MOLECULAR MARKERS OF DRUG RESISTANCE AND CLINICAL OUTCOME IN FALCIPARUM MALARIA IN CAMBODIA AND THE DEMOCRATIC REPUBLIC OF CONGO

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ABSTRACT

Molecular Markers of drug resistance and clinical outcome in falciparum malaria in Cambodia and the Democratic Republic of Congo
(under the direction of Steven R. Meshnick)

Background. Drug resistance is a major obstacle to the control of *Plasmodium falciparum* malaria. Monitoring the efficacy of antimalarials is a critical component to malaria control. One possible surveillance method is to use molecular markers. However, their relationship with clinical resistance needs to be established before they can be used.

Methods. Clinical samples from an in vivo efficacy study of sulfadoxine-pyrimethamine (SP) based therapy in Rutshuru, Democratic Republic of Congo were used to estimate the effect of mutations in dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) on treatment failure. In addition, clinical samples from an in vivo efficacy study of mefloquine-artesunate in Pailin, Cambodia were used to estimate the effect of changes in the *P. falciparum* multidrug resistance-1 (*pfmdr1*) gene on recrudescence. Lastly, a cross-sectional survey of Cambodia was conducted to determine the geographic distribution of the genetic changes in *pfmdr1*.

Results. In Rutshuru, the effect of mutations at *dhps*-437 and *dhps*-540 on SP treatment failure differed by level of parasitemia: for children with low parasitemia, the presence of both mutations was associated with a 17% (95%CI: -3%, 36%) greater absolute risk of treatment failure compared to having neither mutation (baseline risk: 21%). For children with high parasitemia, the risk difference was 50% (95%CI: 35%, 64%; baseline risk: 8%). In Pailin, *pfmdr1* copy number was strongly related to time to recrudescence after mefloquine-

artesunate treatment (adjusted HR = 7.91, 95%CI: 2.38, 26.29). In the cross-sectional study of Cambodia, increased *pfmdr1* copy number was found not only in Pailin, where mefloquine resistance has been well documented, but also in Chumkiri, an area not previously known for drug resistance.

Conclusion. These studies demonstrated *dhps* mutations are associated with SP treatment failure in the DRC and *pfmdr1* copy number is associated with recrudescence after mefloquine-artesunate treatment in Cambodia. The cross-sectional study demonstrates how the use of a molecular marker can be operationalized to detect resistant areas outside of current sentinel surveillance sites. Detecting *dhps* mutations in DRC and *pfmdr1* copy number in Cambodia are potentially cost-effective methods to complement the current in vivo efficacy monitoring of drug resistance in these countries.

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LIST OF ABBREVIATIONS

Ala	Alanine
Arg	Arginine
Asn	Asparagine
CDC	Center for Disease Control and Prevention
CNM	National Center for Parasitology, Entomology and Malaria Control
Cys	Cysteine
dhfr	dihydrofolate reductase
dhps	dihydropteroate synthase
DNA	deoxyribonucleic acid
DRC	Democratic Republic of Congo
Glu	Glutamine
glurp	Glutamine rich protein
Gly	Glycine
Hct	Hematocrit
Hgb	Hemoglobin
HIV	Human Immunodeficiency Virus
HR	Hazard Ratio
IC_{50}	50% inhibitory concentration
Ile	Isoleucine
Lys	lysine
MQ	mefloquine
msp1	merozoite surface protein 1
msp2	merozoite surface protein 2
NAMRU-2	United States Naval Medical Research Unit Number 2
PCR	Polymerase Chain Reaction
pfcrt	*Plasmodium falciparum* chloroquine transporter
pfmdr1	*Plasmodium falciparum* multidrug resistance gene 1
Pgh1	P-glycpprotein homologue 1
Phe	Phenylalanine
RBC	Red Blood Cell
RD	Risk Difference
RR	Risk Ratio
Ser	Serine
SNP	Single Nucleotide Polymorphism
SP	Sulfadoxine-Pyrimethamine
Thr	Threonine
Tyr	Tyrosine
WBC	White Blood Cell
WHO	World Health Organization

CHAPTER I:

REVIEW OF MALARIA AND DRUG RESISTANCE

MALARIA BIOLOGY

Malaria is a mosquito-borne illness caused by parasites in the genus *Plasmodium*. The main *Plasmodium* species that infect humans are: *P. falciparum*, *P. vivax*, *P. ovale,* and *P. malariae. P. falciparum* infection is the most common and the most lethal.

P. falciparum has a complex life cycle (Figure 1.1). An infected mosquito injects sporocytes into the human host during a blood meal. The sporocytes travel to the liver, where they infect hepatocytes. The parasite rapidly divides, which eventually causes the hepatocyte to lyse. Merozoites are released into the circulation, where they infect red blood cells (RBCs) and transform into trophozoites. The trophozoites divide into merozoites, which are released upon lysis of the RBC. Then the merozoites infect other RBCs. A small percentage of trophozoites mature into gametocytes, which are infectious to mosquitoes. When a mosquito takes a blood meal on an infected host, gametocytes are ingested as well. In the gut of the mosquito, the gametocytes undergo sexual reproduction to form sporocytes, which migrate to the salivary glands of the mosquito. At this point, the mosquito is considered infectious and is capable of transmitting the sporocytes to a human during their next blood meal (1).

Malarial infections are often multiclonal, which means there is more than one genetically distinct clone in an infection. Multiclonal infections can be caused by presence of multiple clones in a mosquito or being bitten by more than one infected mosquito. Infections with more than 10 clones do occur, but most infections have 1-3 clones. There are more multiclonal infections in areas of high malaria transmission compared to low transmission areas.

PUBLIC HEALTH IMPACT OF FALCIPARUM MALARIA

Falciparum malaria is a significant public health problem in the developing world. Approximately 40% of the world's population is at risk of malaria (2). Control efforts have eliminated malaria from temperate areas but have been largely unsuccessful in tropical areas. In 2002, there were over 500 million clinical cases of *Plasmodium falciparum* (3). Most of these clinical cases occurred in Africa (70%) and Southeast Asia (25%) (3).

Each year, approximately 1 million people die of *P. falciparum* malaria, most of which are African children (4). Malaria is the leading cause of death in children in sub-Saharan Africa: approximately 18% of all child deaths in this region was attributed to falciparum malaria in 2000 (5).

Falciparum malaria is also a significant source of morbidity. In children, malaria infection can lead to chronic anemia, malnutrition, and impaired growth (6-8). Cerebral malaria can cause long-term neurological and cognitive impairment, including deficits in memory, attention, and language (9). Malaria in pregnant women can lead to intrauterine growth restriction and preterm birth, which are both risk factors for infant mortality (10).

EPIDEMIOLOGY OF FALCIPARUM MALARIA

The epidemiology of malaria differs significantly by the endemicity of the region. In hyperendemic areas, the risk of having a symptomatic illness is highest in children between the ages of 6 months and 5 years (11). Adults rarely have severe disease (because the development of partial immunity). One exception is pregnant women, who are susceptible to strains of *P. falciparum* that bind to the placenta (12). In hypoendemic regions, there is less development of immunity, and as a result, malaria causes clinical disease in all age groups.

The epidemiology of falciparum malaria is also influenced by the vector. Anopheles mosquitoes transmit malaria. The particular species involved in transmission differs by region and also might change by season. Each species differs slightly in behavior, habitat and transmission efficiency, which in turn influence malaria dynamics. The main species complexes involved in transmission in Africa are: *A. gambiae, A. arabiensis, A. funestus, A. nili,* and *A. moucheti* (13). The most common vector, *A. gambiae*, lives and feeds around human settlements. It feeds mostly in the evening and at night. The amount of standing water and the density of animals will influence the mosquito population, and in turn will influence malaria dynamics.

In Southeast Asia, the major vectors are *A. dirus* and *A. minimus*, which are located in forest and forest-fringe areas, respectively (14). The geographic distribution of falciparum malaria in this area can be partially explained by forestation. In addition, traveling to the deep forest is associated with increased risk of malaria.

CLINICAL PRESENTATION OF MALARIA

P. falciparum malaria is predominately a febrile illness, though the fever may fluctuate. Other symptoms include headache, anemia, vomiting, and diarrhea. Repeated malaria infections promote the development of partial immunity (15). Infected individuals with partial immunity are either asymptomatic or experience mild clinical signs and symptoms.

Malaria is diagnosed by detecting parasites in the peripheral blood, either through microscopy, rapid antigen tests, or PCR (16). The density of parasites in the blood (parasitemia) is the main prognostic indicator of clinical outcome.

Severe malaria is defined as having any of the following conditions: hyperparasitemia (>100,000 parasites / μl in hypoendemic regions and >200,000 parasites / μl in hyperendemic regions), impaired consciousness, respiratory distress, or severe anemia (17).

DRUG RESISTANCE: DEFINITION AND CLINICAL MANIFESTATION

The technical definition of antimalarial drug resistance is "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject" (18). To definitively prove that an infection is resistant to therapy, either adequate levels of the drug in the blood have to be demonstrated or resistance of the isolate has to be confirmed in vitro. The falciparum strain present after treatment also has to be shown to be identical to the strain present before treatment (16).

However, many *P. falciparum* infections are neither fully resistant nor completely sensitive to antimalarial drugs (19). Therefore, it is more useful to view resistance as a gradient of increasing tolerance of the parasite to the antimalarial drug.

The normal response of a *P. falciparum* infection to treatment with an effective antimalarial is the abatement of fever and the disappearance of parasites within three days. The first sign of increased resistance is the delayed clearance of parasitemia. Moderate resistance is characterized by an initial disappearance of parasitemia followed by the reappearance of parasites after the concentration of the antimalarial has decreased to below the IC_{50} (20). Antimalarial drugs vary considerably in half-lives, and therefore the time before the reappearance of parasites varies as well; SP failures appear 7-28 days after

treatment while mefloquine failures appear up to 63 days after treatment. A high level of drug resistance manifests as no parasite clearance after treatment.

The clinical manifestation of drug resistance is influenced by immunity. Older children and adults are more likely to clear resistant parasites than younger children because of the higher level of immunity (21). Therefore, in Africa and other highly endemic areas, clinical studies of antimalarial resistance are often restricted to children under 5 years of age.

It is important to note that in vivo treatment failure can be caused by factors other than drug resistance, such as inadequate dosing or absorption, lack of adherence, vomiting, lack of immunity, and the acquisition of a new infection (22).

ANTIMALARIALS AND THE HISTORY OF DRUG RESISTANCE

Chloroquine

Chloroquine was widely used for treatment and prophylaxis in the second half of the 20th century. This drug is cheap, effective, and causes few side effects. Resistance was first recorded in South America and the Thai-Cambodian border in the late 1950's (23). Chloroquine resistance also developed independently in Paupa New Guinea and a second time in South America (24). Chloroquine resistance spread from these four loci to much of the world. In 2000, chloroquine resistance was present in almost all countries with falciparum malaria (25).

In Malawi, there has been a re-emergence of chloroquine-sensitive falciparum malaria 10 years after chloroquine was stopped being used (26). As a result, the re-introduction of chloroquine (in combination with artesunate) is currently being considered in Malawi.

Sulfadoxine-Pyrimethamine

Sulfadoxine-pyrimethamine (SP) was the main replacement of chloroquine in Southeast Asia and Africa. Resistance to this combination emerged quickly: resistance was documented in Southeast Asia one year after its introduction (27). While moderate SP resistance has multiple origins, all parasites with a high level of SP resistance come from a single origin in Southeast Asia (28, 29).

In Cambodia and Thailand, the rapid emergence of SP resistance made this drug unusable by the 1980's. Even though SP has not been used in this area for over 20 years, recent surveys found that the parasites have remained resistant (30, 31).

In Africa, moderate SP resistance is widespread, with a few pockets of high SP resistance (27). The median SP failure rate in Central and South Africa is around 8.9% while the median for Western Africa is slightly lower (32). However, 14-day failure rates greater than 30% at one or more sites have been reported from Tanzania, Rwanda, Burundi, Kenya, and Liberia (33-35).

Mefloquine

Mefloquine has predominately been used in Southeast Asia and South America. In Southeast Asia, mefloquine was first used in the 1980's in combination with SP. Mefloquine resistance was documented a few years later at the Thai-Cambodian border (36). Mefloquine resistance then spread to the Thai-Burmese border (14, 37). The decreasing efficacy of mefloquine prompted policy makers to increase the recommended dose of mefloquine and later to combine mefloquine with artesunate (38). On the Thai-Burmese border, the efficacy of mefloquine-artesunate has remained high since its introduction in 1994 (37). The

incidence of malaria in this region has also declined, possibly due to in part of the effectiveness of this combination. The mefloquine-artesunate combination has had less success on the Thai-Cambodian border. A recent study found that the 28 day failure rate was over 20% in this area, which suggests resistance is still a problem (39).

Amodiaquine

Little is known about resistance to amodiaquine. Amodiaquine has not been used that extensively in the past, however, many African countries have recently switched to amodiaquine-combination therapies (32). Clinical resistance to amodiaquine has been documented: in Rwanda, amodiaquine monotherapy had >25% 28-day failure rates two years after the implementation of-SP for the treatment of uncomplicated malaria (40).

Artemisinin compounds

Clinical resistance to an artemisinin compound, such as artesunate, has yet to be documented (41). These compounds are rarely used in monotherapy for the treatment of uncomplicated malaria because they are not as efficacious as the other antimalarials and often require multiple doses.

Plasmodium falciparum clinical isolates with decreased sensitivity to artesunate and artemether has been documented in Central African Republic, Senegal, and French Guiana (42, 43). In addition, genetically stable *P. chabaudi* clones have been developed with 15 times greater resistance to artesunate (44). These two pieces of evidence suggest *Plasmodium spp.* is capable of developing a high level of resistance to this class of compounds. Therefore, it is not a question of whether resistance will emerge but when will it occur.

STRATEGIES FOR COMPATING DRUG RESISTANCE

One of the main strategies employed to impede the emergence of drug resistance is combination therapy. By simultaneously using two drugs with independent modes of action, the probability of the parasite evolving resistance to both drugs is dramatically decreased (45). In addition, certain drugs are synergistic, and therefore the efficacy of the combination is greater than either drug alone.

The main partner drug using in combination therapies is artesunate. Artesunate is a very potent antimalarial with a rapid half-life that causes a dramatic drop in parasite levels (20). It is not that effective at curing malaria on its own, but increases the efficacy of other antimalarials when given in combination (46). In addition, artesunate is gametocydal, which decreases the transmissibility of malaria and slows down the transmission of drug resistant strains.

Another key element to a successful program to fight drug resistance is the monitoring and evaluation of drug efficacy (18, 47). To decrease the impact of drug resistance on the morbidity and mortality of malaria, policy makers need to make informed decision in a timely manner about when to switch antimalarial regiments and what combinations should be used next. This can only be accomplished if they have access to current data on the effectiveness of antimalarials.

IN VIVO EFFICACY STUDY

The gold standard in monitoring parasite resistance is the in vivo test. This study involves recruiting symptomatic and parasitemic patients, treating them with the drug of interest under

observation, and following them for 14-63 days for evidence of treatment failure (i.e. return of asexual parasites) (17). The World Health Organization (WHO) has standardized the protocol, which varies slightly by the endemicity of the region and the drugs being tested (17, 48). The in vivo efficacy study is used not only to assess the current regimen, but also to determine the efficacy of potential replacement regimens.

The main outcome of the in vivo efficacy study is treatment failure, which is defined as a worsening of the disease or the recurrence of parasites (Table 1.1). Treatment failure is classified according to when the treatment failure occurred (early: within 3 days; late: after 3 days) and whether the subject was symptomatic (clinical: presence of fever or signs of severe disease; parasitologic: no fever or signs of severe disease).

The main advantage of the in vivo efficacy study is that the results are easy to interpret and they appeal to policy makers (49). However, there are numerous disadvantages to these studies. First of all, treatment failure is a rough measure of clinical resistance. Most cases of treatment failure are caused by drug resistance but there are many other contributing causes (i.e. inadequate drug absorption or rapid elimination) (32). In addition, the results are not always applicable to the general population. For example, these studies often take place in areas with adequate health facilities, which are not always representative of a region. In addition, people with severe malaria, who are pregnant, or have infections mixed-species infections, are excluded. An additional disadvantage of the in vivo study is they are time-consuming, especially when a 42-day follow-up is needed.

One of the major complication of the in vivo efficacy study is that reoccurrence of parasites can be caused by both treatment failure (recrudescence) and a new infection. The classic way to distinguish between these two possibilities is the Snounou method. This

approach involves genotyping three variable genes (*msp-1*, *msp-2*, *glurp*) through size-fractionation (50). The appearance of a variant not seen in the enrollment sample is often interpreted as a re-infection while no new variants is considered recrudescence. Even though this method is the standard in the field, the validity of this method has been questioned. For example, there are multiple ways to interpret the results. While some investigators consider a a re-infection as the appearance of a new variant irrespective if the recurrence sample contains variants seen in the original sample, others consider a re-infection when the recurrence sample does not contain any of the variants in the original infection (51). This technique has also been shown to underestimate the genetic complexity of infections (52). Therefore, the second infection might appear genetically identical to the enrollment sample when in fact they are different.

Distinguishing between recrudescence and re-infection is more important in areas in high transmission areas and when the study has a long follow-up period because these factors are associated with increased risk of re-infection. The current WHO recommendation is to conduct molecular analysis of infection/re-infection in studies with greater than 14 days of follow-up in high transmission areas and in studies with greater than 28 days of follow-up in low transmission areas (32).

One of the main criticisms of the implementation of the in vivo efficacy study is that the follow-up time is too short (53). The WHO recommends follow-up periods of 28 days or greater for antimalarials with extended half-lives, such as SP, mefloquine, lumefantrine, amodiaquine, and chloroquine (17, 32). However, shorter durations of follow-up are used in practice. In Africa, the 14-day in vivo efficacy study for SP has been used extensively (17, 34). The 14-day in vivo efficacy study clearly is underestimating the true treatment failure

rate, as SP failures have been documented 28 days after treatment (54). But the 14-day study is still useful in comparing resistance in different regions or over time. In addition, the results can be interpreted without molecular genotyping, as the risk of re-infection is low in the two weeks following treatment.

