40 µl**).

- Add 6 µl of denaturing solution: 0.5 µl 0.5M EDTA pH 8.0
  2.0 µl 10% SDS
  3.5 µl H_20 (Final volume: **46 µl**)

- Incubate at 94 °C for 60 sec
- Chill on ice for 2 min

5. Hybridisation of extended primers

5.1 Preparation of the spotted microarray

- Add 23 µl of the extension reaction mix on the chip
- Add 6 µl of 20 x SSC to each well of the slide

5.2 Hybridisation

- Incubate the chip in a opaque humid chamber at 50 °C for 60-90 min

5.3 Washing procedure after hybridisation

1. 2x SSC + 0.2% SDS: 20 min at room temperature (RT)
2. 2x SSC: 20 min at RT
3. 2xSSC + 2% EtOH: 2 min at RT

Dry the chip with compressed air and store at RT in the dark

6. Data acquisition and analysis

6.1 Base calling

Slides can be scanned by the use of any laser scanner.

IMPORTANT:

1. **Cy3 (wavelength: 532 nm) and Cy5 (wavelength: 635 nm) signals have to be acquired!**
2. **Single signal or combined signal images have to be stored as tif-files for further analysis!**
3. **File names have to include a unique study, slide, experiment and operator identification code**

6.2 Short guide for data analysis

a) Prior to analysis, patient/study identification numbers and their respective position on the slide(s) have to be entered using the galDesigner software:

1. Open **galDesigner** software
2. Load template = **malaria.sti**
3. Enter all 12 patient/study identification numbers in the respective fields on each slide
4. Save each slide (containing 12 patients) as separate **gal-file**

Important: file names have to include a unique **study, slide, experiment and operator** identification code

b) Slide images are analysed using the Axon GenePix® Pro (version 6.0) software (www.axon.com):

5. Open **GenePix® Pro** software

"Image" menu:
6. Open image = **tif-file** (Ctrl+O))
7. Open array list = **gal-file** (Alt+Y)
8. Align array list (by using *Block Mode* and/or *Feature Mode*)
9. Analyse slide (creates image) (Alt+A)

"Results" menu:
10. Save results as GenePix Results Files = **gpr-file** (Alt+U)

"Report" menu:
11. Run report: by using the script: **Triplicates, ratios, with 6 parameters**
12. Start (creates the STI chip report)
13. Export the STI chip report as tab-delimited **txt-file**

Important: file names have to include a unique **study, slide, experiment and operator** identification code

c) Data are converted into a format compatible with any statistical package using fileConverter software:

14. Open **fileConverter** software
15. Transfer results as tab-delimited txt-files into the data folder
16. Run fileConverter
17. Rename and save the outTable.txt file in your personal data folder

7. Appendix

7.1 Buffers

- 500 mM EDTA pH 8.0
- 180 mM phosphate buffer pH 8.0
- 20 x SSC pH 7.0
- 2 x SSC
- 2 x SSC + 0.2% SDS
- 2 x SSC + 2% EtOH
- 10% SDS
- 1 x TE buffer (= 10 mM Tris/HCl pH 8.0)
- 10 mM Tris/HCl pH 7.4

1. Prepare all buffers/solutions according to the protocols in: *Molecular Cloning* (a laboratory manual; Sambrook J., Fritsch E.F. and Maniatis T. 2nd edition; Cold Spring Harbor Laboratory Press, 1989)
2. Store all buffers at **RT**!

7.2 Reagents

• 10 x PCR buffer (=buffer B)	Solis BioDyne, Tartu, Estonia
• 25 mM $MgCl_2$	Solis BioDyne, Tartu, Estonia
• *Taq* polymerase (Firepol®; 5 U/μl)	Solis BioDyne, Tartu, Estonia
• dNTP mix (2mM each):	

Dilute 100 mM stock solutions 1:50 in 10 mM Tris/HCl pH 7.4

dATP 100 mM	Amersham Biosciences: 272050
dTTP 100 mM	Amersham Biosciences: 272080
dCTP 100 mM	Amersham Biosciences: 272060
dGTP 100 mM	Amersham Biosciences: 272070

• 10 x SAP buffer	Amersham Biosciences: 70103
• Shrimp Alkaline Phospothase (SAP; 1U/μl)	Amersham Biosciences: 70092Z
• 10 x Sequenase buffer	Amersham Biosciences: 93-79222
• Thermo Sequenase (Termipol®; 5 U/μl)	Solis BioDyne, Tartu, Estonia

NOTE Store all reagents at **-20°C**!

• Cy3 /Cy5 labelled ddNTP	Perkin Elmer: NEL999

NOTE Store 100 μM ddNTP stock solutions at **-80°C**!

7.3 Oligonucleotides

A. C-7 oligos (Spotting)

500 μM stock solutions in 180 mM phosphate buffer pH 8.0 **(aliquots at -20°C**)	Operon (Amino **C-7 linker** at the 3' end!!!)

B. Extension oligos

100 μM stock solutions in TE buffer pH 8.0 **(aliquots at -20°C**)	Operon (**HPLC**-purified!!!)

7.4 Sequence information

NOTE:

- **All primers and sequences are listed from the 5' to the 3' end of the sequence**
- **Sequences in *italic/underlined* denote flexible primer sequences (=flexi tag)**
- **Neg1-Neg4: negative control oligonucleotides (=conserved sequence stretches from the respective genes)**

7.4.1 *Pfdhfr*

***Pfdhfr* primary PCR primer (Size PCR product: 677 bp)**

P5-1 for:	TTTATGATGGAACAAGTCTGC
P5-1 rev:	TAAATGATAAAATCCAATGTTGTAT

***Pfdhfr* nested PCR primer (Size PCR product: 637 bp)**

P5 for:	ACAAGTCTGCGACGTTTTCGATATTTATG
P5 rev:	AGTATATACATCGCTAACAGA

***Pfdhfr* C-7 primer**

DHFR Neg1 C-7:	AAATATAAGAGATGTAAATATTTAAACAA
16 C-7:	CACATATGGCATAAATATCGAAAACGTC
51 C-7:	TACATTTCCATGGTAATACTCCTTTATTTC
59 C-7:	GTGCAGTTACAACATATGTGAA
10/108 C-7:	GCAGGGAAGCGGGAGCGAAACAGC
11/108B C-7:	AAAACGGGGCACAGCGCGGCGGAA
06/164 C-7:	GGGAGAGCGCAGCAGGCAACAGAG
07/164B C-7:	GACCGCCACCAAGAACAGCACCGG

***Pfdhfr* extension primer**

16:	GACGTTTTCGATATTTATGCCATATGTG
51:	GAAATAAAGGAGTATTACCATGGAAATGTA
59:	TTCACATATGTTGTAACTGCAC
10/108:	*GCTGTTTCGCTCCCGCTTCCCTGC*CAAAATGTTGTAGTTATGGGAAGAACAA
11/108B:	*TTCCGCCGCGCTGTGCCCCGTTTT*AAAGGTTTAAATTTTTTTGGAATGCTTTCCCAG
06/164:	*CTCTGTTGCCTGCTGCGCTCTCCC*GGGAAATTAAATTACTATAAATGTTTTATT
07/164B:	*CCGGTGCTGTTCTTGGTGGCGGTC*TTCTTGATAAACAACGGAACCTCCTA

***Pfdhfr* sequence (Accession number: J04643)**

P5-1 for
P5 for
P5 rev
P5-1 rev

C-7 and extension primer for *dhfr* 16, *dhfr* 51, *dhfr* 59, *dhfr* 108, *dhfr* 164
SNP