Another criticism of the in vivo efficacy study is the use of a per protocol data analysis strategy (55). The per protocol strategy is analyzing the data after all subjects lost to follow-up or excluded are removed from the dataset. Often 10% of the enrolled subjects are excluded or lost to follow-up, yet greater exclusion rates do occur (33). If these exclusions are related to the outcome, then the results may be biased and the resulting treatment failure rate may not reflect the true rate in the population.

Per protocol analysis is even more of a problem when two different drugs are being compared (56, 57). Differential exclusion or loss to follow-up could be occurring in the in vivo efficacy study, as subjects who are feeling ill (possibly due to malaria) are more likely to return for their follow-up visit and also are more likely to take an antimalarial outside the protocol. To prevent these potential biases, in vivo efficacy studies should be analyzed either using intention to treat (which classifies all exclusions or losses to follow-up as treatment failures) or by conducting sensitivity analyses.

IN VITRO METHODS

In vitro tests involve culturing clinical samples in serial dilutions of drug to determine the IC_{50} (the concentration of the drug at which 50% of parasite growth is inhibited). An isolate is considered resistance when the IC_{50} is above a certain threshold. The main advantage of this technique is that it directly measures parasite resistance without being confounded by clinical characteristics. In addition, the actual mechanism of drug resistance does not need to

be known to measure resistance. However, this technique suffers from various flaws. First of all, the assay involves using radioactivity, which is not always available in developing countries. Samples with low parasitemias cannot be reliability assessed. Certain drugs, such as amodiaquine, exert their antimalarial effect partially through metabolites, which is more difficult to reenact in an in vitro system. Technical issues prevent some drugs (i.e. sulfadoxine) from being tested in this manner. Finally, due to selective forces resulting from culturing, the results do not always correspond to the actual situation in vivo (58).

MOLECULAR MARKERS AS A PROPOSED SURVEILLANCE TOOL

Molecular markers are genetic changes in the parasite that are associated with drug resistance. They have been established for many (SP, chloroquine, mefloquine, atovaquone) but not all (artesunate, amodiaquine) drugs used to treat falciparum malaria. New PCR-based technology has increased the capability and affordability of screening a large number of samples for these genetic changes. Therefore, using molecular markers for surveillance instead of in vivo tests could save considerable time and effort. In addition, since only small amounts of blood need to be collected and no follow-up is required, surveillance can be extended beyond sentinel sites. Processing clinical samples directly, instead of culturing them first, eliminates the bias created from the selective pressure from culturing samples.

However, using molecular markers for monitoring drug resistance has at least three disadvantages. First, molecular markers have not been identified for all drugs. Second, drug resistance in a parasite is often caused by more than one genetic change and therefore just looking at one marker might not accurately represent drug resistance. Third, the use of

molecular markers for surveillance needs to be validated with clinical data before the results can be interpreted correctly.

RELATIONSHIP BETWEEN MOLECULAR MARKERS AND RESISTANCE: SUMMARY OF THE LITERATURE

Molecular markers of resistance have been investigated for several antimalarials. Potential molecular markers are often first identified by sequencing of clinical isolates or by performing genetic cross between different laboratory strains and identifying the genetic changes that segregate with drug resistance traits. The relationship between molecular markers and in vivo treatment failure is then investigated to determine if the molecular markers confer clinically relevant resistance. Table 1.2 lists the genes that have been proposed as potential molecular markers. The literature supporting most of these molecular markers is summarized below.

Chloroquine

Chloroquine resistance has been strongly linked to genetic changes in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) (24). *pfcrt*'s function is not fully known, but one hypothesis is that *pfcrt* is a transporter in the digestive vacuole membrane (59). A mutation at codon 76 appears to be the key determinant of chloroquine resistance, though changes at codons 72, 74, 75, and 220 are also important (60). Chloroquine resistance is further modulated by changes in the *Plasmodium falciparum* multi-drug resistance gene-1 (which is described further under molecular markers of mefloquine resistance).

Numerous clinical studies have demonstrated that *pfcrt*-76 and *pfmdr1*-86 are related to chloroquine treatment failure (61-64). For example, in Mali, the presence of the *pfcrt*-76 and *pfmdr1*-86 were independently associated with 14-day chloroquine treatment failure (*pfcrt*-76: OR=16.1 95%CI: 5.7, 45.7; *pfmdr1*-86: OR = 2.5, 95%CI: 1.1, 5.8) (61).

Sulfadoxine-Pyrimethamine

Sulfadoxine and pyrimethamine inhibit dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*), respectively, which are two enzymes involved in folate biosynthesis (Figure 1.2). *Plasmodium spp.* acquire folate primarily from de novo synthesis, though an exogenous folate salvage pathway can be utilized as well (65).

SP resistance is conferred by point mutations in either enzyme, which sterically inhibit the binding of their respective inhibitor (65-69). Mutations in *dhfr* codons 51, 59, and 108 have been associated with pyrimethamine resistance (66, 69). The 108 mutation is the first to appear in an area where SP is being used (70). Additional mutations, which accumulate over time, have a synergistic effect on pyrimethamine resistance.

Sulfadoxine resistance is associated with mutations in *dhps* at codons 436, 437, 540, 581, and 613 (67, 68). However, the role of the mutations at the 436 and 613 codons in conferring sulfadoxine resistance is controversial (65). As with *dhfr*, subsequent mutations in *dhps* synergistically contribute to sulfadoxine resistance (68).

The importance of the *dhfr* mutations in conferring pyrimethamine resistance has been documented in several in vitro studies (71-73). (Due to technical difficulties, in vitro susceptibilities to sulfadoxine cannot be reliably determined). The prevalence of *dhfr* and *dhps* mutations is also associated with the past SP usage in a region (74, 75).

The relationship between the presence of these mutations and in vivo resistance is more tenuous. Most studies have demonstrated an association between in vivo resistance and mutations in *dhfr* and *dhps* (71, 72, 76-90). Though three studies concluded that there was no association (72, 91, 92). Even in the studies that report an association, the strength of the association and the mutations having the biggest impact on resistance varies greatly. There are many reasons to explain these discrepancies. First of all, in vivo resistance depends on other factors beyond the resistance of the parasite, including the parasitemia (density of parasites in the blood) and age (which is a measure of the immune response) (21, 93). These potential confounders and effect measure modifiers vary by region and therefore could affect the apparent relationship between molecular markers and treatment failure. Half of the studies cited above are restricted to children between 6 months and 5 years while the rest vary greatly in the age distribution of the study population. Some studies don't even mention the age eligibility criteria or the age distribution of the participants (85, 89).

In addition, the association between the mutations and clinical resistance is dependent on the prevalence of both treatment failure and the mutations in a particular area. For example, Mockenhaupt et al. (2005) claim that *dhfr* but not *dhps* mutations are associated with treatment failure. However, the only *dhps* mutation observed was *dhps*-437 (86). Another possibility is that the effect of the mutations might be modulated by other parasite characteristics (such as the expression of *dhfr* or *dhps*), which might vary by region as well.

Yet another possible reason for this discrepancy is that the coding of the *dhfr* and *dhps* mutations varies greatly across studies. Some studies combine the haplotypes of *dhfr* and *dhps* while other studies test the two haplotypes separately. Still other studies look at individual mutations or the number of mutations. In addition, two studies excluded all

samples with mixed genotypes (71, 89) while the rest of the studies retained the mixed genotypes by coding them in a variety of ways.

Many of these studies have methodological issues as well. With a few exceptions (78, 82, 84, 85, 90, 94), confounders and effect measure modifiers are not assessed. In addition, the most common analysis approach is to haphazardly try different combinations of the mutations and report ones (or emphasize those) with small p-values. This approach does not account for multiple comparisons and will tend to emphasize the largest effect instead of the most precise or valid estimate.

In summary, many studies have investigated the relationship between *dhfr* and *dhps* mutations and treatment failure (Table 1.3). The results vary greatly, partially due to differences in methodology and partially due to regional differences in the parasite and host. The estimate of the effect of mutations on treatment failure can be improved on by a systematic approach to screen and control for confounders and effect measure modifiers. In addition, the geographic variation suggests the relationship between molecular markers and treatment failure has to be established before they can be used for surveillance in a region.

Mefloquine

Mefloquine is a quinoline compound that is structurally related to chloroquine but has a longer half-life. Mefloquine's mechanism of action has yet to be fully elucidated, but it probably involves blocking hemoglobin breakdown in the parasites' food vacuole (95). Mefloquine drug resistance is associated with genetic changes in the *pfmdr1* gene. This gene encodes for P-glycoprotein homologue-1 (pdh1), an ATP-binding cassette transporter, which is homologous to the multi-drug resistance transporters found in some human cancers (96).

Pgh1 has been localized to the membrane of the parasite's food vacuole. The exact function of pgh1 and how it contributes to drug resistance are unknown but in vitro data suggest it is involved in drug efflux from the vacuole (96). *pfmdr1* has also been associated with chloroquine and artemisinin resistance (97-101).

In vitro studies have demonstrated that mutations at codons 86, 184, 1034, and 1042, along with the amplification of this gene, are associated with mefloquine resistance (97-99, 101, 102). However, the genetic background of the parasite appears to modulate the effect of these mutations on resistance (102). Based on a theoretical structural model of *pfmdr1*, these codons are thought to be in the hydrophobic side of the protein and might modulate the transporter's specificity (101).

pfmdr1 copy number has been related to the increased mRNA expression of the transporter (103), and therefore might confer resistance by increasing the efflux of mefloquine from the food vacuole. Previous studies have found increased *pfmdr1* copy number only in the presence of the wild-type genotype at 86, 1034, and 1042 codons (99, 104). There appears to be no relationship between the genotype at *pfmdr1*-184 and copy number.

In vivo studies comparing *pfmdr1* haplotype and copy number with treatment failure have been quite limited. Pillai et al. 2003 found that the genotypes at *pfmdr1* codons 1034, 1042 and 1246 were not related to treatment failure to mefloquine and mefloquine-artesunate therapy in Peru (105). However, this conclusion is questionable, given that there were no treatment failures. A clinical study in Gabon found *pfmdr1* haplotype was not related to ultra-low dose mefloquine treatment failure (106). Yet the applicability of these results to standard doses of mefloquine is unknown. Two studies have demonstrated that increased copy number of *pfmdr1*, but not the aforementioned mutations, is associated with clinical resistance (104,

107). This relationship persisted when mefloquine was used in combination with artesunate (104). Both these studies took place in Western Thailand and therefore need to be replicated in other areas to determine if the results are generalizable.

Amodiaquine

Little is known about mechanisms of or molecular markers for amodiaquine resistance. Amodiaquine is structurally related to chloroquine. Resistance to these drugs is correlated in clinical isolates, which suggests the mechanism of resistance is similar (108-110). One study found an association between the *pfcrt*-76 mutation and amodiaquine resistance, though this observation was based on very few samples (111).

Artesunate

There is currently a debate about whether changes to *pfmdr1* confer artesunate resistance. Most (97, 101, 102), but not all studies (112, 113) have demonstrated an association between *pfmdr1* haplotype and artesunate IC_{50}. Increased *pfmdr1* copy number, when combined with the haplotype, is also associated with an increased IC_{50} to artemisinin-derivatives (98, 99). However, only small increases in IC_{50} were observed in these studies. One clinical study found that artesunate monotherapy did not select for *pfmdr1* mutations (114). Therefore, the increase in resistance conferred by changes in *pfmdr1* might not be clinically relevant.

In vitro artesunate resistance has also been associated with changes in the translationally controlled tumor protein (115) and a SERCA-type ATPase (116), but it is unclear if these changes are clinically relevant (43, 104).

MALARIA AND DRUG RESISTANCE IN CAMBODIA

Despite a recent decline in incidence in the last 15 years, malaria remains a significant public health problem in Cambodia (117, 118). In 2003, there were over 70,000 reported cases of malaria and approximately 500 reported deaths (118). Since underreporting is likely, the burden of malaria is probably much larger. Most cases of malaria occur near the borders with Laos, Thailand, and Vietnam, while there are virtually no cases in urban areas (119, 120). A majority (89%) of all cases of malaria are caused by *P. falciparum*; the rest of cases are caused by *P. vivax* (118). Malaria disproportionally affects ethnic minorities, plantation workers, gem miners, soldiers and refugees due to their close proximity to mosquito-filled forests. Malaria mostly affects adult men, as traveling into forested areas for work exposes them to malaria-infected mosquitoes.

The Thai-Cambodia border is an epicenter of drug resistance. The second report of chloroquine resistance and the first report of pyrimethamine resistance came from this area (23). Mefloquine was first used in this region in combination with SP in the mid 1980's (25). However, due to the high resistance to SP, this combination was eventually replaced by mefloquine monotherapy. Mefloquine efficacy declined rapidly in the next two decades (121-123). The worst reports of mefloquine efficacy in the world came from this area in the early 1990's (121). In 1999, the national recommended treatment for uncomplicated *P. falciparum* malaria was changed from mefloquine to mefloquine plus artesunate in response to increasing levels of treatment failure in the western areas of the country.

While Western Cambodia is a foci of drug resistance, parasites in the east have remained drug sensitive to chloroquine and mefloquine (119). A previous survey of molecular markers in Cambodia found that mutations in *dhfr*, *dhps*, and *pfcrt* are almost universal (31, 124). In

addition, most parasites had the wild-type genotype at *pfmdr1* codons 86. 1034, 1042, and 1246 while the 184 genotype was more variable (31).

MALARIA AND DRUG RESISTANCE IN DEMOCRATIC REPUBLIC OF CONGO

Malaria is significant cause of morbidity and mortality in children and pregnant women in Sub-Saharan Africa (125). In the DRC, malaria causes 30% of childhood mortality (126). There were over 4 million reported cases of malaria and 16,000 deaths in the DRC in 2003 (126). Civil unrest has compounded the impact of this disease. The eastern part of the country has been the site of much turmoil: over 500,000 Rwandan refugees fled to this area in 1994 and 5 years later it was the foci of the civil war (127). In both of these crises, malaria was a significant source of morbidity and mortality (17, 127, 128).

An in vivo efficacy study that was conducted in the refugee camp in 1994 documented moderate failure rates for both chloroquine and SP (129). However, malnutrition was highly prevalent in this population, and since malnutrition is a risk factor for treatment failure, the failure rates observed in this study likely overestimate the actual level of resistance (64, 130). Observers from the CDC noticed high failure rates of chloroquine in the refuge camps in Eastern Congo. As a result, in 1995 they implemented the use of SP for the treatment of uncomplicated falciparum malaria (P. Bloland, per. com.).

With the building of a health infrastructure in the DRC in the past couple of years, more information on drug resistance in the country is becoming available. In a nation-wide survey conducted in 2000-2001, the overall failure rate for chloroquine and SP was 45.4% and 7.5%, respectively (131). There was substantial geographic heterogeneity in the efficacy of both drugs, with the eastern part of the country having a higher failure rate. In Bukavu, which is

near the Rwandan border, the chloroquine failure rate was 80% and the SP failure rate was 9.3%. Because of the observed high failure rates of chloroquine, the national first line agent for the treatment of uncomplicated malaria was changed to SP in 2001.

There have been no previous studies on the prevalence of molecular markers of drug resistance in the DRC. However, there have been prevalence studies in neighboring countries. In the Republic of Congo, where chloroquine remains the first-line treatment for uncomplicated malaria, a study in Brazzaville and Pointe-Noire found a high prevalence of the triple *dhfr* mutant (mutations at 51, 59, and 108) and the *dhps*-437 mutation but no mutations at *dhps*-540 (132). These results suggest that if the first-line treatment was changed to SP, it would initially be effective but resistance would emerge quickly. In Uganda, a nation-wide survey found the prevalence of samples with the quintuple mutation and the *pfcrt*-76 mutation varied between 61-91% (depending on the site) (133).

Table 1.1 Outcome classification of the in vivo efficacy study*

Outcome	Abbr.	Criteria
Early Treatment Failure	ETF	one of the following: --signs of severe malaria on day 1-3 in the presence of parasitemia --parasitemia on day 2 higher than on day 0 --parasitemia on day 3 greater than 1/4th parasitemia on day 0
Late Clinical Failure	LCF	one of the following: --signs of severe malaria on after day 3 in the presence of parasitemia --presence of parasitemia after day 3 with axillary temp ³ 37.5 □C
Late Parasitological Failure	LPF	--presence of parasitemia after day 3 with axillary temperature < 37.5 □C
Adequate Clinical and Parasitological Response	ACPR	--absence of parasitemia on day 14, irrespective of axillary temperature

*according to the WHO protocol (17)

Table 1.2 *Plasmodium falciparum* genes that are potential molecular markers for antimalarial resistance*

Anti-malarial	Gene	Gene abbreviation
Quinine	*Plasmodium falciparum* multidrug resistance gene-1	*pfmdr1*
Chloroquine	*Plasmodium falciparum* multidrug resistance gene-1	*pfmdr1*
	Plasmodium falciparum chloroquine transporter	*pfcrt*
Mefloquine	*Plasmodium falciparum* multidrug resistance gene-1	*pfmdr1*
Amodiaquine	*Plasmodium falciparum* chloroquine transporter	*pfcrt*
Lumefantrine	*Plasmodium falciparum* multidrug resistance gene-1	*pfmdr1*
Artemisinin compounds	ATPase gene 6	*atp6*
Sulfadoxine	dihydropteroate synthase	*dhps*
Dapsone	dihydropteroate synthase	*dhps*
Pyrimethamine	dihydrofolate reductase	*dhfr*
Proguanil	dihydrofolate reductase	*dhfr*
Atovaquone	cytochrome b	*cytb*

*based on (43, 134).

Table 1.3 Summary of studies investigating the relationship between mutations in *dhfr* and *dhps* and SP treatment failure

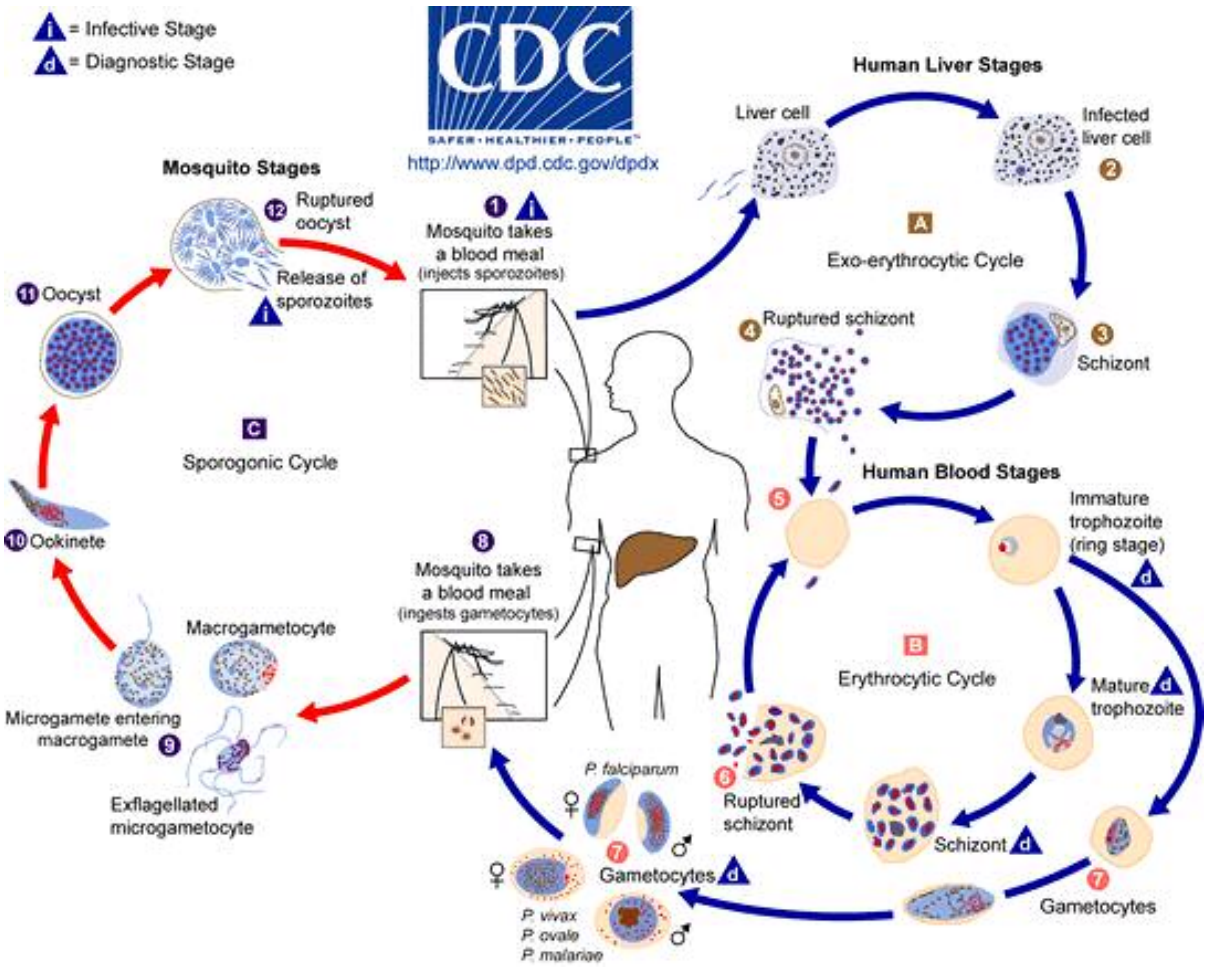
Location	Follow-up (days)	PCR corrected?	Confounders controlled for	Age distribution of subjects*	*dhfr/dhps* associated with TF‡?	What codons were associated with TF?	Ref.
Gabon	28	no		6m-10y	no	.	(72)
Cameroon	28	yes		all ages	possible†	*dhfr*-51,59,108	(71)
Lao PDR	14	no		>1 year	possible	*dhfr*-51, *dhps*-436, 437, 613	(77)
Uganda	14	yes	parasite density	6m-5y	yes	*dhfr*-59, *dhps*-437, 540	(94)
Uganda	28	yes	age, parasite density	6m-5y	yes	*dhfr*-59, *dhps*-540	(78)
Sudan	28	no		10 - 65y	yes	*dhps*-436	(76)
Mozambique	28	no		6m - 5y	yes	*dhps*-437	(76)
Tanzania	14	no		6m - 5y	yes	*dhps*-437	(76)
Nigeria	28	yes		<12 years	yes	*dhfr*-51,59,108, *dhps*-437, 540	(80)
Sudan	28	no		10-65 years	possible	*dhfr*-51, 108, *dhps*-436	(81)
Malawi	28	no	hemoglobin	6m-6y	yes	*dhfr*-51,59,108, *dhps*-437, 540	(82)
Uganda	28	yes	parasite density	6m-5y	yes	*dhfr*-59, *dhps*-437, 540	(84)
Tanzania	14	no		6m-5y	yes	*dhfr*-51,59,108, *dhps*-437, 540	(87)
Kenya	14	no		Not mentioned	yes	*dhfr*-108, 59, *dhps*-437	(89)
Columbia	14	no		Not mentioned	no	.	(91)
Ghana	28	yes	sex, parasite density, previous drug use	6m - 5y	yes	*dhfr*-51,59,108	(86)
Uganda	14	yes		1-37 years	possible	*dhfr*-51,59,108 *dhps*-436, 437	(135)
Uganda	28	yes		> 6 months	yes	*dhfr*-59, *dhps*-437, 540	(88)
Uganda	28	yes	age, prior drug use	6m-5y	yes	*dhfr*-108, 59, *dhps*-437	(90)
Sri Lanka	42	no		Not mentioned	no	.	(92)

* in months (m) or years (y)

† The mutations appeared to be related to treatment failure yet the small sample size of these studies precluded the association from achieving statistical significance

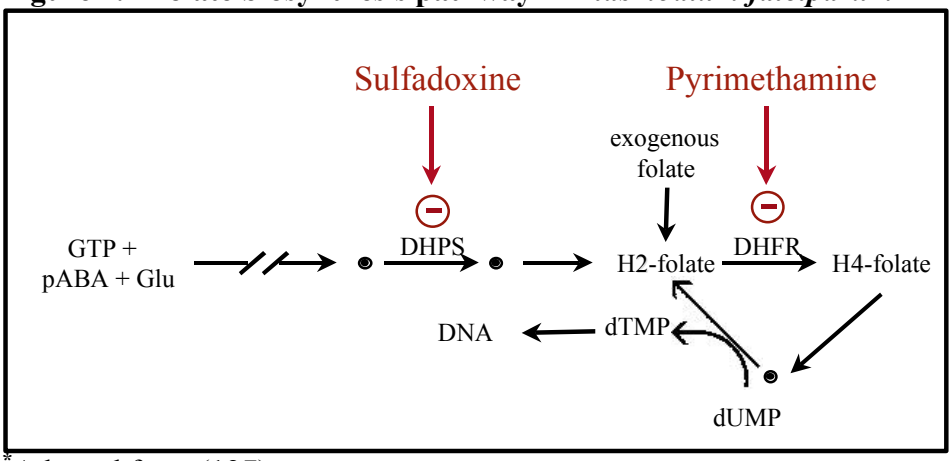
‡ Treatment failure

Figure 1.1 *Plasmodium falciparum* life cycle*



*Reference: (136)

Figure 1.2 Folate biosynthesis pathway in *Plasmodium falciparum*[*]



[*]Adapted from (137).

CHAPTER II:

RATIONALE, OBJECTIVES, AND METHODS

RATIONALE

Malaria is a major public health problem; in 2002, there were over 500 million clinical cases of *Plasmodium falciparum* malaria (3). Malaria is particularly devastating in developing countries with inadequate health infrastructures. For example, in the Democratic Republic of the Congo (DRC), one-third of all child mortality is caused by malaria (126). One of the greatest challenges in the control of malaria is drug resistance, which has contributed to its re-emergence and spread (27). Resistance to chloroquine is nearly universal while resistance to sulfadoxine-pyrimethamine (SP) and mefloquine is increasing.

Monitoring drug efficacy is an important component to a national malaria control program. Only with recent and relevant data on the level of drug resistance, can policy makers decide what drug combinations should be used and what areas should be targeted for interventions. Currently, drug resistance is monitored with in vivo efficacy trials. However, these trials are time consuming, as patients need to be followed for 14-42 days. One alternative to the in vivo efficacy trial is to use molecular markers of drug resistance. These molecular markers have been identified though working with the parasite in culture. However, the effect of these genetic changes on in vivo treatment failure is less clear.

OBJECTIVES

The main purpose of this work is to estimate the relationship between molecular markers of resistance and clinical treatment failure in falciparum malaria. Three specific objectives will be addressed:

1. To determine the relationship between genetic changes in parasites' dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes and clinical resistance to

SP-based combination therapy. To address this question, samples collected from an in vivo efficacy trial in Rutshuru, DRC have been genotyped for mutations in *dhfr* and *dhps*.

2. To establish the association between molecular markers in *pfmdr1* and clinical resistance to mefloquine-artesunate therapy. To accomplish this aim, samples from an in vivo efficacy trial in Pailin, Cambodia were used. Clinical samples have been genotyped for four codons in *pfmdr1* and the number of copies of *pfmdr1* in the genome.
3. To determine the geographic variation in the prevalence of molecular markers in Cambodia. In Cambodia, resistance to mefloquine increases from east to west.

SPECIFIC HYPOTHESES

1. Genetic changes in the *dhfr* and *dhps* gene in *Plasmodium falciparum* are associated with increase risk of SP treatment failure in Rutshuru, DRC.
2. *pfmdr1* copy number and genotype are associated with increase risk of recrudescence after mefloquine-artesunate treatment in Pailin Cambodia
3. *pfmdr1* copy number varies geographically in Cambodia. Specifically, pfmdr1 copy number is highest in the West (which is historically the focus of resistance).

OVERVIEW OF METHODS

Clinical data and samples from three different studies were utilized in this dissertation. Ancillary studies were conducted on two in vivo efficacy trials in Rutshuru, DRC and Pailin, Cambodia. In addition, the main analysis for a cross-sectional study in Cambodia is included.

The methods for each study will be summarized below and will be expanded upon in subsequent chapters. Each study was a collaborative effort. The main collaborators and their contribution will be made clear in the beginning of each section.

IN VIVO EFFICACY STUDY IN RUTSHURU, DRC

Collaborators

Walter Kazadi ran this trial for the Programme National de Lutte contre le Paludisme of DRC, in collaboration with Kutelemeni K. Albert and Antoinette Tshefu (Kinshasa School of Public Health, DRC) and Peter Bloland (Centers for Disease Control, USA). Alisa Alker is responsible for all the laboratory work and all the data analysis.

Study setting and population

Rutshuru is in the North Kivu district of the Democratic Republic of the Congo near the Ugandan and Rwandan border (Figure 2.1). Rutshuru has a long history of conflict: fighting occurred here during the 1993 civil war, Rwandan refugees fled to here in 1995, and the fighting in the most recent civil war occurred near by. Due to the political instability of this area, little is known about the epidemiology of malaria. The study participants came from the surrounding areas and where recruited a four different clinics around Rutshuru.

Clinical Study

A therapeutic efficacy trial in Rutshuru, DRC was conducted to compare the efficacy of 3 regiments: 1. amodiaquine plus SP (AQSP); 2. artesunate plus SP (ASSP); and 3. SP monotherapy (SP). This trial was a randomized, open label study following the 14-day WHO

1996 protocol with the modifications suggested in the 2002 report (48, 138). The study took place between June and September 2002 at four different clinics.

The inclusion criteria included: 1. between the ages of 6 months and 5 years; 2. did not suffer from severe malnutrition; 3. malaria infection with only *P. falciparum* species; 4. a parasitemia between 1,000 to 200,000 parasites / µl; 5. absence of signs of severe disease, which included: prostration, impaired consciousness, respiratory distress, multiple convulsions and abnormal laboratory findings (17).; 6. axillary temperature equal or greater than 37.5 ºC; 7. absence of other fever-causing illnesses; 8. no previous reaction to sulfa-containing drugs; 9. ability to return to clinic for follow-up visits; and 10. consent of the parent or guardian.

At enrollment a brief clinical exam was administered and approximately 50 µl of blood was collected. The blood was placed on IsoCode Stix filter paper (Schleicher & Schuell, Keene, N.H., USA), desiccated, and then transported to UNC Chapel Hill for processing. The subjects were randomized to a treatment arm and given their assigned treatment. Subjects randomized to the ASSP and AQSP received additional doses of artesunate or amodiaquine on the first and second day after enrollment.

Follow-up visits were scheduled for days 1, 2, 3, 7 and 14 after enrollment. They were also encouraged to return to the clinic if the subject's symptoms worsened. Subjects were excluded after enrollment if: 1. they were diagnosed with another illness that would prevent assessment of treatment outcome; 2. they could not be located for follow-up visits; 3. the parents or guardians withdrew consent; 4. they took another antimalarial beyond those given in the study protocol; or 5. non-falciparum malaria infection was detected.

The sample size of this study was calculated in order to estimate the population proportion of treatment failure, while taking in account loss to follow-up according to the WHO protocol (17). In all three arms, the confidence level was 95%, the precision was 10 percentage points, and the loss to follow-up was assumed to be 10%. In the SP arm, with an expected failure proportion of 30%, the required sample size is 90. For the two combination arms, an expected failure proportion of 20% would require a sample size of 80.

249 children under the age of 5 were initially enrolled, though 17 (7%) patients were later excluded. Most of the exclusions were from lost to follow-up. An adequate clinical and parasitological response was observed in 39.4% of the subjects in the SP arm, 68.2% of the AQSP arm, and 78.9% of the ASSP arm. Three subjects died during the course of the study. The exact cause of death was not definitely determined, though in one case the suspected cause was a home-remedy for malaria.

Collection of Clinical Covariates

At enrollment, a brief clinical exam collected information on age, weight, axial temperature, and respiratory rate. A few drops of blood were collected to determine the parasite concentration and hemoglobin level. At all subsequent visits, axial temperature was collected. Parasite and gametocyte concentration was measured on all visits except for day 1. Z-scores of weight for age, which is a measure of malnutrition, were calculated in reference to the NCHS/WHO international dataset (139).

Measuring the presence and concentration of *P. falciparum* parasites is important not only in assessing eligibility but also in determining the outcome. When the patient was being screened for eligibility, two slides were made from drops of blood: a thick smear and a thin

smear. Both were stained with Giemsa. To determine if the patient was eligible for the study, the thick smear was examined for the presence of *P. falciparum* asexual parasites and the relative number of parasites per leukocyte. At least 200 leukocytes were counted. The parasitemia was determined using the following formula: parasitemia (per μl) = # of parasites * 8000 / # leukocytes (140). 100 different fields were examined to ensure the infection was not mixed or that it was truly negative. If the patient was enrolled in the study, the thin smear slides were read to obtain a more accurate measurement of the parasite density.

However, on the second reading of the slides, 18 (8%) people were found to have a parasitemia of greater than 200,000 parasites per μl. These individuals were kept in the dataset because having a high parasitemia does not preclude the estimation of the effect of molecular markers on treatment failure. However, the inclusion of these subjects might make the results difficult to compare to other studies that followed the eligibility criteria more strictly.

Diagnosis of malaria by thick-smear microscopy is the gold standard of the field and is used in most in vivo efficacy trials. While using PCR to detect infection does have increased sensitivity (141), developing countries do not have the infrastructure to support the use PCR in the treatment of patients. The sensitivity limit for using smear slides and microscopy is 8 parasites per μl of blood (140). Since one of the eligibility criteria is having a parasitemia of >1,000 parasites per μl of blood, the limited sensitivity will not affect eligibility. However, since treatment failure is defined as the presence of parasites in the blood, there is the possibility of misclassification of people with low parasitemia as treatment successes.

Genotyping

Out of the 249 children initially enrolled, 212 (85%) were available for genotyping. Samples were available for all the patients that were lost to follow-up or died. The genotyped subjects were more likely to be male, have higher parasitemias, and were more likely to experience treatment failure compared to the subjects that were not genotyped (Table 2.1). The results of t-test or chi-square test for comparing these two groups are presented in Table 2.1.

DNA was extracted according to the IsoCode Stix's protocol. The samples were genotyped for 3 *dhfr* (51, 59, 108) and 4 *dhps* (437, 540, 581, 613) codons using a real-time PCR assay with minor groove binding (MGB) probes (142). The real-time PCR methods are presented in Appendix B. MGB probes consist of an oligonucleotide that is complementary to the sequence of interest, a fluorophore on the 5' end and a quencher on the 3' end. The binding between the oligonucleotide and sequence is very sensitive to single base differences (143, 144). To achieve allelic discrimination, two probes are used: one specific to the mutant sequence and another specific to the wild-type sequence. The two probes are attached to different fluorophores (wild-type: FAM, mutant: VIC). During amplification, the probe that is complementary to the sequence contained in the sample will bind to the amplicon. When the DNA polymerase creates the complimentary strand, its innate 5' nuclease activity will cause the fluorophore to be separated from the quencher, which allows it to fluoresce.

The genotype of a sample can be determined by measuring the change in fluorescence during the PCR reaction: an increase in the FAM signal indicates the presence of the wild-type sequence, an increase in the VIC signal indicates a mutant sequence and an increase of both signals indicates the sample is mixed.

These assays have a few limitations. There are two different mutant forms found in nature of *dhfr*-108 (Asn and Ser) and *dhps*-613 (Thr and Ser). The assays can detect both forms but cannot identify which mutant form is present. Another limitation is that the *dhps*-437 mutation does not work in the presence of a mutation in codon 436. However, the *dhps*-436 mutation, *dhfr*-108-Thr, and *dhps*-613-Thr are rare in Africa, and therefore should have a minimal impact on the genotyping of samples from Rutshuru.

Based on a limited number of samples (n = 5), the *dhfr* and *dhps* MGB assays were found to be 100% sensitive and specific when compared to sequencing (the gold standard) (142). In addition, the assay was able to detect a minor allele when it consisted of at least 10% of the sample. The detection limit ranged from 10 – 1,000 copies of the genome per reaction.

One complication in the genotyping of clinical samples is that many *P. falciparum* infection are multi-clonal. In addition, the relative concentration of clones varies on a daily basis (145). Therefore, at any particular codon, more than one genotype might be present. Once antimalarial therapy is initiated, the drug resistant strains will be selected for. Therefore the presence of these strains, not their relative concentration is most likely to be related to treatment failure.