TTTATGATGGAACAAGTCTGC
ACAAGTCTGCGACGTTTTCGATATTTAT

```
  01 ATGATGGAACAAGTCTGCGACGTTTTCGATATTTATGCCATATGTGCATGTTGTAAGGTT
  61 GAAAGCAAAAATGAGGGGAAAAAAAATGAGGTTTTTAATAACTACACATTTAGAGGTCTA
 121 GGAAATAAAGGAGTATTACCATGGAAATGTAATTCCCTAGATATGAAATATTTTTGTGCA
 181 GTTACAACATATGTGAATGAATCAAAATATGAAAAATTGAAATATAAGAGATGTAAATAT
 241 TTAAACAAAGAAACTGTGGATAATGTAAATGATATGCCTAATTCTAAAAAATTACAAAAT
 301 GTTGTAGTTATGGGAAGAACAAACTGGGAAAGCATTCCAAAAAAATTTAAACCTTTAAGC
 361 AATAGGATAAATGTTATATTGTCTAGAACCTTAAAAAAAGAAGATTTTGATGAAGATGTT
 421 TATATCATTAACAAAGTTGAAGATCTAATAGTTTTACTTGGGAAATTAAATTACTATAAA
 481 TGTTTTATTATAGGAGGTTCCGTTGTTTATCAAGAATTTTTAGAAAAGAAATTAATAAAA
 541 AAAATATATTTTACTAGAATAAATAGTACATATGAATGTGATGTATTTTTTCCAGAAATA
 601 AATGAAAATGAGTATCAAATTATTTCTGTTAGCGATGTATATACTAGTAACAATACAACA
 661 TTGGATTTTATCATTTATAAGAAAACGAATAATAAAATGTTAAATGAACAAAATTGTATA
 721 AAAGGAGAAGAAAAAAATAATGATATGCCTTTAAAGAATGATGACAAAGATACATGTCAT
 781 ATGAAAAAATTAACAGAATTTTACAAAAATGTAGACAAATATAAAATTAATTATGAAAAT
 841 GATGATGATGATGAAGAAGAAGATGATTTTGTTTATTTTAATTTTAATAAAGAAAAAGAA
 901 GAGAAAAATAAAAATTCTATACATCCAAATGATTTTCAAATATATAATAGCTTGAAATAT
 961 AAATATCATCCTGAATACCAATATTTAAATATTATTTATGATATTATGATGAATGGAAAT
1021 AAACAAAGTGATCGAACGGGAGTAGGTGTTTTAAGTAAATTCGGATATATTATGAAATTT
1081 GATTTAAGTCAATATTTCCCATTATTAACTACGAAGAAATTATTTTTAAGAGGAATTATT
1141 GAAGAATTGCTTTGGTTTATTAGAGGAGAAACAAATGGTAATACGTTGTTAAATAAGAAT
1201 GTAAGGATATGGGAAGCTAATGGTACTAGGGAATTTTTAGATAATAGAAAATTATTTCAT
1261 AGAGAAGTTAACGATTTAGGACCTATTTATGGTTTTCAATGGAGACATTTCGGTGCTGAA
1321 TATACAAATATGTATGATAATTATGAAAATAAAGGAGTGGATCAATTAAAAAATATAATA
1381 AATTTAATTAAAAATGATCCTACAAGTAGAAGAATTCTTTTGTGTGCATGGAATGTAAAA
1441 GATCTTGACCAAATGGCATTACCTCCTTGTCATATTTTATGTCAGTTTTATGTTTTCGAT
1501 GGGAAATTATCATGTATTATGTATCAAAGATCATGTGATTTAGGGCTAGGAGTACCTTTT
1561 AATATTGCTTCTTATTCTATTTTTACTCATATGATTGCACAAGTCTGTAATTTGCAACCT
1621 GCGCAGTTCATACACGTTTTAGGAAATGCACATGTTTATAATAATCACATTGATAGTTTA
1681 AAAATTCAACTTAACAGAATACCCTATCCATTCCCAACACTTAAATTAAATCCAGATATT
1741 AAAAATATTGAAGATTTTACAATTTCGGATTTTACAATACAAAATTATGTTCATCATGAA
1801 AAAATTTCAATGGATATGGCTGCTTAA
```

7.4.2 *Pfdhps*

***Pfdhps* primary PCR primer (Size PCR product: 756 bp)**

P8-1 for:	ATTTTTGTTGAACCTAAACGTGCTGTTCA
P8-1 rev:	CTTGTCTTTCCTCATGTAATTCATCT

***Pfdhps* nested PCR primer (Size PCR product: 686 bp)**

P8 for:	TTGAAATGATAAATGAAGGTGCTAGT
P8 rev:	CCAATTGTGTGATTTGTCCA

***Pfdhps* C-7 primer**

DHPS Neg4 C-7:	AACAAAAATTACATGATGAACAACAAAAT
436 C-7:	GGATTCTCCACCTATATCTATAA
437 C-7:	TCCTTTTGTTATACCTAATCCAA
540 C-7:	ATCCATTGTATGTGGATTTCCA
581 C-7:	CAAATCCTAATCCAATATCAAATAGTATCC
613 C-7:	AATAAATCTTTTTCTTGAATATCC
09/613B C-7:	CGCGCACAGAAGGGCGAGAGACGA
640 C7:	TTGTGGACAAATCACACAATTG
645 C7:	GTGATTTGTCCACAATATTTTTAT

***Pfdhps* extension primer**

436:	TTATAGATATAGGTGGAGAATCC
437:	TTGGATTAGGTATAACAAAAGGA
540:	AGGAAATCCACATACAATGGAT
581:	GGATACTATTTGATATTGGATTAGGATTTG
613:	GGATATTCAAGAAAAAGATTTATT
09/613B:	*TCGTCTCTCGCCCTTCTGTGCGCG*ATTTTGATCATTCATGCAATGGG
640:	CAATTGTGTGATTTGTCCACAA
645:	ATAAAAATATTGTGGACAAATCAC

***Pfdhps* sequence (Accession Number: Z30659)**

P8-1 for
P8 for
P8 rev
P8-1 rev

C-7 and extension primer *dhps* 436, *dhps* 437, *dhps* 540, *dhps* 581, *dhps* 613, *dhps* 640, *dhps* 645
SNP

```
  01 TGATACCCGAATATAAGCATAATGTTTTAAATAATACCATCAGATGTTTATATAACAAAT
  61 ATGTGAGTAGGATGAAAGAACAATATAATATAAATATTAAAGAAAATAATAAAAGGATAT
 121 ATGTATTAAAAGATAGAATTTCTTATTTAAAAGAAAAAACAAATATTGTTGGAATATTAA
 181 ATGTTAATTATGATTCTTTTTCAGATGGAGGTATTTTTGTTGAACCTAAACGTGCTGTTC
 241 AAAGAATGTTTGAAATGATAAATGAAGGTGCTAGTGTTATAGATATAGGTGGAGAATCCG
 301 CTGGTCCTTTTGTTATACCTAATCCAAAAATTAGTGAAAGAGATTTAGTAGTACCTGTAT
 361 TACAATTATTTCAAAAAGAATGGAATGATATAAAAAATAAAATTGTTAAATGTGATGCGA
 421 AACCAATTATAAGTATTGATACAATTAACTATAATGTTTTTAAAGAATGTGTTGATAATG
 481 ATTTAGTTGATATATTAAATGATATTAGTGCTTGTACAAATAATCCAGAAATTATAAAAT
 541 TATTAAAAAAAAAAAAACAAATTCTATAGTGTAGTTCTAATGCATAAAAGAGGAAATCCAC
 601 ATACAATGGATAAACTAACAAATTATGATAATCTAGTTTATGATATAAAAAATTATTTAG
 661 AACAAAGATTAAATTTTCTTGTATTAAATGGAATACCTCGTTATAGGATACTATTTGATA
 721 TTGGATTAGGATTTGCGAAGAAACATGATCAATCTATTAAACTCTTACAAAATATACATG
 781 TATATGATGAGTATCCACTTTTTATTGGATATTCAAGAAAAAGATTTATTGCCCATTGCA
 841 TGAATGATCAAAATGTTGTAATAAATACACAACAAAAATTACATGATGAACAACAAAATG
 901 AAAATAAAAATATTGTGGACAAATCACACAATTGGATGTTTCAGATGAATTACATGAGGA
 961 AAGACAAGGATCAACTTTTATATCAAAAAAATATATGTGGTGTGTTTAAAAAAAAAAAAA
1021 AATTCAAATGAGTATACAAAAGTAACAATTCTATATATGTTACATATAAAATATAAATAA
1081 TATATATTCATGTATATGTATTTATGTATTTCTTTTCAGGTGGATTAGCAATTGCTTCCT
1141 ACAGCTATTATAAAAAGGTAGATCTAATAAGAGTTCATGACGTTTTAGAAACAAAATCGG
1201 TTTTGGATGTTTTAACAAAAATAGACCAAGTGTAATTTACAAAAGGAAAGTGCAAACATG
1261 TGATTAAAC
```