Another difficulty is the determining the haplotypes present in a sample. The genotype at a single nucleotide polymorphism may be important, but the combinations of SNPs often interact in a complex manner to produce the phenotype. Therefore, in human genetics, a lot of effort is put into determining the haplotype (i.e. combination of SNPs) of both alleles. *P. falciparum* is haploid for most of the lifecycle (and therefore each parasite will have only one allele). Yet the multiclonal nature of infections might make haplotype determination difficult. For example, if a sample was mixed at all three *dhfr* codons, it is unclear if the *dhfr*-51, -59,

and 108 mutations are present on the same allele or if there are three separate alleles each with one mutation.

To address this issue, Paul Wilson took three samples from the Rutshuru study with the following *dhfr*-51, -59, and -108 genotypes (which I determined by real-time PCR): 1. all mutant, 2. all wild-type, 3. mixed wild-type/mutant at each codon (146). These samples were subcloned and sequenced. For the wild-type sample, all of the 55 clones sequenced were wild-type. Similarly, all 90 clones from the mutant sample were mutant at *dhfr*-51, 59, 108. For the 33 cloned sequenced from the mixed sample, 9 were mutant and 24 were wild-type at *dhfr*-51, 59, 108. No single or double mutants (at codons 51, 59, 108) were observed. These results suggest that when there is a mutant genotype present at *dhfr*-51, 59, and 108, we can assume that the triple mutant haplotype is present.

One last complication of using real-time PCR for the genotyping of clinical samples is that these assays will only detect mutations at *dhfr*-51, 59, 108 and *dhps*-436, 437, 540, 581, and 613. In contrast, sequencing will pick up any alteration in these genes. Polymorphisms at other codons have been shown to be related to pyrimethamine resistance (such as *dhfr* codons 16, 50, and 164), though these mutations are rare in Africa and are not the main determinants of pyrimethamine resistance (65). Other genetic modifications of *dhfr* have been noted in clinical samples. In fact, the subcloning of the thee Rutshuru samples found non-synonymous mutations at *dhfr* codons 19, 29, and 98. The role of these mutations in conferring resistance is unclear. In summary, the real-time PCR assays detect the major determinants of SP resistance. Other genetic alterations might be present, but most likely play a minor role in SP resistance.

Analysis

For the purpose of the data analysis, the exposure was coded as the presence of the mutant genotype, even if it is not the major component of the sample.

The main outcome of the analysis is any type of treatment failure by day 14. The presence of parasites on day 3 was investigated as a secondary outcome. (The presence of parasites on the third day after treatment is a sign of drug resistance). For the main analysis, children who died or were not assigned an outcome were not included.

To investigate the independent contributions of the *dhfr* and *dhps* mutations on treatment failure, linear risk regression was used. Interaction terms between mutations were evaluated using forward selection. Forward selection was used instead of backwards selection because the small sample size of this dataset limits the power to detect interactions.

To estimate the effect of *dhfr* and *dhps* mutations on treatment failure, linear risk regression was used. Covariates that were evaluated as potential effect modifiers and confounders were: hemoglobin, parasitemia, age, gender, clinic, treatment arm, and Z-score of weight for age. Effect modifiers were first evaluated by including an interaction term in the model and using the Wald test to determine if it was significantly contributing to the fit of the model. Interaction terms with $p < 0.05$ were retained in the model. All non-effect modifiers were then evaluated as potential confounders using the DAG (147). The same modeling technique was used to evaluate the effect of mutations on the presence of parasites on day 3.

To determine the impact of the loss to follow-up on the effect estimate of mutations on treatment failure, a simple sensitivity analysis was conducted. The effect estimates were re-calculated under two scenarios: 1. All the excluded subjects were assumed to have failed

treatment and 2. All the excluded subjects were assumed to have not experienced treatment failure.

IN VIVO EFFICACY STUDY IN PAILIN, CAMBODIA

Collaborators

This in vivo efficacy study was part of the monitoring program conducted by the Cambodian National Center for Parasitology, Entomology and Malaria Control (CNM) in collaboration with the World Health Organization (WHO) and the U.S. Naval Medical Research Unit No. 2 (NAMRU-2). Poravuth Yi (CNM), Denis Mey Bouth (WHO), Reiko Tsuyoka (WHO), Jason Maguire (NAMRU-2) and Chansuda Wongsrichanalai (NAMRU-2) were all involved in running this efficacy study. Pharath Lim, of the Pasteur Institute in Cambodia, genotyped the samples for *msp1*, *msp2*, and *glurp*. Thierry Fandeur and Federick Ariey at Pasteur supervised this work. Rithy Sem read the thin smear microscopy slides to determine the parasitemia, extracted the samples and genotyped 56 of the samples for *pfmdr1* mutations. Naman Shah developed the new *pfmdr1* copy number assay and ran all samples for copy number determination. Alisa Alker genotyped 24 samples for *pfmdr1* mutations, trained and supervised Rithy Sem and Naman Shah, carried out quality control on all laboratory work, and conducted the data analysis. Both Steve Meshnick and Chansuda Wongsrichanalai supervised the genotyping work and analysis.

Clinical Study

In 2004, an in vivo efficacy study was conducted in Pailin, Cambodia for surveillance of mefloquine-artesunate, which is the current first line treatment for uncomplicated malaria in

Cambodia. Between June and August 2004, 25 children and 56 adults were enrolled. The sample size was calculated in the same manner as described for the Rutshuru study. Briefly, with a 95% confidence interval and a precision of 10 percentage points, 20% loss to follow-up and an expected prevalence of treatment failure of 20%, the required sample size is 73 people (17, 148).

The eligibility criteria for this study were 1. being older than 6 years, 2. having a slide-confirmed *P. falciparum* infection (with no other species of *Plasmodium* present), 3. having an initial parasite density ≤ 100,000 asexual parasites/µl, 4. having a measured axillary temperature ≥ 37.5 ºC, 5. providing informed consent (by parent or guardian, when appropriate), and 8. willing to return for follow-up. Exclusion criteria included: 1. having a previous adverse reaction to mefloquine or artesunate, 2. having a severe chronic illness (such as HIV or kidney disease), 3. being pregnant, or 4. having signs of severe disease. The cutoff level of parasitemia in this study is lower than in the Rutshuru study because Cambodia is a low transmission area, where people have less immunity towards in malaria. As a result, the parasitemia threshold for severe disease is lower.

The parasitemia was first determined by thick smear microscopy, as previously described for the Rutshuru study. The thin smear slides were read after the study ended to confirm the parasitemia. However, the think smear slides revealed that 18 people (23%) had a parasitemia greater than 100,000 parasites per µl. These individuals were kept in the dataset because having a high parasitemia does not preclude the estimation of the effect of molecular markers on recrudescence. However, the inclusion of these subjects might make the results difficult to compare to other studies that followed the eligibility criteria more strictly.

At enrollment, a brief clinical exam was performed and a questionnaire was administrated. Approximately 2 ml of blood was taken and frozen. The subjects will be treated according to the national policy: they were initially given 1 dose of mefloquine (20 mg/kg) and 1 dose of artesunate (20 mg/kg). Additional doses of artesunate (10 mg/kg) were administered under observation at 24 and 48 hours. The patients stayed at the clinic for 3 days for observation. They also returned for follow-up visits at 7, 14, 21, 28, 35, and 42 days after the date of enrollment. Clinic staff actively sought out people who did not return for the follow-up visits.

Nine adults and one child were eventually lost to follow-up, though all subjects were followed for at least two weeks. Out of the remaining 70 people, 24 people (34.3%) had a reoccurrence of *P. falciparum* parasitemia within 42 days of treatment. Half of these cases were classified as recrudescence, which results in a failure rate of 17.1%. The other half were classified as re-infections. The failure rate was substantially higher in children (43.8%) than in adults (11.9%).

Study Population and Recruitment

The Pailin referral hospital, where this study took place, serves the city of Pailin and also the surrounding rural areas. The clientele includes patients referred by medical clinics in the area and people who come to the clinic directly because they suspect they have malaria.

The regular clientele at this clinic was invited to participate in this study. In addition, a staff member went to surrounding villages and asked if anyone had a fever. The staff member drove people with a fever to the clinic for evaluation. (Transportation in this area is very difficult and is a limiting factor in people seeking healthcare).

Collection of Clinical Covariates

The study doctor administered a brief questionnaire, which contained questions on prior medical history, use of antimalarials in the last month, and basic sociodemographic information. At each follow-up visit, the patient was screened for malaria (via thick smear microscopy), and the axial temperature was recorded.

Parasite concentration was determined as previously described in section 2.4.4 with the following exception. At high parasitemia, counting the number of parasites relative to the number of RBC is more accurate than counting relative to the number of WBCs (C. Wongsrichanalai, per. com.). Therefore, when the parasitemia was determined to be greater than 10,000 parasites/μl using the WBC method, the counting was re-done in relation to RBCs. The number of parasites per 5,000 RBCs were counted and converted into the parasitemia by assuming there are 4,500,000 RBCs per μl.

Biological Specimen Collection

At enrollment, approximately 2 ml of peripheral blood was collected and frozen. Subsequent blood samples were taken after treatment and at any recurrent of parasitemia. These blood samples were collected from finger pricks and stored on 3M Whatman filter paper.

Laboratory Procedures

DNA extraction from whole blood and filter paper was performed using the Qiagen Qiamp DNA mini kit. All samples taken at enrollment and at reoccurrence of disease were

genotyped for *pfmdr1* codons 86, 184, 1032, and 1042 using a real-time PCR assay based on MGB probes (58). These assays are very similar to those described for the *dhfr* and *dhps* mutations in section 2.4.5. When compared to sequencing, these assays were all 100% sensitive and specific except for 184, whose sensitivity was 92% (58). However, this was based on a limited number of samples (n = 22).

pfmdr1 copy number was assessed for all samples using a new assay. This assay consisted of combining in one reaction both the primers and a FAM-TAMRA probe specific to a conserved region of *pfmdr1* and the primers and a VIC-TAMRA probe specific probe to *β-tubulin*. The primer and probes had been designed previously (99, 104). There exists only one copy of the *β-tubulin* gene per genome. Therefore, by comparing the amount of *pfmdr1* relative to *β-tubulin,* the *pfmdr1* copy number can be assessed.

In real-time PCR, quantification of DNA concentration is accomplished through the measurement of the cycle threshold (CT). The CT is the cycle at which the fluorescence rises above a threshold. Therefore, for each well, the CT was measured for both the *β-tubulin* VIC dye and the *pfmdr1* FAM dye

pfmdr1 copy number was calculated according to the following formula: copy number = $(1 + E_{btubulin})^{CT(btubulin)} / (1 + E_{pfmdr1})^{CT(pfmdr1)}$. The efficiency (E) of the b-tubulin was assumed to be 1. The efficiency of the *pfmdr1* reaction was calculated for each plate by back-calculating the efficiency from the formula above and a copy number of 1. DNA from the *P. falciparum* strain Dd2 was included on every plate as a control. Dd2 was previously determined to have 4 *pfmdr1* copies. In our assay, Dd2 had a mean copy number of 4.27 and a coefficient of variation of 5.77%. The end result is a continuous variable representing the average *pfmdr1* copy number for a particular sample.

Pharath Lim of the Pasteur Institute used paired samples taken at enrollment and reoccurrence of disease to classify the reoccurrence as either recrudescence (return of the original infection) or reinfection (a new infection). The number of variants in 3 polymorphic genes (*msp1*, *msp2*, and *glurp*) was determined using size fractionization in agarose gel electrophoresis, as described previously (124). If the second sample contained a sub-sample or the same variants as the first sample, the infection was classified as recrudescence. If the sample did not contain any of the original variants, then it was considered a re-infection.

Analysis

The main outcome of the analysis was time to recrudescence. Since the subjects were assessed at distinct time points (1, 2, 3, 7, 14, 21, 28, 35, and 42 days), the actual time of recrudescence is interval censored. Subjects who were parasite-free on day 42 were considered treatment successes. Patients that were lost to follow-up, who developed a *P. vivax* infection, or who developed a *P. falciparum* infection that was determined to be a re-infection were censored on the last day of disease-free follow-up.

The secondary outcome assessed was time to parasite clearance. Parasite clearance time is the amount of time between treatment and the disappearance of *P. falciparum* asexual parasites from the peripheral blood.

To determine the relationship between *pfmdr1* haplotype and copy number and time to recrudescence, survival analysis was used. Discrete Cox proportional hazards model was used to take in account the interval censoring of the failure time. In Stata, discrete Cox proportional hazards model is estimated by a general linear model with the complementary log-log link (149). The different time periods are represented by indicator variables in the

model and the constant term is not included. The hazard ratio of the exposure is obtained by exponentiating the corresponding coefficient. The results of the discrete proportional hazards model was compared to the results obtained from a continuous Cox proportional hazards model using 3 different methods for the treatment of ties (efron, exact marginal likelihood, and exact partial likelihood). All variables were tested for the proportional hazards assumption.

The following covariates were evaluated as potential effect measure modifiers and confounders: previous antimalarial drug use, age, parasite density, hematocrit, and gender. These variables were coded to reflect their distribution and to maintain their relationship with treatment failure. Hazard ratio modification was determined by testing the interaction term between copy number and the covariate using the Wald test. If the p-value < 0.05, the covariate was considered an effect measure modifier. All non-modifiers were then evaluated in a directed acyclic diagram (DAG). This same modeling strategy was used to explore the relationship between genetic changes in *pfmdr1* and parasite clearance time.

To determine whether mefloquine-artesunate treatment selects for increased copy number and *pfmdr1* haplotype, the genotype of the samples taken at enrollment and recurrence were compared. The Wilcoxon signed rank test (the non-parametric version of the paired t-test) was utilized to determine if the difference was statistically significant.

CAMBODIAN CROSS-SECTIONAL STUDY

Collaborators

This study was conducted by Chansuda Wongsrichanalai of NAMRU-2 in collaboration with the Cambodian national malaria control program and the Pasteur Institute of Cambodia.

Rithy Sem read the microscope slides for parasite determination, genotyped all the samples for *pfmdr1* haplotype and ran approximately 50 samples on the *pfmdr1* copy number assay. Naman Shah ran the rest of the samples on the copy number assay. Alisa Alker conducted quality assurance on the laboratory work and completed all the data analysis.

Study Design

Clinical *P. falciparum* samples were collected between 2004-2005. The samples were taken before antimalarials were administered. The inclusion criteria included: symptomatic or asymptomatic *P. falciparum* malaria, being 18 years or older (changed to > 5 in 2005), and informed consent. Exclusion criteria included any contraindications to giving blood and severe disease. Basic clinical and demographic information were recorded. Samples were collected at malaria clinics in 5 towns: Pailin, Rattanakiri, Memut, Kampong Seila, and Chumkiri. A subset of samples taken at Pailin and Rattanakiri were from people enrolled in in vivo efficacy studies. The in vivo efficacy study in Pailin was described in section 2.5.2. The in vivo efficacy study in Rattanakiri was conducted using the same protocol but no one experienced a reoccurrence of parasitemia. The enrollment criteria for in vivo studies was more strict that the cross-sectional study: mixed species infections and asymptomatic disease were excluded in the in vivo but not the cross sectional study. In addition, children were eligible for the Pailin in vivo study.

Questionnaire and Clinical Assessment

The study nurse administered a brief questionnaire, which contained questions on prior medical history, previous use of antimalarials, previous episodes of malaria, and basic sociodemographic information. Of note, the information on previous use of antimalarials and previous episodes of malaria are hard to interpret because they are based on self-report. In addition, fake antimalarials are common in Cambodia, which further decreases the reliability of this measure (150). Parasitemia was determined as described in section 2.5.4.

Genotyping

Clinical samples were processed and genotyped as described in section 2.5.6.

Analysis

The main purpose of this study was to describe the distribution of *pfmdr1* haplotype and copy number in Cambodia. Therefore, the mean copy number and the prevalence of the different *pfmdr1* haplotypes were calculated for each site.

The secondary purpose of this analysis was to test whether *pfmdr1* copy number varies by site. The assumptions of parametric statistics (normally distributed and homogeneity of variances) could not be met, even though numerous transformations of copy number were tried. Therefore, non-parametric statistics were used. The Kruskal-Wallis test was used to determine if copy number varied by site. To investigate whether copy number is related to any clinical characteristics (such a gender, parasite density, age, presence of mixed species, or the presence of gametocytes, either the Kruskal Wallis or the Spearman Correlation was

used. Lastly, to determine if copy number still varies by site when controlling for the clinical characteristics that are also related to copy number, a rank ANCOVA was used.

Table 2.1 Comparison between the full cohort, the genotyped subjects, and the non-genotyped subjects in Rutshuru, DRC

Characteristic	Genotyped	Not genotyped	p-value
# patients	212	37	
# men (%)	115 (55.0%)	14 (37.9%)	0.047
median age in months (range)	25 (6-59)	23 (8-53)	0.304
parasitemia (geo. mean)	38,249	23,849	0.049
range of parasitemia	(1043-454664)	(1734, 280000)	
mean hemoglobin	10	10.1	0.994
mean temperature (ºC)	38.7	38.9	0.252
Number excluded	17	0	1.000
% Treatment failure	40.10%	21.60%	0.015

Figure 2.1 Map of Democratic Republic of Congo

CHAPTER III:

DHFR AND *DHPS* GENOTYPE AND IN VIVO RESISTANCE TO SULFADOXINE-PYRIMETHAMINE IN CHILDREN WITH FALCIPARUM MALARIA IN THE DEMOCRATIC REPUBLIC OF THE CONGO

by

Alisa P. Alker, Walter M. Kazadi, Albert K. Kutelemeni, Peter B. Bloland, Antoinette K. Tshefu, and Steven R. Meshnick

ABSTRACT

Malaria is endemic in the Democratic Republic of the Congo (DRC) and is a significant source of morbidity and mortality. Between June and September 2002, a therapeutic efficacy trial was conducted in four clinics in Rutshuru, Eastern DRC, comparing SP, amodiaquine and SP (AQSP), and artesunate and SP (ASSP) regimens for treating malaria in children. In the SP arm, 60.6% of the subjects experienced treatment failure after 14 days. The failure rate was lower in the combination arms (AQSP: 32%, ASSP: 21%). We genotyped 212 samples from this study for mutations associated with SP resistance and investigated their association with treatment failure. The *dhfr*-108 and *dhfr*-51 mutations were nearly universal while 89% of the samples had at least one additional mutation at *dhfr*-59, *dhps*-437, or *dhps*-540. The relationship between *dhps* mutations and treatment failure differed by initial parasite density: for children with a parasite density less than 45,000 parasites/μl, the risk of treatment failure was 37% for children with mutations at *dhps*-437 and *dhps*-540 mutation and 21% for children with neither mutation (risk difference (RD) = 17%, 95%CI: -4%, 36%; risk ratio (RR) = 1.9, 96%CI: 0.7, 5.7). In children with a parasite density greater than 45,000 parasites/μl, the treatment failure risk was 59% and 8% for children with both mutations or neither mutation, respectively (RD = 51%, 95%CI: 34%, 67%; RR = 10.9, 95%CI: 2.8, 42.4). The high failure rate of SP from this and other studies, in addition to the high frequency of *dhfr*/*dhps* mutations, are indications that SP-based regimens are not an appropriate treatment for falciparum malaria in Eastern DRC.

INTRODUCTION

A major obstacle in the control of *Plasmodium falciparum* malaria is the development of

drug resistance. Drug resistance has increased malaria-related morbidity and mortality (151, 152). It has also amplified the cost of malaria control, as second line drugs and combination therapy are often more expensive. Sulfadoxine-pyrimethamine (SP) has been used widely in Sub-Saharan African for the treatment of uncomplicated malaria. However, resistance to SP has been documented in several Sub-Saharan countries (27, 34). As a result of the emergence and rapid spread of resistance to cheap antimalarial drugs, countries have been urged to shift to combination therapies. More over, SP is the only recommended drug for use for Intermittent Preventive Treatment in pregnant women (IPTp) for the time being. Therefore, monitoring of SP resistance is essential for determining whether or not an SP-based combination therapy regimen should be introduced as first line antimalarial drug for uncomplicated malaria or kept as an option for IPTp.

SP resistance is conferred by mutations in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes, which encode for the drugs' targets. The *dhfr*-108-Ser mutation is the first to occur; subsequent mutations in *dhfr* and *dhps* incrementally add to the level of resistance (93). The presence of mutations at *dhfr*-51, *dhfr*-59, *dhfr*-108, *dhps*-437, and *dhps*-540 has been previously associated with SP treatment failure (77, 78, 80, 82, 86-90). The role of mutations at *dhps*-436, *dhps*-581, and *dhps*-613 in treatment failure is less clear, as these mutations have received less attention due to their low prevalence in Africa.

Molecular markers of drug resistance, such as *dhfr* and *dhps*, can be used for surveillance of drug resistance (49, 93). For example, the prevalence of molecular markers of chloroquine resistance (*pfcrt*-76 and *pfmdr1*-86) and SP resistance were used assess drug efficacy in regions where in vivo efficacy studies were unfeasible (153, 154).

In the Democratic Republic of Congo (DRC), SP replaced chloroquine as the national first

line drug in 2001 as an interim strategy (131). However, SP has been in use in the Eastern region of the country since 1995 (P. Bloland, unpublished data). In vivo efficacy studies have shown that parasites in the East are more resistant to SP and chloroquine than the rest of the country (131). Methods to extend the current in vivo surveillance is greatly needed in DRC because of its large geographic size, limited health infrastructure, and political instability. We report here the prevalence of *dhfr* and *dhps* mutations in Eastern DRC and their relationship to 14 day risk of SP treatment failure in order to evaluate the use of these markers for surveillance of SP resistance.

METHODS

Study Design

A therapeutic efficacy trial was conducted in Rutshuru, a district near the Rwandan border in Eastern DRC, to compare the efficacy of 3 regiments: 1. amodiaquine plus SP (AQSP); 2. artesunate plus SP (ASSP); and 3. SP alone (SP). The details of this study will be reported elsewhere. Briefly, this trial was a randomized, open label study following the WHO 1996 protocol with the modifications suggested in the 2002 report (48, 131, 138). The study took place between June and September 2002 at four different clinics. Children were included in the study if they met the following criteria: 1. they were between the ages of 6 to 59 months; 2. they did not suffer from severe malnutrition; 3. they had a *P. falciparum* infection with no other *Plasmodium* species detected; 4. their parasitemia was between 2,000 to 200,000 parasites / µl; 5. there were no signs of severe disease; 6. the axillary temperature was equal or greater than 37.5 ºC; 7. there was no evidence of fever caused by illnesses other than malaria; 8. there was no history of allergic reactions to sulfa-containing drugs; 9. they were

able to return to clinic for follow-up visits; and 10. parent or guardian consent.

249 children were initially enrolled and randomized to a treatment arm (AQSP: 75, ASSP: 82, SP: 92). All subjects received SP at enrollment (25 mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine). Children in the combination arms received either amodiaquine (10 mg/kg/day) or artesunate (4 mg/kg/day) for 3 days. Follow-up visits occurred 1, 2, 3, 7, and 14 days after enrollment, though the subjects were encouraged to return sooner if they were symptomatic. Subjects were excluded after enrollment if they did not return for scheduled visits, they reported using an antimalarial drug other than those given in the study, they developed another disease or their parent or guardian withdrew consent.

Sample Processing and genotyping

Approximately 50 µl of blood was collected from 212 patients at enrollment. The blood was placed on IsoCode Stix filter paper (Schleicher & Schuell, Keene, N.H., USA), desiccated, and then transported to UNC Chapel Hill for processing. DNA was extracted according to the protocol included within IsoCode Stix' packaging.

All samples were genotyped at 3 *dhfr* (51, 59, 108) and 4 *dhps* (437, 540, 581, 613) codons using real-time PCR and MGB probes, as described previously (142). A subset of samples was genotyped for *pfmdr1*-86, *pfmdr1*-184, and for *pfmdr1* copy number (58, 99). Genotyping of *dhps*-437 does not work in the presence of a mutation at *dhps*-436 (142). Therefore, all samples that were negative in the *dhps*-437 assay were amplified and sequenced, as previously described (142).

Analysis

All statistical analyses were performed in Stata 8.2 (College Station, TX). The main outcome being investigated was 14 day treatment failure, as classified by the WHO protocol for in vivo efficacy studies in intense transmission areas (138). Early treatment failure is defined as having any of the following conditions: 1. development of severe malaria on days 1-3; 2. parasitemia on day 2 higher than the parasitemia on day 0; 3. day 3 parasitemia that is greater than 25% of the day 0 parasitemia; or 4. presence of parasitemia on day 3 in the presence of fever (axillary temperature ≥ 37.5 °C). Late clinical failure is defined as either 1. development of severe malaria after day 3, or 2. presence of parasitemia on day 4-14 in the presence of fever (axillary temperature ≥ 37.5 °C). Late parasitological failure is defined as the presence of parasites on day 4-14 with an axillary temperature < 37.5 °C. If none of these criteria were met, the subject was considered to have an adequate clinical and parasitological response to treatment. In the statistical analyses, all types of treatment failure were combined.

The main exposure of this analysis was the *dhfr* and *dhps* genotype. For each individual codon, a binary variable was created that reflected whether the mutant genotype was present. The following genotypes are considered mutant: *dhfr*-51-Ile *dhfr*-59-Arg, *dhfr*-108-Asn/Thr, *dhps*-437-Gly, *dhps*-540-Glu, *dhps*-581-Gly, *dhps*-613-Ser/Thr, *pfmdr1*-86-Tyr and *pfmdr1*-184-Phe.

For all analyses, risk differences were estimated using linear risk regression (general linear model with the identity link and a binomial error distribution). Linear risk regression is more appropriate than log risk or logistic regression in this study because risk differences more accurately reflect the magnitude of the effect when the outcome is common. In addition, linear risk regression is more appropriate for evaluating effect modification because it is on

the additive scale (155, 156). Risk differences have been used before in the evaluation of molecular markers on treatment failure in malaria (88). However, since most other studies use ratio measures of effect, RR will also be calculated to aid in the comparison to other studies.

To evaluate the independent effect of each mutation on treatment failure, *dhfr*-59, *dhps*-437, and *dhps*-540 were all included in the model *a priori*. Interactions between codons were then evaluated using a forward selection technique based on a p-value of 0.05. *dhfr*-108, *dhfr*-51, *dhps*-581, and *dhps*-613 were not included in this analysis because they are homogeneous in this population.

Adjusted effects of these mutations were estimated by including confounders and effect measure modifiers in the linear risk model. Potential covariates included: initial parasite density (coded as a binary variable with a cut point at the median (45,000 parasites/μl)), age (in years), hemoglobin (coded as a binary variable with the cut point at the mean (10)), fever, z-score of weight for age (coded as a binary variable with the cut point of –2), clinic, and treatment arm. Covariates were first evaluated as potential effect measure modifiers using forward selection. The interaction term was retained in the model and the covariate was considered an effect measure modifier when $p \leq 0.05$ in the Wald test. All non-effect measure modifiers were evaluated in a directed acyclic diagram to select a sufficient set of covariates that should be included in the model to control for confounding (147).

Sensitivity analyses were performed to quantify the uncertainty caused by the 20 exclusions (including the 3 deaths). The data were re-analyzed under the following scenarios: 1. all the excluded patients failed treatment and 2. all the excluded patients did not fail treatment.

The effect of *dhfr* and *dhps* mutations on the presence of parasites on day 3 was also investigated because parasite resistance can manifest clinically as delayed parasite clearance (65). The same modeling strategy was employed as described above.

Ethics

The original study was reviewed by the Ministry of Health Technical Panel on behalf of the ethics committee. The Institutional Review Board at UNC Chapel Hill School of Public Health approved the genotyping and data analysis.

RESULTS

Efficacy study

The results of the entire cohort study will be presented elsewhere (W. Kazadi, unpublished data). For the 212 subjects that were genotyped, 17 patients were excluded, mostly due to loss to follow-up (AQSP: 3, ASSP: 1, SP: 13). In addition, three children died during the course of the study (AQSP: 0, ASSP: 1, SP: 2). The exact cause of death was not determined, though in one case a home remedy for malaria was suspected. In the remaining 192 subjects, the treatment failure was high (AQSP: 33%, ASSP: 24%, SP: 63%,). The majority of the treatment failures occurred after day 3 (late clinical failures & late parasitological failures), except for the SP arm in which 10 out of the 42 failures were classified as early treatment failures (Figure 3.1). The cause of treatment failure (i.e. recrudescence of the original infection or a new infection) was not assessed.

For study participants whose samples were genotyped, the average age was 28.7 months (range: 6-59). The mean parasitemia at enrollment was 74,173 parasites per µl (range: 1,043-

454,664) while the mean hemoglobin was 10.0 gm/dl (range: 5.6-14.5 gm/dl). A quarter of the subjects were underweight (weight for age z-scores ≤ -2 in reference to the NCHS/WHO international dataset (157)).

The genotyped sub-cohort (n = 212) was similar in these clinical characteristics to subjects to the subject that were not genotyped (n = 37, results not shown). However, the treatment failure rate was slightly higher in the genotyped subjects compared to the entire cohort (Entire cohort failure rate: AQSP: 32% ASSP: 22% SP: 61%).

Genotyping

All genotyping was successful except for 10 samples for *dhps*-437. Sequencing revealed one sample contained 436-Ser 437-Gly, eight samples were 436-Ala 437-Ala, and one sample had a 436-Cys 437-Ala *dhps* genotype. The 436-Cys mutation, which is a two nucleotide difference from the wild-type sequence, has only been reported once before (71).

The prevalences of mutant and mixed (mutant and wild-type) genotypes in *dhfr*, *dhps,* and *pfmdr1* are presented in Figure 3.2. Most samples had a mutant *dhfr*-108 and *dhfr*-51 component. Mutations at *dhfr*-59, *dhps*-437 and *dhps*-540 were very common (*dhfr*-59: 66%, *dhps*-437: 72% *dhps*-540: 67%). No mutation at *dhps*-613 was observed. Only one sample (1%) was wild-type at all *dhfr* and *dhps* codons while 92 samples (43%) contained the quintuple mutant (mutation at *dhfr*-108, -51, -59, *dhps*-437 and -540).

Individual *dhfr* and *dhps* mutations and treatment failure

Since all patients received SP or an SP containing regimen, data on all patients were pooled. *dhps*-437 and –540 mutations were strongly associated with increased risk of

treatment failure (Table 3.1). Interestingly, the presence of both these mutations was associated with a slightly smaller increase in risk in treatment failure than with either of these mutations alone (interaction term between 437 and 540: $\chi^2 = 4.84$, p=0.023). This suggests that these two mutations are antagonistic. *dhfr*-59 was only weakly associated with treatment failure. These results did not substantially change when only subjects in the SP arm were analyzed.

dhps-437/540 mutations multivariable modeling of treatment failure

Because *dhps*-437 and *dhps*-540 had the biggest impact on treatment failure, they were evaluated further in multivariable modeling. The purpose of this analysis was to test for effect measure modifiers and to obtain an unconfounded estimate effect of *dhps* mutations on treatment failure. Information on the genotype of these two mutations was combined into a categorical variable with 3 levels: having neither mutation, having one mutation, and having both *dhps*-437 and –540 mutation.

In the effect measure modifier screening, initial parasite density was the only covariate identified ($\chi^2 = 10.70$, p=0.005). This result implies that the effect of *dhps*-437 and *dhps*-540 differs by parasitemia. Of particular interest, neither treatment arm nor age were significant effect measure modifiers (treatment arm: $\chi^2 = 2.03$, p=0.567; age: $\chi^2 = 0.12$, p=0.940), which implies that the effect of the *dhps* mutations does not differ by regimen or by age.

In subjects with parasitemia less than 45,000 parasites/µl, the *dhps*-437 and -540 mutations were associated with a small increase in risk of treatment failure compared to subjects with neither mutation (for one mutation: RD = 19%, 95%CI: 14%, 53%; for both mutations: RD = 17%, 95%CI: -4%, 36%). The corresponding RR is 1.9 (95%CI: 0.7, 5.7) for one mutation

and 1.8 (95%CI: 0.8, 4.2) for both mutations. In subjects with parasitemia equal or greater than 45,000 parasites/µl, the risk difference was 80% (95% CI: 54%, 105%) for one mutation and 51% (95%CI: 34% , 67%) for both mutations. The corresponding RR for one mutation is 10.9 (95%CI: 2.8, 42.4) and for both mutations is 7.3 (95%CI: 1.9, 28.2). The adjusted risk differences were similar to the crude estimates (Table 3.2). The sensitivity analysis revealed that the risk differences changed little by assuming either all the excluded children failed or were all successfully treated (Table 3.3).

dhfr/dhps genotype and presence of parasites on day 3

The children with both *dhps*-437 and *dhps*-540 mutations were less likely to be parasitemic on day 3 compared to children without these mutations (31% versus 39%; RD= -8, 95%CI: -23, 8). The corresponding relative risk is 0.8 (95%CI: 0.5, 1.2). The adjusted RD and RR were similar and magnitude and precision compared to the crude estimates (Table 3.4). Thus, there was no association between *dhps*-437/540 mutations on the presence of parasites on day 3.

DISCUSSION

In this study investigating the molecular determinants of SP treatment failure, mutations at *dhps*-437 and *dhps*-540 were strongly associated with treatment failure. However, this relationship differed by parasitemia level. In the low parasitemic group, subjects with either *dhps*-437/540 mutation had a 19% greater absolute risk of 14 day treatment failure than subjects with neither mutation. In contrast, among those with high parasitemia, the absolute risk of treatment failure was 80% greater in subjects with either *dhps*-437/540 mutation.

The differential effect of genetic markers of resistance by parasitemia has been reported before: higher parasitemia was related to decreased ability to clear infections with *pfcrt*-76 and *pfmdr1*-86 mutations after chloroquine treatment (63). High parasitemia is a sign of low partial immunity. Therefore, the larger effect of these mutations at high parasitmias might be caused by the inability of the immune system to clear resistant parasites. These results suggest that resistance might have a greater impact on severe disease. In addition, clinical information, such as parasite density, might need to be collected when monitoring for drug resistance in order for the prevalence of mutations to accurately depict the treatment failure rate.

Interestingly, the effect of *dhps*-437 and 540 did not vary by treatment arm, even though the different regimens had different failure rates. This suggests that *dhfr* and *dhps* mutations are important determinants of treatment failure, even when SP is used in combination.

In the evaluation of the independent effects of *dhfr* and *dhps* mutations, both *dhps*-437 and *dhps*-540 were strongly related to risk of treatment failure. *dhfr*-59 was only weakly associated. This is in contrast to a previous study that found these three mutations have similar independent effects (88). However, the effect of mutations has been shown to vary by region (76, 89), which is likely caused by variation in the prevalence of treatment failure, the presence of other *dhfr/dhps* mutations, and the prevalence of other risk factors for treatment failure across sites.

Despite the fact that delayed parasite clearance is a clinical manifestation of parasite resistance, we did not find an association between *dhps*-437/540 and the presence of parasites on day 3. In fact, an opposite trend was found. This finding is in contrast to a study in Columbia that found the presence of both *dhfr*-108 and *dhfr*-51 mutations was associated

with delayed parasite clearance (85). However, delayed parasite clearance was more common in Rutshuru, even in subjects without *dhps*-437 and -540 mutations. Therefore, other determinants of resistance might be masking the effects of these mutations.

The prevalence of *dhfr* and *dhps* mutations in Rutshuru is higher than previous reports from the Republic of Congo (132) but similar to Uganda (158). The high prevalence of the *pfcrt*-76 and *pfmdr1*-86 mutation in this cohort suggests these parasites are also resistant to chloroquine (159). The high prevalence of mutations associated with drug resistance confirms the presence of multi-drug resistant *P. falciparum* in this area.

While the mean *pfmdr1* copy number in these samples was low (1.18), 14% of these samples contained greater than 1.5 copies. Increased *pfmdr1* copy number has previously been associated with mefloquine and lumefantrine resistance (104, 107, 160) and has been seen in Africa before (100, 161). Therefore the use of lumefantrine and mefloquine in Africa should be implemented carefully to minimize the selection of resistant parasites, which are already present (though at low frequencies).