7.4.3 *Pfmdr1*

PCR I:

***Pfmdr1* primary PCR I primer (Size PCR product: 613 bp)**

P1-1 for: TTAAATGTTTACCTGCACAACATAGAAAATT
P1-1 rev : CTCCACAATAACTTGCAACAGTTCTTA

***Pfmdr1* nested PCR I primer (Size PCR product: 526 bp)**

P1 for: TGTATGTGCTGTATTATCAGGA
P1 rev: CTCTTCTATAATGGACATGGTA

***Pfmdr1* I C-7 primer**

MDR Neg2 C-7: AAAACTACAGCAATCGTTGGAGAAACAGGT

86 C-7: CATGTTCTTTAATATTACACCAAA
13/184 C-7: GGGAACGACACAGACAAGCCGGGG

***Pfmdr1* I extension primer**

86: TTTGGTGTAATATTAAAGAACATG
13/184: *CCCCGGCTTGTCTGTGTCGTTCCC*TGCCAGTTCCTTTTTAGGTTTAT

***Pfmdr1* sequence I (Accession Number: from S53996)**

P1-1 for
P1 for
P1 rev
P1-1 rev

C-7 and extension primer *mdr* 86, *mdr* 184
SNP

```
  01 ATGGGTAAAGAGCAGAAAGAGAAAAAAGATGGTAACCTCAGTATCAAAGAAGAGGTTGAA
  61 AAAGAGTTGAACAAAAAGAGTACCGCTGAATTATTTAGAAAAATAAAGAATGAGAAAATA
 121 TCATTTTTTTTACCGTTTAAATGTTTACCTGCACAACATAGAAAATTATTATTTATATCA
 181 TTTGTATGTGCTGTATTATCAGGAGGAACATTACCTTTTTTTATATCTGTGTTTGGTGTA
 241 ATATTAAAGAACATGAATTTAGGTGATGATATTAATCCTATAATATTATCATTAGTATCT
 301 ATAGGTTTAGTACAATTTATATTATCAATGATATCAAGTTATTGTATGGATGTAATTACA
 361 TCAAAAATATTAAAAACTTTAAAGCTTGAATATTTAAGAAGTGTTTTTTATCAAGATGGA
 421 CAATTTCATGATAATAATCCTGGATCTAAATTAAGATCTGATTTAGATTTTTATTTAGAA
 481 CAAGTGAGTTCAGGAATTGGTACGAAATTTATAACAATTTTTACATATGCCAGTTCCTTT
 541 TTAGGTTTATATATTTGGTCATTAATAAAAAATGCACGTTTGACTTTATGTATTACTTGC
 601 GTTTTTCCGTTAATTTATGTTTGTGGTGTCATATGTAATAAGAAAGTAAAATTAAATAAA
 661 AAAACATCTTTGTTATATAATAACAATACCATGTCCATTATAGAAGAGGCTTTAATGGGA
 721 ATAAGAACTGTTGCAAGTTATTGTGGAGAAAAGACTATATTAAACAAATTTAATTTGTCC
 781 GAAACTTTTTATAGTAAATATATTTTAAAAGCTAATTTTGTAGAAGCATTACATATAGGT
 841 TTAATAAATGGTTTAATTTTAGTTTCTTATGCATTCGGTTTTTGGTATGGTACAAGAATT
 901 ATTATAAATAGTGCAACGAATCAATACCCCAATAATGATTTTAATGGTGCCTCAGTTATA
 961 TCCATTTTATTAGGTGTACTTATTAGTATGTTTATGTTAACAATTATCTTACCAAATATA
1021 ACAGAATATATGAAAGCTTTAGAAGCAACAAATAGTTTATATGAAATAATAAATCGAAAA
```

PCR II:

***Pfmdr1* primary PCR II primer (Size PCR product: 880 bp)**

P3-1 for: AATTTGATAGAAAAAGCTATTGATTATAA
P3-1 rev: TATTTGGTAATGATTCGATAAATTCATC

***Pfmdr1* nested PCR II primer (Size PCR product: 799 bp)**

P3 for: GAATTATTGTAAATGCAGCTTTA
P3 rev: GCAGCAAACTTACTAACACG

***Pfmdr1* II C-7 primer**

1034 C-7: GAATCCCCATAAAGCTGCATTTACAAT
1042 C-7: ATAGTTTTGCCTATTGGTTTGGATCCTTCT
1246 C-7: TCTTAAGTTATAATCACATATATTA

***Pfmdr1* II extension primer**

1034: ATTGTAAATGCAGCTTTATGGGGATTC
1042: AGAAGGATCCAAACCAATAGGCAAAACTAT
1246: TAATATATGTGATTATAACTTAAGA

***Pfmdr1* sequence II (Accession Number: from S53996)**

P3-1 for
P3 for
P3 rev
P3-1 rev

C-7 and extension primer *mdr* 1034, *mdr* 1042, *mdr* 1246
SNP

```
2701 AAAACGGGTTTAGTAAATAATATTGTTATTTTCTCTCATTTCATAATGCTCTTTCTGGTT
2761 AGCATGGTTATGTCCTTTTATTTTTGTCCAATTGTTGCAGCTGTATTAACTTTTATATAT
2821 TTTATTAATATGCGTGTATTTGCTGTAAGAGCTAGATTAACCAAAAGTAAAGAAATTGAG
2881 AAAAAAGAAAATATGTCAAGCGGAGTTTTTGCATTTAGTTCAGATGATGAAATGTTTAAA
2941 GATCCAAGTTTTTTAATACAGGAAGCATTTTATAATATGCATACTGTTATTAATTATGGT
3001 TTAGAAGATTATTTCTGTAATTTGATAGAAAAAGCTATTGATTATAAAAATAAAGGACAA
3061 AAAAGAAGAATTATTGTAAATGCAGCTTTATGGGGATTCAGTCAAAGCGCTCAATTATTT
3121 ATTAATAGTTTTGCCTATTGGTTTGGATCCTTCTTAATTAAAAGAGGTACTATATTAGTT
3181 GATGACTTTATGAAATCCTTATTTACTTTTATATTTACTGGTAGTTATGCTGGAAAATTA
3241 ATGTCCTTAAAAGGAGATTCAGAAAATGCAAAATTATCATTTGAGAAATATTATCCATTA
3301 ATGATTAGAAAATCAAATATTGATGTAAGAGATGATGGTGGAATAAGAATAAATAAAAAT
3361 TTAATAAAAGGTAAAGTTGATATTAAAGATGTAAATTTCCGTTATATTTCAAGACCAAAT
3421 GTACCTATTTATAAAAATTTATCTTTTACATGTGATAGTAAAAAAACTACAGCAATCGTT
3481 GGAGAAACAGGTAGTGGAAAATCAACTTTTATGAATCTCTTATTAAGATTTTATGACTTG
3541 AAAAATGATCACATTATATTAAAAAATGATATGACAAATTTTCAAGATTATCAAAATAAT
3601 AATAATAATTCATTGGTTTTAAAAAATGTAAATGAATTTTCAAACCAATCTGGATCTGCA
3661 GAAGATTATACTGTATTTAATAATAATGGAGAAATATTATTAGATGATATTAATATATGT
3721 GATTATAACTTAAGAGATCTTAGAAACTTATTTTCAATAGTTAGTCAAGAACCCATGTTA
3781 TTTAATATGTCCATATATGAAAATATCAAATTTGGAAGAGAAGATGCAAATTGGAAGATC
3841 GTTAAACGTGTTAGTAAGTTTGCTGCTATAGATGAATTTATCGAATCATTACCAAATAAA
3901 TATGATACAAATGTTGGACCATATGGTAAAAGCTTATCAGGTGGACAAAAACAGAGAATA
```