The main strength of this study is analytical technique: effect measure modifiers were systematically screened, potential confounders were controlled for, and the uncertainty caused by exclusions was quantified. In addition, the genotyping of these samples by real-time PCR assay has previously been validated: three samples whose genotype was determined by real-time PCR were cloned and sequenced to look for minor alleles. The genotype at *dhfr*-108, -59, and 51 from over 30 clones per sample was consistent with the real-time PCR genotyping of the entire sample (146).

The main limitation of this study is the limited follow-up. SP failures have occurred up to 28 days after treatment (54) and therefore it is possible that some subjects failed after the 14-

day follow-up ended. A recent review of in vivo efficacy studies found that, in general, the 14-day follow-up period has limited sensitivity in detecting treatment failures (53). However, the 14-day follow-up had the best sensitivity (~80%) in areas of high resistance and high levels of transmission (Figures 3C and 4 in (53)). For example, Checchi et al. conducted an in vivo efficacy study with SP in Liberia in a high resistance, high transmission area(33). After 14 days, 33 (out of 68) people had experienced recurrent episodes. Between days 14 and 28, 13 additional people experienced recurrent episodes. However, it was estimated that most of these were due to re-infection. After PCR correction, only 36 people total were considered to be true treatment failure. For this study, the sensitivity of the 14-day assessment in detecting treatment failure was 94%. Therefore, in the Rutshuru study, it is likely that most treatment failures were captured.

Another limitation to the interpretation of data is the lack of PCR correction to distinguish true recrudescence from re-infections However, the risk of re-infection is low during the first two weeks after treatment. Stephniewska et al. found that 73% of trials that conducted PCR genotyping before day 14 found that all instances of treatment failure were due to recrudescence .

Since outcome misclassification caused by the lack of PCR correction and the limited follow-up is nondifferential with respect to the exposure (*dhfr/dhps* genotype), these potential biases would likely bias the effect estimate towards the null (162). Another limitation of this study is that not all determinants of treatment failure were measured, such as drug pharmacokinetics, HIV status, and host genetic factors.

The high failure rates of SP and SP-combination therapy indicates that SP should no longer be used in this area. It was based on this and evidence from other sentinel site in DRC

that the country recently decided to shift from SP to the combination of amodiaquine plus artesunate (AQ+AS) as the new first line antimalarial. However, when a new regiment is introduced, *dhfr* and *dhps* could provide a complementary technique to efficacy trials to explore the geographic and temporal changes in resistance as far as SP is still used for IPTp. This study suggests that the collection of clinical information, such as parasite density, might be needed to use molecular markers for the monitoring of drug resistance.

Table 3.1 Risk differences and 95% confidence intervals for the independent effect of *dhfr* and *dhps* mutations on 14 day treatment failure in Rutshuru, DRC. The referent group for all comparisons is parasites with the wild-type genotype at *dhfr*-59, *dhps*-437, and *dhps*-540. RD = risk differences; CI = confidence interval.

Mutation	RD (%)	CI (%)
dhfr-59	9	-4, 22
dhps-437	44	15, 73
dhps-540	50	9, 91
dhps-437 and -540	30	15, 45

Table 3.2 Risk differences and 95% confidence intervals for the association between *dhps* mutations and 14 day risk of treatment failure by parasitemia in Rutshuru, DRC.

RD = risk differences; CI = confidence interval.

	mutation at	Failed	Total	Risk	Crude		Adjusted[†]	
Parasitemia	**dhps-437 + 540**	**(n)**	**(n)**	**(%)**	**RD**	**95%CI**	**RD**	**95%CI**
< 45,000	none	5	24	20.8	0.*		0.	
	1 mutation	4	10	40.0	19	-15, 53	19	-14, 53
	both mutations	25	67	37.3	17	-4 , 36	17	-3, 36
≥ 45,000	none	2	25	8.0	0.		0.	
	1 mutation	7	8	87.5	80	54, 105	85	71, 98
	both mutations	34	58	58.6	51	34, 67	49	35, 64

[*]Referent level

[†]Adjusted for clinic, age, and z-score of weight for age

Table 3.3 Sensitivity analysis of the effect of *dhps* mutations on treatment failure by parasitemia in Rutshuru, DRC. Unadjusted risk differences (RD) and 95% confidence intervals (CI) were calculated.

Scenario	Parasitemia	*dhps*-437/540	Risk (%)	RD	CI
All excluded failed treatment	< 45,000	none	24.0	0.*	
		1 mutation	40.0	16	-19, 51
		both mutations	40.8	17	-3, 37
	≥ 45,000	none	23.3	0.	
		1 mutation	90.0	67	43, 91
		both mutations	63.6	40	21, 59
All excluded were successfully treated	< 45,000	none	20.0	0.	
		1 mutation	40.0	20	-14, 54
		both mutations	35.2	15	-4, 34
	≥ 45,000	none	6.7	0.	
		1 mutation	70.0	63	34, 93
		both mutations	51.5	45	30, 60

*referent level

Table 3.4 Risk differences and 95% confidence intervals for the association between *dhps* mutations and risk of day 3 parasitemia, Rutshuru, DRC. RD = risk differences; CI = confidence interval.

Mutation at *dhps*-437/540	Parasitemic on day 3 (n)	Total (n)	Risk (%)	Crude		Adjusted[†]	
				RD	CI	RD	CI
none	20	52	38.5	0.*		0	
one	8	20	40.0	2	-24, 27	-2	-27, 23
both	40	130	30.8	-8	-23, 8	-7	-22, 8

[*]Referent level

[†]adjusted for initial parasite density, age, clinic, and z-score weight for age

Figure 3.1 Flowchart for the in vivo efficacy trial in Rutshuru, DRC. Clinical outcome was classified according to the WHO in vivo efficacy protocol for intense transmission areas (138). ACPR = adequate clinical and parasitological response.

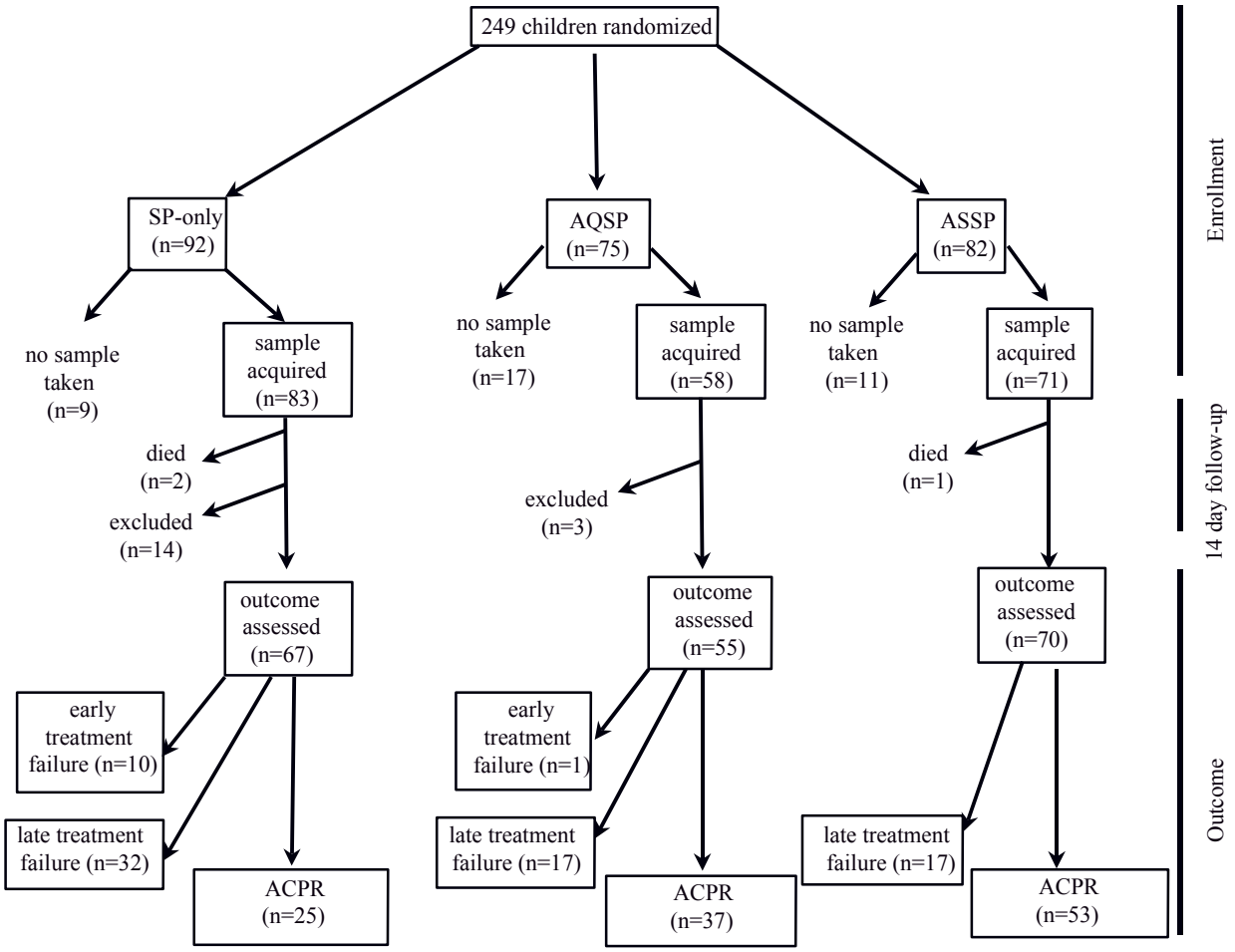
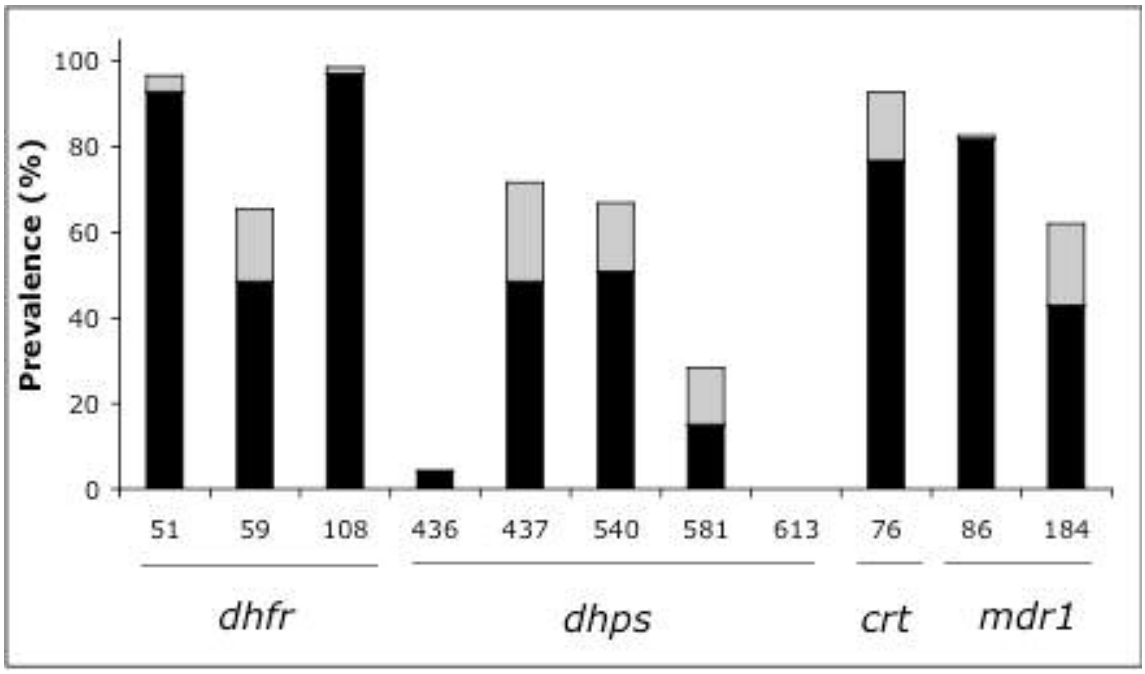


Figure 3.2 Prevalence of mutant and mixed genotypes at codons associated with drug resistance. The following genes were genotyped: dihydrofolate reductase (*dhfr)*, dihydropteroate synthase (*dhps),* chloroquine resistance transporter *(pfcrt*), and multidrug resistance gene-1 *(pfmdr1).* The *pfcrt* 76 genotype from these samples was previously reported (159). Sample size is 212 except for *pfcrt* (n=56) and *pfmdr1* (n=58). Black = mutant, gray = mixed.



CHAPTER IV:

PFMDR1 AND IN VIVO RESISTANCE TO MEFLOQUINE-ARTESUNATE IN FALCIPARUM MALARIA ON THE THAI-CAMBODIAN BORDER

by

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ABSTRACT

Background. Artemisinin Combination Therapies (ACT) has recently been adopted as first-line therapy for *Plasmodium falciparum* infections in most malaria-endemic countries. One ACT, artesunate-mefloquine, has been used in Southeast Asia since 1994. In this study, we determine whether artesunate-mefloquine failures are associated with genetic changes in the biomarker, *pfmdr1*.

Methods. Blood samples were acquired from 80 patients enrolled in a 2004 in vivo efficacy study of artesunate-mefloquine in Pailin, Cambodia, and genotyped for *pfmdr1* copy number and haplotype.

Results. Having three or more copies of *pfmdr1* was strongly associated with recrudescence (hazard ratio (HR) = 8.30, 95%CI: 2.60, 26.43). This relationship was maintained when controlling for initial parasite density and hematocrit (HR = 7.91, 95%CI: 2.38, 26.29). Isolates from patients who had recurrent episodes after artesunate-mefloquine treatment had higher copy numbers than the paired enrollment sample (Wilcoxon Rank test, $p = 0.040$).

Conclusion. *pfmdr1* copy number was strongly associated with recrudescence to artesunate-mefloquine therapy. Treatment selected for parasites with increased *pfmdr1* copy number. *pfmdr1* copy number should be evaluated further as a surveillance tool for artesunate-mefloquine resistance in Cambodia.

INTRODUCTION

One of the challenges in the treatment and control of falciparum malaria is drug resistance. The Thai-Cambodian border, in particular, is a hotspot for the emergence of antimalarial resistance: some of the earliest reports of chloroquine and pyrimethamine resistance came from this area (23). In addition, high levels failure rates of mefloquine monotherapy were documented in the 1990's (121).

Artesmisinin combination therapy (ACT) has been promoted to be an effective strategy to combat the emergence and spread of resistance (163). Currently, 38 countries have adopted ACT as first-line regimens for the treatment of malaria (32). However, a recent in vivo efficacy study on Thailand's border with Cambodia reported a artesunate-mefloquine 28-day cure rate of 78.6% (39). In addition, an in vivo study on the Cambodian side of the border documented a 28-day cure rate of 86.7% in 2002 (164). These studies suggest declining susceptibility to the artesunate-mefloquine combination in this sub-region. However, it is not yet known whether these reported ACT failures are due to true parasite resistance, or other factors such as drug pharmacokinetics.

Because antimalarial resistance can emerge quickly, surveillance is a key element to a successful malaria control program (18, 47). While the gold standard for monitoring drug efficacy is the in vivo trial, molecular markers could provide an inexpensive complementary tool. However, before they can be used, the relationship between specific molecular markers and in vivo resistance must be established.

One possible molecular marker is *Plasmodium falciparum* multidrug resistance gene-1 (*pfmdr1).* Single nucleotide polymorphisms (SNPs) and gene copy number have previously been associated with in vitro resistance to a wide array of antimalarials, including

chloroquine, lumefantrine, quinine, halofantrine, mefloquine and artesunate (31, 97-99, 101, 102, 107, 165). But in vitro and in vivo resistance are not always correlated (107). The purpose of this study was to determine whether *pfmdr1* genetic changes are associated with in vivo evidence of resistance to artesunate-mefloquine in Cambodia.

MATERIAL AND METHODS

Clinical study

As part of Cambodia's National Malaria Control Program, 25 children and 56 adults with uncomplicated malaria were treated with artesunate and mefloquine between June and September, 2004. The details of this study will be published elsewhere (148). Informed consent was obtained from the subject or guardian. The WHO 2003 protocol for low transmission areas was followed (17). At enrollment, a brief clinical exam was performed and a questionnaire was administrated.

Approximately 2 ml of venous blood was collected and frozen. Subjects were treated according to the national policy: they were given 25 mg/kg mefloquine and 12 mg/kg artesunate over 3 days. Subjects stayed at the clinic for 3-4 days (depending on when the parasites cleared) and had follow-up visits at 7, 14, 21, 28, 35, and 42 days after the date of enrollment. People who did not return on their own were actively sought out. In instances of reoccurrence of parasitemia, an additional blood sample was acquired and stored on 3M Whatman filter paper.

Genotyping

DNA was extracted using the Qiamp DNA mini kit (Qiagen, Hilden, Germany). Samples taken at enrollment, day one, and recurrence of parasitemia were used to distinguish between recrudescence and reinfection. The number of variants in 3 polymorphic genes *(msp1, msp2, and glurp)* was determined using size fractionization (124). If the recurrence specimen contained one or more of the variants seen in the enrollment or day one specimen, the infection was classified as a recrudescence.

Enrollment and recurrence samples were genotyped for *pfmdr1* codons 86, 184, 1034, and 1042 using real-time PCR. The protocol by Purfield et al. (58) was followed, except that the concentration of probes was changed to 200 nM.

pfmdr1 copy number was assessed for all samples using a modification of two previous assays (99, 104). The primers and a FAM-TAMRA probe specific to a conserved region of pfmdr1 (from (99)) and the primers and a VIC-TAMRA probe specific to β-tubulin (from (104)) were multiplexed so that both genes could be assayed in the same well. The primers were synthesized by MWG Biotech (High Point, NC) and the probes were synthesized by Applied Biosystems (ABI, Foster City, California).