7.4.4 *Pfcrt*

PCR I:

***Pfcrt* primary PCR I primer (Size PCR product: 280 bp)**

P10-1 for: TTGTCGACCTTAACAGATGGCTCAC
P10-1 rev: AATTTCCCTTTTTATTTCCAAATAAGGA

***Pfcrt* nested PCR I primer (Size PCR product: 200 bp)**

P10-for: CTTGTCTTGGTAAATGTGCTC
P10-rev: GAACATAATCATACAAATAAAGT

***Pfcrt* I C-7 primer**

CRT Neg 3 C-7: AAACTTATTTTTAAAGAGATTAAGGATAA

01/72 C-7: ACGCCGGAACGCCGGAACGCCGGA
75B1 C-7: AATTACACATACACTTAAATAAATAATACTTAA
02/74 C-7: ACGGGGCAACGGGGCAACGGGGCA
76 C-7: AATTTTTGCTAAAAGAACTTTAAACAAA
97 C-7: TGAGTTTCGGATGTTACAAAAC

***Pfcrt* I extension primer**

01/72: *TCCGGCGTTCCGGCGTTCCGGCGT*TTTTAAGTATTATTTATTTAAGTGTA
75B1: TTAAGTATTATTTATTTAAGTGTATGTGTAATT
02/74: *TGCCCCGTTGCCCCGTTGCCCCGT*TATTATTTATTTAAGTGTATGTGTAAT
76: TTTGTTTAAAGTTCTTTTAGCAAAAATT
97: GTTTTGTAACATCCGAAACTCA

***Pfcrt* sequence I (Accession Number: from AF030694)**

P10-1 for
P10 for
P10 rev
P10-1 rev

C-7 and extension primer *crt* 72, *crt* 74, *crt* 75, *crt* 76, *crt* 97
SNP

```
  01 AAATTCGCAAGTAAAAAAAATAATCAAAAAAATTCAAGCAAAAATGACGAGCGTTATAGA
  61 GAATTAGATAATTTAGTACAAGAAGGAAGTAAGTATCCAAAAATGGAAATATTGAATGAT
 121 ATAAATGAATAGATAAATCAACCTATTGGATATATATATATATATATATATATATATATA
 181 TATGTATACCCATATGTATTAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCC
 241 CTTGTCGACCTTAACAGATGGCTCACGTTTAGGTGGAGGTTCTTGTCTTGGTAAATGTGC
 301 TCATGTGTTTAAACTTATTTTTAAAGAGATTAAGGATAATATTTTTATTTATATTTTAAG
 361 TATTATTTATTTAAGTGTATGTGTAATTGAAACAATTTTTGCTAAAAGAACTTTAAACAA
 421 AATTGGTAACTATAGTTTTGTAACATCCGAAACTCACAACTTTATTTGTATGATTATGTT
 481 CTTTATTGTTTATTCCTTATTTGGAAATAAAAAGGGAAATTCAAAAGTAAGATAAATCAA
 541 TATATTAAAATGATGGATTTATAAGAGAATCTATTCCACCTACCAATATAAAACATTACA
 601 CATATATATATATATATATATATATATATATGTATGTATGTTGATTAATTTGTTTATATA
 661 TTTATATTTATTTCTTATGACCTTTTTAGGAACGACACCGAAGCTTTAATTTACAATTTT
 721 TTGCTATATCCATGTTAGATGCCTGTTCAGTCATTTTGGCCTTCATAGGTCTTACAAGAA
 781 CTACTGGAAATATCCAATCATTTGTTCTTCAATTAAGTATTCCTATTAATATGTTCTTCT
 841 GCTTTTTAATATTAAGATATAGGTAAGTATACTATTTTAAATTACAAAAAAAAAAAAAAA
 901 AAAAAAAAAAAAAAAAATATAAAATATATATATATATATATATATATATATATATATA
```

PCR II:

***Pfcrt* primary PCR II primer (Size PCR product: 605 bp)**

P18-1 for: ACTTTATTTGTATGATTATGTTC
P18-1 rev: TAACTGCTCCGAGATAATTGT

***Pfcrt* nested PCR II primer (Size PCR product: 548 bp)**

P18 for: TCCTTATTTGGAAATAAAAAGGGAAATT
P18 rev: TAAGTGATATCTAAAAAGGAGTAAAT

***Pfcrt* II C-7 primer**

152 C-7: AGTTCTTGTAAGACCTATGAAGG
163 C-7: CTTAATTGAAGAACAAATGATTGGAT

***Pfcrt* II extension primer**

152: CCTTCATAGGTCTTACAAGAACT
163: ATCCAATCATTTGTTCTTCAATTAAG

***Pfcrt* sequence II (Accession Number: from AF030694)**

P18.1 for
P18 for
P18 rev
P18.1 rev

C-7 and extension primer *crt* 152, *crt* 163
SNP

```
 421 AATTGGTAACTATAGTTTTGTAACATCCGAAACTCACAACTTTATTTGTATGATTATGTT
 481 CTTTATTGTTTATTCCTTATTTGGAAATAAAAAGGGAAATTCAAAAGTAAGATAAATCAA
 541 TATATTAAAATGATGGATTTATAAGAGAATCTATTCCACCTACCAATATAAAACATTACA
 601 CATATATATATATATATATATATATATATGTATGTATGTTGATTAATTTGTTTATATA
 661 TTTATATTTATTTCTTATGACCTTTTTAGGAACGACACCGAAGCTTTAATTTACAATTTT
 721 TTGCTATATCCATGTTAGATGCCTGTTCAGTCATTTTGGCCTTCATAGGTCTTACAAGAA
 781 CTACTGGAAATATCCAATCATTTGTTCTTCAATTAAGTATTCCTATTAATATGTTCTTCT
 841 GCTTTTTAATATTAAGATATAGGTAAGTATACTATTTTAAATTACAAAAAAAAAAAAAAA
 901 AAAAAAAAAAAAAAAAATATAAAATATATATATATATATATATATATATATATATA
 961 TATATTTATATATATTTATTTATATATTTATTTATTTATTTATTTATATTTATTTATTTA
1021 CTCCTTTTTAGATATCACTTATACAATTATCTCGGAGCAGTTATTATTGTTGTAACAATA
```

PCR III:

***Pfcrt* primary PCR III primer (Size PCR product: 360 bp)**

P11-1 for: ATTTACTCCTTTTTAGATATCACTTA
P11-1 rev: TTATATTTTTTAAAAACTATTTCCCTTG

***Pfcrt* nested PCR III primer (Size PCR product: 304 bp)**

P11-for: ACAATTATCTCGGAGCAGTTA
P11-rev: CATGTTTGAAAAGCATACAGGC

***Pfcrt* III C-7 Primer**

220 C-7: ACTAATTAAGACAAGATTAAATATGATAGAA

***Pfcrt* III extension primer**

220: TTCTATCATATTTAATCTTGTCTTAATTAGT

***Pfcrt* sequence III (Accession Number: from AF030694)**

P11-1 for
P11 for
P11 rev
P11-1 rev

C-7 and extension primer *crt* 220,
SNP

```
 961 TATATTTATATATATTTATTTATATATTTATTTATTTATTTATTTATATTTATTTATTTA
1021 CTCCTTTTTAGATATCACTTATACAATTATCTCGGAGCAGTTATTATTGTTGTAACAATA
1081 GCTCTTGTAGAAATGAAATTATCTTTTGAAACACAAGAAGAAAATTCTATCATATTTAAT
1141 CTTGTCTTAATTAGTTCCTTAATTGTAAGAAAACAAAATATATAAATAAATAAATATATA
1201 TATATATATATATATATATATATATTGTAATATTTAATATATATTAACACTTAAACTTTG
1261 TTTTTATTATATTAATTTATATTCTTTATCATTTTTTTTTTTTTTTTCCTTCTTTTTTTT
1321 TTTAGCCTGTATGCTTTTCAAACATGACAAGGGAAATAGTTTTTAAAAAATATAAGATTG
1381 ACATTTTAAGATTAAATGTAAGAAGAAATATATAATAATAATAATATATATATTATATAT
```

PCR IV:

***Pfcrt* primary PCR IV primer (Size PCR product: 697 bp)**

P16-1 for:	TCTGTTATTTTTATTTCTTATAGGCTAT
P16-1 rev:	CTTGTATGTATCAACGTTTTTCATCC

***Pfcrt* nested PCR IV primer (Size PCR product: 630 bp)**

P16-for:	CTTTTTCCAATTGTTCACTTCTTG
P16-rev:	TCTTACATAGCTGGTTATTAAAT

***Pfcrt* IV C-7 primer**

271 C-7:	TTTTAAAAATGGAAGGGTGTATA
326 C-7:	AAAGAAGGAAAACAATGCGAAGGTTT
326B C-7:	CATTTGTGATAATTTAATAACCAGCTATGT

***Pfcrt* IV extension primer**

271:	TATACACCCTTCCATTTTTAAAA
326:	AAACCTTCGCATTGTTTTCCTTCTTT
326B:	ACATAGCTGGTTATTAAATTATCACAAATG

***Pfcrt* sequence IV (Accession Number: from AF030694)**

P16-1for
P16 for
P16 rev
P16-1 rev

C-7 and extension primer *crt* 271, *crt* 326, *crt* 326B
SNP

1441 ATATTTCCTTTTTTACCACTTTTTTTTTTTTTATTCCTATAACGCATTATAATTATT**TCT**
1501 **GTTATTTTTATTTCTTATAGGCTAT**GGTATC**CTTTTTCCAATTGTTCACTTCTTG**TCTTA
1561 TATTACCTG**TATACACCCTTCCATTTTTAAAA**GAACGTAAGAATTAATTAGGAAAGAAAA
1621 AATAAATAAATGAATGTGCCCATATATATATATATATATATATATATATATGTATGTA
1681 TGTATAATTTTCCCTTTTTAGTTCATTTACCATATAATGAAATATGGACAAATATAAAAA
1741 ATGGTTTCGCATGTTTATTCTTGGGAAGAAACACAGTCGTAGAGGTAAAATAGGATTTTC
1801 ATTATATATTAAAAATTACTACTTATTATGTTAATAAAAAAATATGTTTTTTAATGTTCA
1861 ATTTGTTTTATTTTAATTATTTTTTTTTTTTTGTTTTGTTTCCTCTTCAGAATTGTGGTC
1921 TTGGTATGGCTAAGTTATGTGATGATTGTGACGGAGCATGGGTAAGAAGCTTATAATAAA
1981 ATTTCAAAATTATAAGAGACATTTATATATATTTTAACAATAATAATTAAATAAAACAAT
2041 ATTATATATATTATATATATTATTATTTTATTTATTATTTTTTTTTTTTTTTTTTTAGAA
2101 AACCTTCGCATTGTTTTCCTTCTTT**AG**CATTTGTGATA**ATTTAATAACCAGCTATGTAAG**
2161 **A**ATAAAAA**GGATGAAAAACGTTGATACATACAAG**

PCR V:

***Pfcrt* primary PCR V primer (Size PCR product: 626 bp)**

P12-1 for: AGGAAATAAATATGGGAATGTTTAATTGA
P12-1 rev: TTCTAAGATAATATTTCCTACACGGT

***Pfcrt* nested PCR V primer (Size PCR product: 476 bp)**

P12-for: ACCATGACATATACTATTGTTAG
P12-rev: TTATAGAACCAAATAGGTAGCC

***Pfcrt* V C-7 primer**

356 C-7: TGCTGGACCTTGTATACAACTAACAA
356B C-7: AGCAATTGCTTATTACTTTAAATTCTTAGCC
371 C-7: TTACAACATCACCCTATAAAATTAAAAAG

***Pfcrt* V extension primer**

356: TTGTTAGTTGTATACAAGGTCCAGCA
356B: GGCTAAGAATTTAAAGTAATAAGCAATTGCT
371: CTTTTTAATTTTATAGGGTGATGTTGTAA

***Pfcrt* sequence V (Accession Number: from AF030694)**

P12-1 for
P12 for
P12 rev
P12-1 rev

C-7 and extension primer *crt* 356, *crt* 356B, *crt* 371
SNP

```
                                                AGGAAATAAATATGGGAATGTTTAAT
2221 TGAATTAAGATATATATATATATATATATATATATATATGTAACCATATAATTTTTCATT
2281 TTCTATCTTTTTTATAGATTATCGACAAATTTTCTACCATGACATATACTATTGTTAGTT
2341 GTATACAAGGTCCAGCAACAGCAATTGCTTATTACTTTAAATTCTTAGCCGTAAGAATTA
2401 AAAAGATATAAATATATAAATATATATGTGAAATATATGATATATATGATATATATTTTT
2461 TATATGTAATGTTTTTTTTTTTTTTTCACAATATACATTTAAATGTTTATGATGGTACAA
2521 CGTATCATATTTTATAATAATTTTATGCATTCATGTATATTATTTTTACTTTTTAATTTT
2581 ATAGGGTGATGTTGTAATAGAACCAAGATTATTAGATTTCGTAACTTTGGTAAGTGTGAA
2641 ATTAAAAAAATGAATTTTTTTTTTTTTTTTTTTTTTTATGAACAAAATAATGTGTATATAA
2701 TATGTGTGTAATATCAAATGGCTTGTTCGTTATAAATATTATATTTTTTTTTTTTTTTTT
2761 TTACAGTTTGGCTACCTATTTGGTTCTATAATTTACCGTGTAGGAAATATTATCTTAGAA
2821 AGTAATACAAAAATAAGATAAAAAAATATAATATATAAAATATGTATATTGTTCTTATAT
2881 ATTTTGTTCATATATATATATATATATATATATTTTTATATTTCCATCTGTCTTTTTA
2941 TTCTATTGTTATAATTTATCATAAATTTTTTTTAAATTTGTTTACATTAGGAAAAAAAAT
3001 GAGAAATGAAGAAAATGAAGATTCCGAAGGAGAATTAACCAACGTCGATTCAATTATTAC
3061 ACAATAA
```

7.4.5 *PfATPase6*

***PfATPase6* primary PCR primer (Size PCR product: 896 bp)**

P17-1 for	AATATTGTTATTCAGAATATGATTATAA
P17-1 rev	TGGATCAATAATACCTAATCCACCTA

***PfATPase6* nested PCR primer (Size PCR product: 798 bp)**

P17 for	AGCAAATATTTTCTGTAACGATAATA
P17 rev	TGTTCTAATTTATAATAATCATCTGT

***PfATPase6* C-7 primer**

538 C-7:	ACCGAATTAGCTTTATTACATTT
574 C-7:	GTACAGGTGTTGTATTTTTTTCA
623 C-7:	CCTGAGCTGTAGTATAATTAGAATGGTT
683 C-7:	TTTCTCCAAGAAGAAATACATTCA
769 C-7:	TTAATTTTTTATAAGCAAAGCTAAGT
769B C-7	TAGTAAAGATTTAAATATTAAGAATACAG

***PfATPase6* extension primer**

538:	AAATGTAATAAAGCTAATTCGGT
574:	TGAAAAAAATACAACACCTGTAC
623:	AACCATTCTAATTATACTACAGCTCAGG
683:	TGAATGTATTTCTTCTTGGAGAAA
769:	ACTTAGCTTTGCTTATAAAAAATTAA
769B	CTGTATTCTTAATATTTAAATCTTTACTA

***PfATPase6* sequence (Accession Number: AJ532679)**

P17-1 for
P17 for
P17 rev
P17-1 rev

C-7 and extension primer *ATPase6 538*, *ATPase6* 574, *ATPase6* 623, *ATPase6* 683, *ATPase6* 769
SNP

```
   1 AATATATATATATATATATATATATATATATATATATATATATGTGTTTATTTTATATTATT
  61 TAATTTATTTCGTTGAACTTATTATATCTTTGTCATTCGTGAAATTATTTATTATTATAC
 121 ATAATATTTTGGTTTGTATATAAAGAATGGAAGAGGTTATTAAGAATGCTCATACATACG
 181 ATGTTGAGGATGTACTAAAATTTTTGGATGTAAACAAAGATAATGGTTTAAAGAATGAGG
 241 AATTGGATGATAGAAGATTAAAATATGGTTTGAATGAATTAGAAGTAGAAAAGAAGAAAA
 301 GTATTTTTGAATTGATATTAAATCAATTTGATGATTTATTAGTAAAGATATTATTACTAG
 361 CTGCATTCATTAGTTTCGTGTTAACTTTATTAGATATGAAACATAAAAAAATAGAAATAT
 421 GTGATTTTATTGAACCATTAGTTATAGTATTAATATTAATATTAAATGCTGCCGTAGGTG
 481 TATGGCAAGAATGTAATGCTGAAAAATCTTTAGAAGCTTTAAAAGAATTACAACCTACCA
 541 AAGCTAAAGTATTACGAGATGGGAAGTGGGAAATTATTGATAGTAAATATTTATATGTTG
 601 GTGATATTATTGAATTGAGTGTTGGTAATAAAACTCCCGCTGATGCAAGAATAATTAAAA
 661 TATATTCAACAAGTTTAAAAGTTGAACAGAGTATGTTAACAGGAGAATCCTGTTCAGTTG
 721 ACAAATATGCTGAAAAAATGGAAGATAGTTATAAAAATTGTGAAATACAGTTGAAAAAAA
 781 ATATTTTATTTTCATCTACCGCTATTGTATGTGGTAGATGTATAGCTGTTGTAATCAACA
 841 TAGGTATGAAGACTGAAATAGGTCATATTCAGCATGCTGTTATAGAATCAAATAGTGAAG
 901 ATACTCAAACACCTTTACAAATAAAAATCGATTTATTTGGTCAACAATTATCAAAAATCA
 961 TTTTTGTAATATGTGTAACTGTATGGATTATTAATTTTAAACATTTCTCAGATCCAATTC
1021 ATGGTTCATTTTTATATGGTTGTTTATATTATTTTAAAATTAGTGTTGCTTTAGCTGTTG
1081 CTGCTATACCAGAAGGATTGCCAGCAGTCATAACAACTTGTTTAGCTTTAGGAACAAGAA
1141 GAATGGTAAAAAAAAATGCTATAGTAAGAAAATTACAAAGTGTTGAGACGTTAGGATGTA
1201 CAACGGTTATATGTTCTGATAAAACAGGTACCCTTACAACAAATCAAATGACAACAACCG
1261 TGTTTCATTTGTTTAGAGAATCTGATTCTTTAACAGAATACCAACTATGTCAAAAAGGGG
1321 ATACCTATTACTTTTATGAAAGTTCAAACTTAACAAATGATATATATGCAGGTGAATCAT
1381 CTTTTTTTAATAAATTAAAAGATGAAGGAAATGTTGAAGCTTTAACGGATGATGGAGAAG
1441 AAGGATCAATTGATGAAGCTGATCCATATAGTGATTATTTTTCTAGTGATAGTAAGAAAA
1501 TGAAAAATGATTTAAACAACAACAATAATAATAATAATAGTAGTAGGAGTGGTGCTA
1561 AGAGGAATATTCCTTTAAAAGAAATGAAATCAAATGAAAATACAATAATAAGTAGAGGTA
1621 GTAAAATATTAGAAGATAAAATTAATAAATATTGTTATTCAGAATATGATTATAATTTTT
1681 ATATGTGTTTAGTAAATTGTAATGAAGCAAATATTTTCTGTAACGATAATAGTCAAATAG
1741 TAAAAAAATTTGGAGACAGTACCGAATTAGCTTTATTACATTTTGTACATAATTTTGATA
1801 TATTACCAACATTCTCTAAAAATAATAAAATGCCAGCAGAATATGAAAAAAATACAACAC
1861 CTGTACAATCATCAAATAAGAAGGATAAATCACCAAGGGGTATCAACAAATTCTTTAGTT
1921 CAAAAAATGATAACAGTCATATTACCAGTACATTGAATGAAAATGATAAGAATTTAAAGA
1981 ATGCTAACCATTCTAATTATACTACAGCTCAGGCAACAACAAATGGATATGAAGCTATAG
2041 GAGAAAATACATTTGAGCATGGCACAAGTTTTGAAAATTGTTTCCACTCAAAATTGGGTA
2101 ATAAAATAAATACCACATCAACACATAATAATAATAACAACAATAATAATAATAGTAATA
2161 GTGTTCCAAGTGAATGTATTTCTTCTTGGAGAAATGAATGTAAACAAATAAAAATTATTG
2221 AATTCACTAGAGAAAGGAAACTTATGAGTGTTATTGTTGAAAATAAAAAAAAAGAAATAA
2281 TATTGTATTGTAAAGGTGCACCTGAGAATATAATAAAAAATTGTAAATATTATTTAACGA
2341 AAAATGATATACGTCCATTAAATGAAACTTTAAAAAATGAAATTCATAATAAGATTCAAA
2401 ATATGGGAAAAAGAGCATTAAGAACACTTAGCTTTGCTTATAAAAAATTAAGTAGTAAAG
2461 ATTTAAATATTAAGAATACAGATGATTATTATAAATTAGAACAAGATTTAATTTATTTAG
2521 GTGGATTAGGTATTATTGATCCACCACGTAAATATGTAGGAAGAGCAATTAGATTATGCC
2581 ATATGGCTGGTATACGTGTATTTATGATTACAGGTGATAATATTAATACGGCCAGAGCTA
2641 TAGCTAAAGAAATTAATATATTAAATAAAAATGAAGGAGATGATGAAAAGGATAATTATA
2701 CAAATAATAAAAATACACAAATATGTTGTTATAATGGAAGAGAATTTGAAGATTTTTCAT
2761 TAGAAAAGCAAAAACATATTTTAAAAAATACACCAAGAATTGTTTTCTGTAGAACTGAAC
2821 CTAAACATAAAAAACAAATAGTAAAAGTATTAAAAGACTTAGGAGAAACAGTTGCTATGA
2881 CAGGTGATGGTGTAAATGATGCACCAGCATTGAAATCAGCTGACATAGGAATAGCTATGG
2941 GTATTAATGGAACGGAGGTAGCTAAAGAAGCATCAGATATTGTTTTAGCTGATGATAATT
3001 TTAATACTATAGTTGAAGCAATTAAAGAAGGAAGATGTATATATAATAATATGAAAGCAT
```

7.4.6 Position controls

BIN CONTROL Cy3: TAATTATGAAAATAAAGGAG

BIN CONTROL Cy5: TAATTATGAAAATAAAGGAG

7.4.5 Flexible primers

NOTE:

- Regular: C-7 flexi tags free
- *Italic*: flexi extension tags free
- **Bold**: flexi C-7 tags in use!!!
- *Italic underlined*: flexi extension tags in use!!!