PCR reactions were performed on either the ABI Prism 7000 or ABI Prism 7300. Each reaction consisted of: *pfmdr1* probe (150 nM), *pfmdr1* primers (300 nM), β-tubulin probe (100 nM), *β-tubulin* primers (100 nM), Abgene QPCR Matermix (1 x), DNA (2 µl) and water up to 25 µl. If amplification of a sample was unsuccessful (no amplification, replicates being greater than 50% different, or a copy number < 0.6), it was repeated with 4 µl DNA. The reaction conditions consisted of: 95°C for 15 minutes and then 50 cycles of 95°C for 15 seconds and 60°C for one minute. The cycle threshold (CT) was calculated with the SDS software (ABI). DNA from strains 3D7 and Dd2 were included on each plate. The 3D7 DNA

was extracted from a laboratory grown culture and Dd2 DNA was acquired from MR4 (MRA-387, ATCC Manassas Virginia).

pfmdr1 copy number was calculated according to the following formula: copy number = $(E_{\beta tubulin})^{CT(\beta tubulin)} / (E_{pfmdr1})^{CT(pfmdr1).}$ The efficiency (E) of *β-tubulin*, which was higher than that of *pfmdr1*, was assumed to be 2. *Pfmdr1*'s efficiency, relative to that of *β-tubulin,* was calculated for each plate by assuming the 3D7 control has 1 *pfmdr1* copy. Dd2, an additional control, was previously determined to have approximately 4 *pfmdr1* copies (166, 167). In our assay, Dd2 had a mean copy number of 4.27 and a coefficient of variation of 5.77%.

Statistical analysis

The clinical data were entered into Excel (Microsoft) and analyzed with Stata 8.2 (StataCorp, College Station, Texas). The clinical outcome of each patient was classified according to the WHO protocol (17). Since there were no early treatment failures, the main outcome for this analysis was the recurrence of *P. falciparum* parasites in the peripheral blood. Recurrences of parasitemia were further categorized as reinfections or recrudescence by the *msp1*, *msp2* and *glurp* genotyping.

The copy number of samples taken before treatment and those taken at recurrence was compared using the Wilcoxon signed-rank test.

The relationship between molecular changes in *pfmdr1* and treatment failure was estimated using survival analysis. Since the subjects were assessed at distinct time points, the failure time is interval-censored. Discrete Cox proportional hazards model was used to take this censoring in account. This model was estimated using regression with a complementary log-log link (149). Indicator variables representing four time periods (0-21, 22-28, 29-35, and 36-

42 days) were included while a constant term was not estimated. All variables were assessed for the proportional hazard assumption. The main outcome was time to recrudescence. Subjects that were lost to follow-up were censored on their last visit. Subjects who developed a *P. vivax* infection or a *P. falciparum* re-infection were censored on the day of that second diagnosis. The precision of all hazard ratio estimates were evaluated by calculating the confidence limits ratio (CLR) (168).

The genotype at codons 86, 184, 1034, and 1042 in *pfmdr1* were combined into haplotypes, as previously described (99). Samples that were a mixture of haplotypes I and III were coded as haplotype III. The relationship between copy number and time to recrudescence was not linear on the log hazard scale. Therefore, copy number was coded as a binary variable of <3 copies and ≥ 3 copies to reflect the relationship between copy number and time to recrudescence and to meet the proportional hazards assumption of the model.

The following covariates were evaluated as potential effect measure modifiers and confounders: previous use of antimalarials, initial parasite density, age, sex, and hematocrit. Initial parasite density was coded as a binary variable, with the cutoff point of 80,000 parasites/μl. Effect measure modification was assessed with the Wald test for the interaction term in the model ($p \leq 0.1$ was considered significant). All non-effect measure modifiers were evaluated in a directed acyclic diagram to select a sufficient set of covariates to control for confounding (147).

The population attributable rate fraction (AF_p) and 95% confidence intervals were calculated according to (169). The specific formula for AF_p was $((HR_{adj} - 1)*p) / HR_{adj}$ where HR_{adj} = adjusted hazard ratio, and p = proportion of cases that were exposed.

The relationship between molecular change in *pfmdr1* and parasite clearance time was assessed using discrete Cox proportional hazards model in the same manner as described above.

Ethics

The *pfmdr1* genotyping and data analysis were approved by the Institutional Review Boards at the U.S. Naval Medical Research Unit No. 2 and at UNC Chapel Hill School of Public Health. Both the genotyping and the efficacy study were approved by the National Ethics Committee of Cambodia.

RESULTS

Efficacy study

Out of the 81 people enrolled, 10 were lost to follow-up, 7 were diagnosed with *P. vivax*, and 25 people experienced late treatment failure between days 14-42 (Figure 4.1). *msp1, msp2*, and *glurp* genotyping determined that 13 out of the 25 recurrences were due to recrudescence of the original infection. In all the enrollment samples, there were 2 *msp1* variants, 5 *msp2* variants, and 5 *glurp* variants. The prevalence of the most common *msp1/msp2/glurp* genotype was 8%.

Enrollment samples were available for *pfmdr1* genotyping for all but one subject. This subject was a child who recrudesced on day 14. Clinical and demographic information for the remaining 80 subjects are presented in Table 4.1.

Genotyping of enrollment samples

Out of the 80 enrollment samples, 75 (93.8%) were successfully genotyped at all four *pfmdr1* codons. Of the remaining five samples, genotyping of one sample was unsuccessful at *pfmdr1*-86, one was unsuccessful at *pfmdr1*-1034, and three were unsuccessful at *pfmdr1*-1042. Most samples had either the I or III haplotype (Table 4.2). *pfmdr1*-86-Tyr was not observed.

pfmdr1 copy number was successfully determined for all enrollment samples. The median copy number was 1.50 (range: 0.60 – 6.28) and 18.8% contained 3 or more copies. Elevated copy number was only observed in the presence of 86-Asn, 1034-Ser and 1042-Asn (haplotypes I and III). There was no association between copy number and *pfmdr1*-184 genotype (ANOVA, F=0.51, p=0.479).

Comparison of enrollment and recurrence *pfmdr1* genotypes

pfmdr1 copy number was determined for all recrudescent (n=12) and all re-infection (n=12) samples. 75.0% (9/12) of the re-infection samples and 66.7% (8/12) of the recrudescent samples had a higher copy number than the corresponding enrollment sample. These differences were not statistically significant (Wilcoxon signed rank test, for recrudescent: $z = 1.26$, $p = 0.209$; for re-infection: $z = 1.57$, $p = 0.117$). However, when comparing all enrollment and recurrent samples, the difference was statistically significant (Wilcoxon signed rank test, $z = 2.06$, $p = 0.040$). Therefore, artesunate-mefloquine treatment appears to select for increased *pfmdr1* copy number.

Four recrudescent and 10 re-infection samples were genotyped for *pfmdr1* polymorphisms. When comparing the haplotypes at enrollment and recurrence for the reinfection samples,

five were identical, one sample changed from haplotype IV to III, two samples changed from being mixed (haplotypes I and III) to haplotype III and two samples changed from having haplotype III to being mixed (haplotypes I and III). The four recrudescent samples had identical haplotypes to the enrollment sample (three of these paired samples had haplotype III and one had haplotype I). Thus, there appears to be no selection of a particular *pfmdr1* haplotype due to artesunate-mefloquine treatment.

pfmdr1 and time to recrudescence

Subjects with increased *pfmdr1* copy number recrudesced sooner and more often than subjects with low copy number (Figure 4.2 and Table 4.3). The crude hazard ratio for increased copy number (≥ 3 copies compared to < 3 copies) was 8.30 (95%CI: 2.60, 26.43). Adjusting for confounders decreased the hazard ratio to 7.91 (95%CI: 2.38, 26.29). These results were not dependent on the statistical model, as the hazard ratio from a continuous Cox proportional model was similar in size and precision. Thus, *pfmdr1* copy number is strongly related to time to recrudescence.

For *pfmdr1* haplotype, 11 out of 12 recrudescences occurred in subjects with haplotypes I and III. One subject with an incomplete haplotype also recrudesced. None of the four subjects with haplotype IV recrudesced, however the small sample size prevents further evaluation of this haplotype. The crude hazard ratio comparing haplotype III to I was 2.22 (95% CI: 0.28, 17.34). Adjusting for confounders resulted in a slightly lower hazard ratio (1.88, 95%CI: 0.24, 15.00). There appears to be little difference between haplotype III and I, however the estimate is very imprecise (CLR = 61.36).

To investigate the impact of increased *pfmdr1* on recrudescence in the entire cohort, the population attributable fraction was calculated. Using the prevalence of > 3 *pfmdr1* copies in the cases (58.3%) and the adjusted hazard ratio (7.91), the population attributable fraction of increased *pfmdr1* copy number was 50.9% (95% CI: 4.1%, 74.9%). *pfmdr1* copy number appears to contribute to the cause of half the cases of recrudescence observed in this study.

When the outcome of all recurrences of parasites (recrudescence + reinfection) was used, the estimate of effect for copy number was lower (unadjusted HR = 4.51, 95%CI: 1.99, 10.23; adjusted HR = 4.02, 95%CI: 1.73, 9.34). In contrast, the estimate for haplotype III was higher but very imprecise (unadjusted HR = 4.78, 95%CI: 0.64, 35.64; adjusted HR = 4.40, 95%CI: 0.59, 33.02). Therefore, the estimated effect of *pfmdr1* haplotype but not copy number changes depending on whether the treatment failures were PCR corrected. This suggests that if some misclassification of recrudescence and reinfection had occurred, the interpretation of the effects would not be substantially different.

pfmdr1 copy number is a potential screening tool for drug resistance. Greater than 3 copies identified cases of recrudescence with a sensitivity and specificity of 58.3% and 88.2%, respectively, and a positive predictive value of 66.7%. Including information on either *pfmdr1* haplotype or initial parasite density increased the specificity but decreased the sensitivity (≥ 3 copies + haplotype III: sensitivity = 50.0%, specificity = 91.2%; ≥ 3 copies + parasitemia ≥80,000: sensitivity = 50.0%, specificity = 91.2%).

pfmdr1 and parasite clearance time

Out of the 80 subjects, 4 (5.0%) cleared the parasitemia by day 1, 41 (51.3%) cleared by day 2, and 27 (33.8%) cleared by day 3, and 8 (10.0%) cleared by day 4. The parasite

clearance times observed in this study are longer than reported previously for artesunate-mefloquine (170).

Increased copy number was not associated with delayed parasite clearance when controlling for parasite density and hematocrit (Table 4.4). There was a small association between *pfmdr1* haplotype and delayed parasite clearance time, though the estimate was imprecise.

DISCUSSION

In this study, *pfmdr1* copy number was strongly associated with recrudescence after treatment with artesunate-mefloquine. After adjusting for confounding, subjects with greater than 3 copies of *pfmdr1* had 7.91 times the risk of recrudescence as subjects with less than 3 copies. *pfmdr1* copy number was an important determinant of recrudescence, as demonstrated by the population attributable fraction of 50.9%. In addition, artesunate-mefloquine treatment was found to select for increased *pfmdr1* copy number, since parasites from recurrent infections had significantly higher copy numbers than parasites from those same patients on enrollment. These data demonstrate that artesunate-mefloquine failures are associated with a specific drug-resistance biomarker, and that treatment may increase the prevalence of this biomarker in the general population.

Our observed association between *pfmdr1* and failure is consistent with previous *in vivo* studies that looked at mefloquine monotherapy (107) and artesunate-mefloquine combination therapy (104). In both studies, associations between *pfmdr1* copy number and drug failure were found, but no association was seen between *pfmdr1* haplotype and drug failure. In contrast, numerous in vitro studies have found associations between both copy number and

haplotype and in vitro IC50 (104-107, 114). There are 3 possible reasons for this. First, the effect of these mutations might be too small to be seen in vivo. Second, determinations of SNPs in complex polyclonal infections could result in misclassification. Third, in Pailin, the *pfmdr1* genotype had minimal variation, with some sensitive genotypes being either rare or absent, so the effect might have been missed.

It has been suggested that increased *pfmdr1* copy number might cause artesunate and mefloquine resistance (98, 99, 107, 165, 171). The observed drug failures are probably due to mefloquine resistance, since a study in Pailin in 2001-2002 found the clinical isolates had high mefloquine IC50's and low artesunate IC50's (119). Also, parasite clearance is predominantly due to artesunate, and no effect of *pfmdr1* copy number on parasite clearance time was observed in this study. Therefore, the effect *pfmdr1* copy number on recrudescence is most likely due to the effect of *pfmdr1* on mefloquine sensitivity, not artesunate sensitivity [21].

This is the first observation of an increase in *pfmdr1* copy number in parasites isolated after artesunate-mefloquine treatment. Since mefloquine has a much longer half-life than artesunate (10-14 days versus 1 hour) (172, 173), resistant parasites were probably selected by exposure to subtherapeutic concentrations of the single drug, mefloquine (174). Selection for mefloquine resistance in Pailin may have also been facilitated by the pre-existence of resistance to this drug (123, 175). This observation suggests that ACT selects for resistance to the non-artemisinin partner drug.

Strengths of this study include the fact that genotyping was done directly on peripheral blood samples, which negates the potential bias caused by culturing (58). Another strength is its 42-day follow-up with PCR correction (53). The main limitation of this study is the small

sample size, which affected the power and the precision of the effect estimates, especially for *pfmdr1* haplotypes. Another limitation is that not all predictors of recrudescence were measured, such as pharmacokinetics, host genetics, immunity and nutritional status.

Molecular markers of drug-resistant malaria, such as *pfmdr1*, are promising tools for the surveillance of drug resistance. *pfmdr1* copy number is not only strongly associated with recrudescence to artesunate-mefloquine but also with Coartem® failure (artemether-lumefantrine) (176). Surveillance for increased *pfmdr1* copy number could aid malaria control efforts by pinpointing areas where these drugs may be failing.

Table 4.1 Clinical characteristics of the subjects in the Pailin in vivo efficacy study that were genotyped for *pfmdr1*. Sample size is 80, unless otherwise specified.

Characteristic	Number
men (%)	57 (71.3%)
median age (range)	20 (6 - 65)
geo. mean parasitemia[*] (SD[†])	18887.7 (116410)
mean hematocrit (SD)	38.7 (5.3)
previous use of antimalarials[‡] (%)	28 (37.8%)

[*]parasites / μl
[†]standard deviation
[‡]in the past month by self-report, n=74

Table 4.2 *pfmdr1* haplotypes of samples taken at enrollment with successful genotyping at all codons. Amino acids representing the mutant genotype are in bold.

86	184	1034	1042	Haplotype*	Number
Asn	Tyr	Ser	Asn	I	13
Asn	**Phe**	Ser	Asn	III	57†
Asn	**Phe**	Ser	**Asp**	IV	4‡
Asn	Tyr	**Cys**	Asn	V	1

*from (99)
†5 samples were mixed at 184
‡1 sample was mixed at 1042

Table 4.3 Hazard ratios and 95% confidence intervals for time to recrudescence by *pfmdr1* copy number and haplotype. HR = hazard ratio and CI = confidence interval.

		No.	No.	Crude Hazard Ratio			Adj[†] Hazard Ratio		
	Level	**Subjects**	**Recrudesced**	**HR**	**CI**	**CLR[*]**	**HR**	**CI**	**CLR**
Copy number	< 3	65	5	1.[‡]			1.		
	≥ 3	15	7	8.30	2.60, 26.43	10.17	7.91	2.38, 26.29	11.04
pfmdr1 haplotype	I	12	1	1.			1.		
	III	47	10	2.22	0.28, 17.34	61.93	1.88	0.24, 15.00	62.50
Hematocrit	< 40	39	7	1.					
	≥ 40	41	5	0.68	0.22, 2.15	9.8			
Parasite density	< 80,000	58	6	1.					
	≥ 80,000	22	6	3.09	1.00, 9.61	9.61			

[*]confidence limit ratio = upper limit / lower limit
[†]adjusted for initial parasite density and hematocrit
[‡]referent level

Table 4.4 Hazard ratios and 95% confidence intervals for the relationship between parasite clearance time and *pfmdr1* copy number and haplotype. HR = hazard ratio, CI = confidence interval and PCT = parasite clearance time.

		Median PCT	Crude Hazard Ratio			Adj† Hazard Ratio		
	Level	**(hours)**	**HR**	**CI**	**CLR***	**HR**	**CI**	**CLR**
Copy number	< 3	48	1.‡			1.		
	≥ 3	72	0.65	0.34, 1.24	3.65	0.98	0.48, 1.98	4.13
pfmdr1 haplotype	I	48	1.			1		
	III	48	0.81	0.40, 1.63	4.08	0.83	0.41, 1.69	4.12
Hematocrit	< 40	48	1.					
	≥ 40	48	1.23	0.76, 2.00	2.63			
Parasite density	< 80,000	48	1.					
	≥ 80,000	72	0.26	0.14, 0.49	3.50			

*confidence limit ratio (CLR) = upper limit / lower limit
†adjusted for initial parasite density and hematocrit
‡referent level

Figure 4.1 Flowchart describing the results of the in vivo efficacy trial in 2004 in Pailin, Cambodia.