- **Flexible C-7 primers**

01 C-7:	**ACGCCGGAACGCCGGAACGCCGGA**	**(*crt* 72)**
02 C-7:	**ACGGGGCAACGGGGCAACGGGGCA**	**(*crt* 74)**
03 C-7:	CAAGGCGCCAAGGCGCCAAGGCGC	
04 C-7:	CAGCGGCACAGCGGCACAGCGGCA	
05 C-7:	CCACACGGCCACACGGCCACACGG	
06 C-7:	**GGGAGAGCGCAGCAGGCAACAGAG**	**(*dhfr* 164)**
07 C-7:	**GACCGCCACCAAGAACAGCACCGG**	**(*dhfr* 164B)**
08 C-7:	GCGCCAACGCAGACCGGAAGACCA	
09 C-7:	**CGCGCACAGAAGGGCGAGAGACGA**	**(*dhps* 613B)**
010 C-7:	**GCAGGGAAGCGGGAGCGAAACAGC**	**(*dhfr* 108)**
011 C-7:	**AAAACGGGGCACAGCGCGGCGGAA**	**(*dhfr* 108B)**
12 C-7:	CCCCGAGAACGCCCGAAGCACAAG	
13 C-7:	**GGGAACGACACAGACAAGCCGGGG**	**(*mdr1* 184)**
14 C-7:	GGCGGGAACCGAAGACAGGGGAAG	
15 C-7:	GACAACGGGCAGCGACCGGACCAA	
16 C-7:	CCCAAAGCCCGCAACCCGACCAAC	
17 C-7:	*CAGGAACCAAGCCAGCCAGAGGCC*	
18 C-7:	ACACCACAGGACACACGCCCCAGG	

- **Flexible extension primers**

01/72:	*TCCGGCGTTCCGGCGTTCCGGCGT*TTTTAAGTATTATTTATTTAAGTGTA
02/74:	*TGCCCCGTTGCCCCGTTGCCCCGT*TATTATTTATTTAAGTGTATGTGTAAT
03/:	*GTTCCGCGGTTCCGCGGTTCCGCG*
04/:	*GTCGCCGTGTCGCCGTGTCGCCGT*
05/:	*GGTGTGCCGGTGTGCCGGTGTGCC*
06/164:	*CTCTGTTGCCTGCTGCGCTCTCCC*GGGAAATTAAATTACTATAAATGTTTTATT
07/164B:	*CCGGTGCTGTTCTTGGTGGCGGTC*TTCTTGATAAACAACGGAACCTCCTA
08/:	*TGGTCTTCCGGTCTGCGTTGGCGC*
09/613B:	*TCGTCTCTCGCCCTTCTGTGCGCG*ATTTTGATCATTCATGCAATGGG
10/108:	*GCTGTTTCGCTCCCGCTTCCCTGC*CAAAATGTTGTAGTTATGGGAAGAACAA
11/108B:	*TTCCGCCGCGCTGTGCCCCGTTTT*AAAGGTTTAAATTTTTTTGGAATGCTTTCCCAG
12/:	*CTTGTGCTTCGGGCGTTCTCGGGG*
13/184:	*CCCCGGCTTGTCTGTGTCGTTCCC*TGCCAGTTCCTTTTTAGGTTTAT
14/:	*CTTCCCCTGTCTTCGGTTCCCGCC*
15/:	*TTGGTCCGGTCGCTGCCCGTTGTC*
16/:	*GTTGGTCGGGTTGCGGGCTTTGGG*
17/:	*GGCCTCTGGCTGGCTTGGTTCCTG*
18/:	*CCTGGGGCGTGTGTCCTGTGGTGT*

7.5 Oligonucleotide array on the chip

Cy5 cont.	86	13/184	1034	1042	1246	436	437	540	581	613
09/613B	640	645	16	51	59	10/108	11/108B	06/164	07/164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg2	CRT Neg3	DHPSNeg4	Cy3 cont.
769B	02/74	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*
Cy5 cont.	86	13/184	1034	1042	1246	436	437	540	581	613
09/613B	640	645	16	51	59	10/108	11/108B	06/164	07/164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg2	CRT Neg3	DHPSNeg4	Cy3 cont.
769B	02/74	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*
Cy5 cont.	86	13/184	1034	1042	1246	436	437	540	581	613
09/613B	640	645	16	51	59	10/108	11/108B	06/164	07/164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg2	CRT Neg3	DHPSNeg4	Cy3 cont.
769B	02/74	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*	*Empty!*

- galDesigner software: Template saved as ***malaria.sti***

7.5b Oligonucleotide array on the chip

(Intermediate batch designed by Jutta in May 2006 = last batch of slides from *Genopole*!)

Cy5 cont.	86	184	1034	1042	1246	436	437	540	581	613
613B	640	645	16	51	59	108	108B	164	164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg 2	CRT Neg 3	DHPS Neg 4	Cy3 cont.
769B	02/74	13/184/	09/613B	10/108	11/108B	06/164	07/164B	12/	*Empty!*	*Empty!*
Cy5 cont.	86	184	1034	1042	1246	436	437	540	581	613
613B	640	645	16	51	59	108	108B	164	164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg 2	CRT Neg 3	DHPS Neg 4	Cy3 cont.
769B	02/74	13/184/	09/613B	10/108	11/108B	06/164	07/164B	12/	*Empty!*	*Empty!*
Cy5 cont.	86	184	1034	1042	1246	436	437	540	581	613
613B	640	645	16	51	59	108	108B	164	164B	01/72
DHFR Neg1	75B1	76	97	152	163	220	271	326	326B	371
356	356B	538	574	623	683	769	MDR Neg 2	CRT Neg 3	DHPS Neg 4	Cy3 cont.
769B	02/74	13/184/	09/613B	10/108	11/108B	06/164	07/164B	12/	*Empty!*	*Empty!*

- galDesigner software: Template saved as ***genopole_May06.sti***

For slide printing, 0.5 nl of a 50 μM solution of the C-7 primers are used per spot (Prior to printing, the 500 μM C7-primer stock solutions have to be diluted 1:10 in 180 mM phosphate buffer pH 8.0).

7.6 SNPs on the chip

Pfdhps	**Polymorphisms**			**CHIP**				
436	TCT→**G**CT/T**T**T	Ser→**Ala/Phe**	S→A/F	TCT→**G**CT	CII	**S→A**	Cy3=WT	Cy5=MUT1
437* **	GCT→G**G**T	Ala→**Gly**	A→G	GCT→G**G**T	CI	**A→G**	Cy3=MUT	Cy5=WT
540	AAA→**G**AA	Lys→**Glu**	K→E	AAA→**G**AA	CI	**K→E**	Cy3=WT	Cy5=MUT
581	GCG→G**G**G	Ala→**Gly**	A→G	GCG→G**G**G	CI	**A→G**	Cy3=WT	Cy5=MUT
613	GCC→**A**CC	Ala→**Thr**	A→T	GCC→**A**CC	CI	**A→T**	Cy3=MutA	Cy5=WT or MutB
613B* **	GCC→**T**CC	Ala→**Ser**	A→S	GCC→**T**CC	CII	**A→S**	Cy3=WT or MutA	Cy5=MutB
640* **	ATT→**T**TT	Ile→**Phe**	I→F	ATT→**T**TT	C I	**I→F**	Cy3=MUT	Cy5=WT
645	CAC→C**C**C	His→**Pro**	H→P	CAC→C**C**C	CII	**H→P**	Cy3=MUT	Cy5=WT
Pfdhfr	**Polymorphisms**			**CHIP**				
16	GCA→G**T**A	Ala→**Val**	A→V	GCA→G**T**A	CI	**A→V**	Cy3=WT	Cy5=MUT
51	AAT→A**T**T	Asn→**Ile**	N→I	AAT→A**T**T	CI	**N→I**	Cy3=WT	Cy5=MUT
59* **	TGT→**C**GT	Cys→**Arg**	C→R	TGT→**C**GT	CI	**C→R**	Cy3=WT	Cy5=MUT
108	AGC→A**A**C	Ser→**Asn**	S→N	AGC→A**A**C	CI	**S→N**	Cy3=MutA or MutB	Cy5=WT
108B* **	AGC→A**C**C	Ser→**Thr**	S→T	AGC→A**C**C	CII	**S→T**	Cy3=WT or MutA	Cy5=MutB
164	ATA→**T**TA	Ile→**Leu**	I→L	ATA→**T**TA	CI	**I→L**	Cy3=WT	Cy5=MUT
164B* **	ATA→**T**TA	Ile→**Leu**	I→L	ATA→**T**TA	CII	**I→L**	Cy3=WT	Cy5=MUT
Pfmdr1	**Polymorphisms**			**CHIP**				
86	AAT→**T**AT	Asn→**Tyr**	N→Y	AAT→**T**AT	CI	**N→Y**	Cy3=WT	Cy5=MUT
184	TAT→T**T**T	Tyr→**Phe**	Y→F	TAT→T**T**T	CI	**Y→F**	Cy3=WT	Cy5=MUT
1034	AGT→**T**GT	Ser→**Cys**	S→C	AGT→**T**GT	CI	**S→C**	Cy3=WT	Cy5=MUT
1042* **	AAT→**G**AT	Asn→**Asp**	N→D	AAT→**G**AT	CI	**N→D**	Cy3=MUT	Cy5=WT
1246	GAT→**T**AT	Asp→**Tyr**	D→Y	GAT→**T**AT	CII	**D→Y**	Cy3=MUT	Cy5=WT
Pfcrt	**Polymorphisms**			**CHIP**				
72	TGT→**A**GT	Cys→**Ser**	C→S	TGT→**A**GT	CI	**C→S**	Cy3=MUT	Cy5=WT
74	ATG→AT**T**	Met→**Ile**	M→I	ATG→ATT	CII	**M→I**	Cy3=MUT	Cy5=WT
75B1	AAT→**G**AT/**GA**A	Asn→**Asp/Glu**	N→D/E	AAT→**G**AT/**G**AA	CI	**N→D or E**	Cy3=WT	Cy5=MUT
76* **	AAA→A**C**A	Lys→**Thr**	K→T	AAA→A**C**A	CII	**K→T**	Cy3=WT	Cy5=MUT
97	CAC→CA**A**	His→**Gln**	H→Q	CAC→CA**A**	CII	**H→Q**	Cy3=WT	Cy5=MUT
152	ACT→**G**CT	Thr→Ala	T→A	ACT→**G**CT	CI	**T→A**	Cy3=WT	Cy5=MUT
163	AGT→AG**G**	Ser→Arg	S→R	AGT→AG**G**	CII	**S→R**	Cy3=WT	Cy5=MUT
220	GCC→**T**CC	Ala→**Ser**	A→S	GCC→**T**CC	CII	**A→S**	Cy3=MUT	Cy5=WT
271	CAA→**G**AA	Gln→**Glu**	Q→E	CAA→**G**AA	CI	**Q→E**	Cy3=WT	Cy5=MUT
326	AAC→**G**AC	Asn→**Asp**	N→D	AAC→**G**AC	CI	**N→D**	Cy3=WT	Cy5=MUT1
326B* **	AAC→A**G**C	Asn→**Ser**	N→S	AAC→A**G**C	CI	**N→S**	Cy3=MUT2	Cy5=WT
356	ATA→**T**TA	Ile→**Leu**	I→L	ATA→**T**TA	CI	**I→L**	Cy3=WT	Cy5=MUT1
356B* **	ATA→A**C**A	Ile→**Thr**	I→T	ATA→A**C**A	CI	**I→T**	Cy3=WT	Cy5=MUT2
371	AGA→A**T**A	Arg→**Ile**	R→I	AGA→A**T**A	CII	**R→I**	Cy3=MUT	Cy5=WT
PfATPase6	**Polymorphisms**			**CHIP**				
538* **	AGT→AG**C**	Ser→**Arg**	S→R	AGT→AG**C**	CI	**S→R**	Cy3=MUT	Cy5=WT
574	CAA→C**C**A	Gln→**Pro**	Q→P	CAA→C**C**A	CII	**Q→P**	Cy3=MUT	Cy5=WT
623	GCA→G**A**A	Ala→**Glu**	A→E	GCA→G**A**A	CII	**A→E**	Cy3=WT	Cy5=MUT
683	AAT→AA**G**	Asn→**Lys**	N→K	AAT→AA**G**	CII	**N→K**	Cy3=WT	Cy5=MUT
769	AGT→A**A**T	Ser→**Asn**	S→N	AGT→A**A**T	CI	**S→N**	Cy3=MUT	Cy5=WT
769B* **	AGT→A**A**T	Ser→**Asn**	S→N	AGT→A**A**T	CI	**S→N**	Cy3=WT	Cy5=MUT

(*** = extension on antisense strand)

7.7 PCR conditions for QIAGEN® *Taq* polymerase

7.7.1 Primary PCR mix

Reagents	**1 reaction**	**14 reactions**
H_2O	33.25 µl	465.5 µl
10 x buffer (**containing 15 mM $MgCl_2$!**)	5.0 µl	70.0 µl
dNTP mix (2mM)	5.0 µl	70.0 µl
$MgCl_2$ (25mM)	3.0 µl	42.0 µl
Primary PCR primer mix (10 µM each)	1.0 µl	14.0 µl
Taq Polymerase 5U/ µl	0.25 µl	3.5 µl
Final Volume	**47.5 µl**	**665.0 µl**
DNA	2.5 µl	
Final Volume	**50.0 µl**	

6.7.2 Primary PCR program

96 °C 180 Sec

96 °C 30 sec
52 °C 90 sec **<u>20 cycles</u> for clinical (symptomatic) samples**
72 °C 90 sec **<u>25 cycles</u> for community (asymptomatic) samples**

Hold at 4 °C

7.7.3 Nested PCR mix

Reagents	**1 reaction**	**14 reactions**
H_2O	66.5 µl	931.0 µl
10 x buffer (**containing 15 mM $MgCl_2$!**)	10.0 µl	140.0 µl
dNTP mix (2mM)	10.0 µl	140.0 µl
$MgCl_2$ (25mM)	6.0 µl	84.0 µl
Nested PCR primer mix (10 µM each)	2.0 µl	28.0 µl
Taq Polymerase 5U/ µl	0.5 µl	7.0 µl
Final Volume	**95.0 µl**	**1330.0 µl**
Primary PCR product	5.0 µl	
Final Volume	**100.0 µl**	

6.7.4 Nested PCR program

96 °C 180 sec

96 °C 30 sec
52 °C 90 sec **<u>20 cycles</u> for clinical (symptomatic) samples**
72 °C 90 sec **<u>25 cycles</u> for community (asymptomatic) samples**

Hold at 4 ° C

Pfmsp2 primers

- S2-for: GAA GGT AAT TAA AAC ATT GTC
- S3-rev: GAG GGA TGT TGC TGC TCC ACA G

- S1-for: GAG TAT AAG GAG AAG TAT
- S4-rev: CTA GAA CAA TGC ATA TGT CC

- M5-rev: GCA TTG CCA GAA CTT GAA
- N5-rev: CTGA AGA GGT ACT GGT AGA
- S_{TAIL}-for: 7bpTail-GCT TAT AAT ATG AGT ATA AGG AGA A