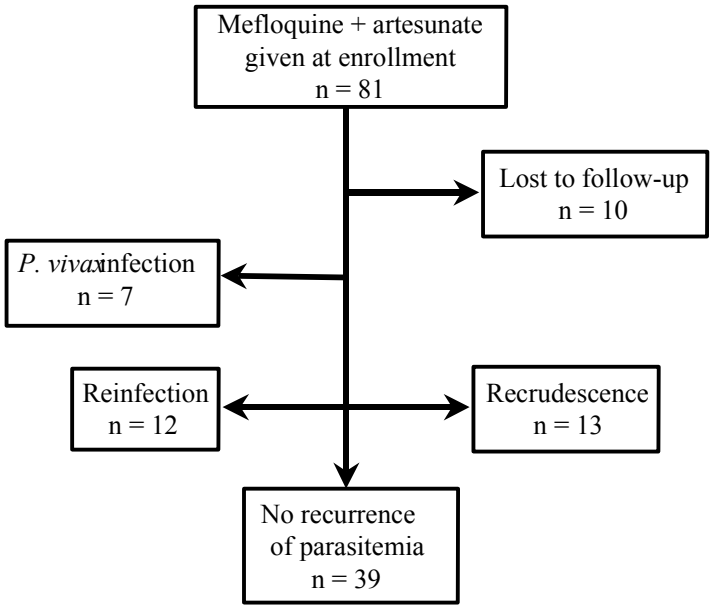
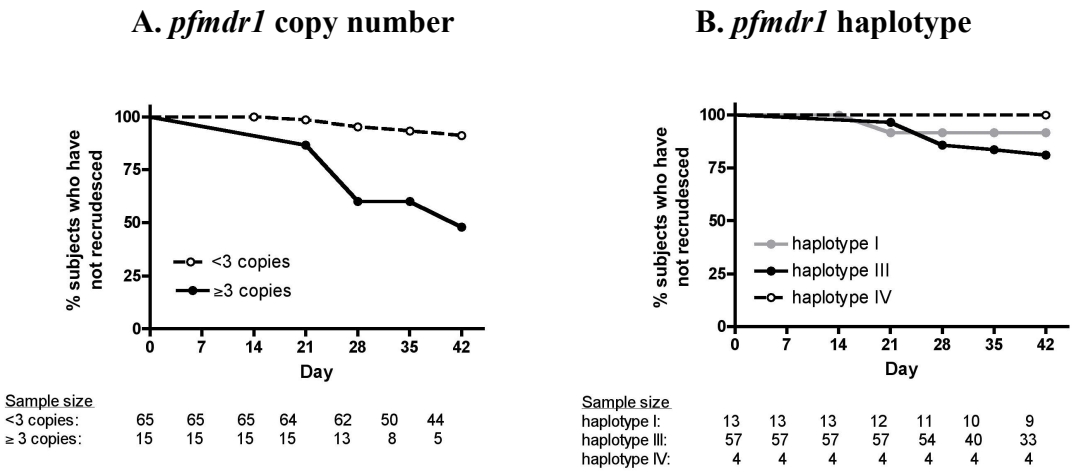


Figure 4.2 The percentage of subjects who have not recrudesced over time after mefloquine + artesunate treatment in Pailin, Cambodia. Copy number was rounded to the nearest integer. Only samples with haplotypes I, III, and IV were included. The haplotypes are defined in Table 4.2.



CHAPTER V:

GEOGRAPHIC DISTRIBUTION OF *PFMDR1* MUTATIONS AND COPY NUMBER IN *PLASMODIUM FALCIPARUM* IN CAMBODIA

by

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ABSTRACT

Plasmodium falciparum malaria is endemic in Cambodia. Because of widespread multi-drug resistant malaria in the region, the national first-line treatment was changed to mefloquine-artesunate in 2001. *pfmdr1* single nucleotide polymorphisms (SNPs) and increased copy number have been associated with mefloquine resistance. To determine the geographic distribution of these genetic changes, we genotyped 457 clinical samples for *pfmdr1* copy number and 255 samples for *pfmdr1* SNPs from 5 geographically disparate clinics. Most samples had the following *pfmdr1* genotype: 86-Asn, 1034-Ser, 1042-Asn. There was more variability at codon 184. *pfmdr1*-184-Phe was more common near the Thai-Cambodian border than in Eastern Cambodia. *pfmdr1* copy number also varied geographically. In addition to Pailin near the Thai border, Chumkiri in Central Cambodia also had a high mean *pfmdr1* copy number, which suggests mefloquine resistance is more widespread than previously thought.

INTRODUCTION

Plasmodium falciparum causes over 500 million clinical cases of malaria each year, most of which are in developing countries (3). One of the major obstacles in controlling this disease is drug resistance. Drug resistance evolves more quickly in areas of low transmission, such as in Asia and South America. In fact, sulfadoxine-pyrimethamine resistance in Africa appears to have arose from the importation of parasites from Southeast Asia (28). Therefore, even though the majority of the malaria burden is in Africa, the control of drug resistance in Asia is essential for malaria endemic countries worldwide.

The Thai-Cambodian border region is a hotspot for the emergence of drug resistance in Asia. One of the first reports of chloroquine resistance in *P. falciparum* came from this area in 1961 (23). Since then, these parasites have developed high levels of resistance to chloroquine and SP, making this region one of the most drug resistant areas in the world (27, 177). Mefloquine was first used in this region in combination with SP in the 1980s and later as monotherapy in the 1990s (25). High levels of mefloquine resistance was documented in the 1990's (38, 123). As a result, mefloquine-artesunate replaced mefloquine monotherapy in 1995 on the Thai side of the border and in 2001 on the Cambodian side of the border. Recent clinical studies indicate the efficacy of the mefloquine-artesunate combination is decreasing at the Thai-Cambodian border ((39) and Mey Bouth et al. in review). The decreasing efficacy of mefloquine-artesunate is most likely due to mefloquine resistance, as clinical samples taken from Pailin were resistant to mefloquine but sensitive to artesunate in in vitro assays (119).

In contrast to the situation at the Thai border, *P. falciparum* in Eastern Cambodia remains sensitive to both mefloquine and chloroquine (38). The level of resistance in Central Cambodia appears to be variable, though only a few in vivo efficacy studies have been conducted in this area (38). The increasing mefloquine-artesunate treatment failure and the geographic variation in resistance suggest expanded surveillance is greatly needed in Cambodia.

One method to extend surveillance of drug resistance beyond sentinel sites is to measure molecular markers of drug resistance. In particular, detecting genetic changes in the *Plasmodium falciparum* multi-drug resistance gene-1 *(pfmdr1)* might be a useful measure because it has been associated with resistance to a wide array of antimalarials. *pfmdr1* copy

number has been associated with increased in vitro resistance to mefloquine, lumefantrine, and quinine, and decreased sensitivity to artesunate (98, 99, 101, 178-180). In addition, increased *pfmdr1* copy number predicts in vivo treatment failure to mefloquine and mefloquine-artesunate combination therapy (104, 107, 181). Yet increased *pfmdr1* copy number is not associated with increased chloroquine resistance. In fact, some studies suggest the opposite trend (99, 178).

Single nucleotide polymorphisms (SNPs) at codons 86, 184, 1034, 1042, and 1246 in *pfmdr1* have been related to in vitro resistance to mefloquine, chloroquine, lumefantrine, artesunate, and quinine (97-99, 101, 102, 182). *pfmdr1*-86 has been associated with clinical outcome after chloroquine treatment (though it is not the main determinant of chloroquine resistance) (64, 183). No association has been demonstrated between *pfmdr1* haplotype and mefloquine treatment failure (104-107).

The purpose of this study was to determine the geographic distribution of *pfmdr1* SNPs and copy number at 5 distinct sites in Cambodia in order to explore the usefulness of *pfmdr1* as a method for drug resistance surveillance.

METHODS

Clinical study

Clinical samples of *Plasmodium falciparum* were acquired from five sites in Cambodia (Pailin, Memut, Kg. Seila, Rattanakiri, and Chumkiri) between 2004-2005 (Figure 5.1). Eligibility criteria included: having uncomplicated falciparum malaria, being 18 years or older (changed to 5+ years in 2005), having no contraindications to giving blood and providing informed consent. At Pailin and Rattanakiri, samples from on going in vivo

efficacy studies were also included (Mey Bouth et al in review, chapter 4 in this dissertation). These studies had slightly different eligibility criteria (mixed *Plasmodium* species infections were excluded and the Pailin efficacy study included children older than 5 years old). In the Pailin in vivo study, mefloquine-artesunate had an 88.5% 42-day failure rate (when PCR corrected for reinfections). The in vivo study at Rattanakiri had a 100% cure rate after 28 days.

A drop of peripheral blood was collected to make thick and thin smear slides. The thick smear slide was used to assess eligibility criteria. The slides were transported to Phnom Penh where trained microscopists used the thin smear slides to measure the parasite density, gametocyte density, and the presence of non-falciparum species.

A questionnaire was administered to all subjects, which collected information on demographics and past medical history. Approximately 2 µl of blood was collected, frozen, and transported to Phnom Penh for processing. All subjects were treated according to the national policy (25 mg/kg mefloquine and 12 mg/kg artesunate over 3 days).

The *pfmdr1* genotyping and data analysis were approved by the Institutional Review Boards at the U.S. Naval Medical Research Unit No. 2 (NAMRU-2) and at UNC Chapel Hill School of Public Health. The National Ethics Committee of Cambodia approved both the genotyping and the in vivo efficacy studies.

Genotyping

All frozen blood samples were extracted using the Qiamp DNA extraction kit. *pfmdr1* copy number was determined for all samples, as previously described (181). In addition, a subset of samples were genotyped for *pfmdr1* codons 86, 184, 1034, and 1042 at Pailin,

Rattanakiri, Memut, and Kg. Seila, as previously described (58, 181). (The Chumkiri samples have not yet been genotyped for mutations in *pfmdr1*). The samples were not genotyped at *pfmdr1*-1246 because previous surveys of Cambodia and Thailand did not find this mutation (31, 98, 184).

Analysis

The main questions being addressed in the analysis were: if genetic changes in *pfmdr1* vary by site and whether any clinical characteristics of the subset are associated with these genetic changes in the parasite. *pfmdr1* copy number is not normally distributed and the site by site variances were heterogeneous. Multiple transformations of copy number were tried, yet none allowed *pfmdr1* copy number to be normally distributed and have homogenous variances. Therefore, non-parametric statistical tests were employed, which do not rely on these assumptions.

Kruskal-Wallis and Spearman Correlations were used to determine if *pfmdr1* copy number varies by site and by clinical characteristics. A rank ANCOVA was used to test if copy number varied by site when controlling for clinical characteristics. To assess whether *pfmdr1*-184 genotype varies by site and by clinical characteristics, logistic regression was used. Statistical analyses were conducted in Stata 8.2 (StataCorp, College Station, Texas) except for the rank ANCOVA test, which was carried out in SAS 9.13 (SAS Institute, Cary, North Carolina).

RESULTS

In total, blood samples were collected from 457 subjects with uncomplicated falciparum malaria between 2004-2005. The clinical characteristics of the subjects are presented in Table 5.1. The distribution of age, gender, parasitemia, and prevalence of mixed infections varied by site. In particular, Chumkiri had the highest geometric mean parasitemia, the highest prevalence of mixed species and the lowest proportion of women.

pfmdr1 copy number was successfully determined for 434 out of 457 samples (success rate: 95%). *pfmdr1* copy number ranged from 0.60 to 6.28. 16.6% of the samples had 2 or more copies.

Out of 255 samples ran in the *pfmdr1* allelic discrimination assays, 238 samples were successful at all four codons and 10 samples were successfully genotyped in at least one codon. There was little variation in genotype at codons 86, 1034 and 1042. Most samples had the genotype 86-Asn 1034-Ser 1042-Asn (Table 5.2).

Samples with 2 or greater *pfmdr1* copies all had the following genotype: 86-Asn 1034-Ser and 1042-Asn but they varied in 184 genotype. There was no relation between increased copy number (defined as having 2 or more copies) and *pfmdr1*-184 genotype when controlling for site (logistic regression, OR = 1.09, 95%CI: 0.40, 2.98).

pfmdr1 copy number varied substantially by site (Kruskal-Wallis test, $\chi^2 = 100.98$, $p < 0.001$). Higher copy numbers were seen in Pailin and Chumkiri compared to K. Seila, Memut, and Rattanakiri (Figure 5.2). The variance of copy number also differed by site: the sites with the highest copy number also had the highest variation in copy number (Levene's test of homogeneity, $W = 54.39$, $p < 0.0001$).

pfmdr1 copy number was higher in men and in mixed species infections (Table 5.3). In addition, parasite density was positively associated with *pfmdr1* copy number. When gender, mixed infections and parasite density was controlled for, site was still a significant predictor of *pfmdr1* copy number (rank ANCOVA, Chi-square = 59.21, df = 4, $p < 0.0001$).

The prevalence of *pfmdr1*-184-Phe also varied significantly by site (Chi-square = 71.59, df = 3, $p < 0.0001$). 184-Phe was more common in West (Pailin) than in the East (Rattanakiri) (Figure 5.3). Age, parasitemia, gender, presence of gametocytes, mixed infection were not related to *pfmdr1*-184 genotype

DISCUSSION

In this cross-sectional study at five different sites in Cambodia, we found substantial heterogeneity in *pfmdr1* copy number. Pailin (near the Thai border) and Chumkiri (in central Cambodia) both had high *pfmdr1* copy number while Memut (in central Cambodia) and Rattanakiri (near the Vietnam border) had relatively low *pfmdr1* copy number. There was very little variation in the genotype at *pfmdr1* codons 86, 1034 and 1042. However, the prevalence of *pfmdr1*-184-Phe decreased from West to East.

Previous in vivo and in vitro studies have demonstrated an increasing level of mefloquine resistance near the Thai border while the Eastern part of the country has remained relatively mefloquine sensitive (38, 119). Surveillance for drug resistance has been limited to 8 sites throughout Cambodia, most of which are near the international borders. However, the increased *pfmdr1* copy number at Chumkiri, which is in the middle of the country, demonstrates that the geographic distribution of drug resistance is more complex than

previously thought. Further investigation of the level of mefloquine resistance, either through in vivo or in vitro methods, should be employed in Chumkiri to confirm this finding.

In contrast to *pfmdr1* copy number, *pfmdr1* haplotype varied little across the five clinics. Most of the samples had the following *pfmdr1* haplotype: 86-Asn 1034-Ser and 1042-Asn, though there was substantial variation at *pfmdr1*-184. A previous Cambodian study found similar prevalences of 86-Asn, 1034-Ser, and 184-Phe genotypes, but they found that 18% of the samples contained 1042-Asp. In contrast, in this currently study, 1042-Asp was only found in 5.4% of the samples. This inconsistency is probably due to the limited sample size of the previous study and slight differences in the geographic origin of the samples.

The presence of the *pfmdr1*-184 mutation increased from West to East. The reason for this trend is unclear because the role of the *pfmdr1*-184 genotype on mefloquine and chloroquine resistance is ambiguous. Khim et al. demonstrate that the presence of the *pfmdr1*-184 mutation is associated with mefloquine resistance (31). However, most studies found no relation between 184 and either mefloquine or chloroquine resistance (98, 99, 104, 107, 112, 182, 185, 186). It is possible that the East-West trend of *pfmdr1*-184 is due to increasing selective pressure by mefloquine or chloroquine. This trend is more likely due to *pfmdr*1-184 being in disequilibrium with an allele that is affected by drug pressure.

This is the first report of an association between *pfmdr1* copy number and clinical characteristics (gender, age, parasite density, and the presence of a non-falciparum *Plasmodium* species). The presence of *dhfr* and *dhps* mutations has been associated with age in African children (187). The reason for the increased copy number in mixed species infections is unclear, though might be related to the decreased number of *P. falciparum* clones in the sample (due to competition with the other *Plasmodium* species).

This study demonstrates the utility of molecular markers for surveillance of drug resistance. A large number of samples can be processed in a relatively short amount of time. In addition, surveillance can be expanded beyond sentinel sites. The main weakness of this project is that treatment outcome was not determined for most of the participants. Therefore, we cannot determine if the relationship between genetic changes in *pfmdr1* and treatment failure varies by region. Another limitation of this study is the limited sample size, especially at Kg. Seila. Nonetheless, measuring *pfmdr1* copy number should be evaluated further as a technique to complement the current in vivo efficacy monitoring in Cambodia and in other regions where quinoline compounds are being used.

Table 5.1 Clinical characteristics of study participants at 5 clinics in Cambodia

	Pailin	Kg. Seila	Chumkiri	Memut	Rattanakiri
Total, N (%)	112 (24.5)	11 (2.4)	78 (17.1)	174 (38.1)	82 (17.9)
Female, N (%)	35 (31.2)	2 (18.2)	12 (15.4)	63 (36.2)	31 (37.8)
Age, mean (range)	25.1 (6-65)	23.7 (18-40)	27.8 (16-57)	30.0 (18-63)	27.7 (18-60)
Parasitemia, geometric mean	14123	4975	24907	17125	5443
Parasitemia > 50,000*, N (%)	37 (33.0)	4 (36.4)	30 (47.6)	68 (39.8)	13 (15.9)
Gametocytemia, N (%)	4 (4.6)	1 (9.1)	4 (5.1)	11 (6.3)	1 (1.3)
Mixed infection, N (%)	6 (6.8)	1 (9.1)	46 (59.0)	5 (2.9)	4 (5.1)

*parasites / µl

Table 5.2 *pfmdr1* haplotype and mefloquine phenotype at Pailin, Kg. Seila, Memut, and Rattanakiri. Only samples with complete haplotype are included in this table. The sample size at each site is: 106 at Pailin, 9 at Kg. Seila, 73 at Memut, and 50 at Rattanakiri.

							Frequency (%)		
Haplotype*	**86†**	**184**	**1034**	**1042**	**in vitro MQ phenotype‡**	**Pailin**	**Kg. Seila**	**Memut**	**Rattanakiri**
I	Asn	Tyr	Ser	Asn	R/S	19.8	88.9	78.8	89.8
II	**Tyr**	Tyr	Ser	Asn	S	0	0	1.5	6.1
III	Asn	**Phe**	Ser	Asn	R	75.00	11.1	12.1	4.1
IV	Asn	**Phe**	**Cys**	**Asp**	S	0	0	1.5	0
IV	Asn	**Phe**	**Cys**	Asn	S	3.1	0	6.1	0
IV	Asn	**Phe**	Ser	**Asp**	R/S	1.0	0	0	0
V	Asn	Tyr	**Cys**	Asn	?	1.0	0	0	0

*Haplotype based on categorization from (99)
†Amino acids in bold represent the mutant genotype
‡ From (31, 99). MQ = mefloquine, R = resistant and S = sensitive. R/S indicates the studies had contrasting results.

Table 5.3 Clinical and demographic factors and their relationship with *pfmdr1* copy number

Covariate	Test	Chi-square/ Spearman's Rho	p-value
Site	Kruskal-Wallis	100.98	< 0.001
in vivo efficacy participant	Kruskal-Wallis	3.57	0.059
Presence of gametocytes	Kruskal-Wallis	0.353	0.552
Presence of non-falciparum *Plasmodium* species	Kruskal-Wallis	23.91	< 0.001
Parasite density	Spearman Correlation	0.116	0.018
Age	Spearman Correlation	0.088	0.070
Gender	Kruskal-Wallis	5.277	0.022

Figure 5.1 Map of Cambodia showing the 5 collection sites

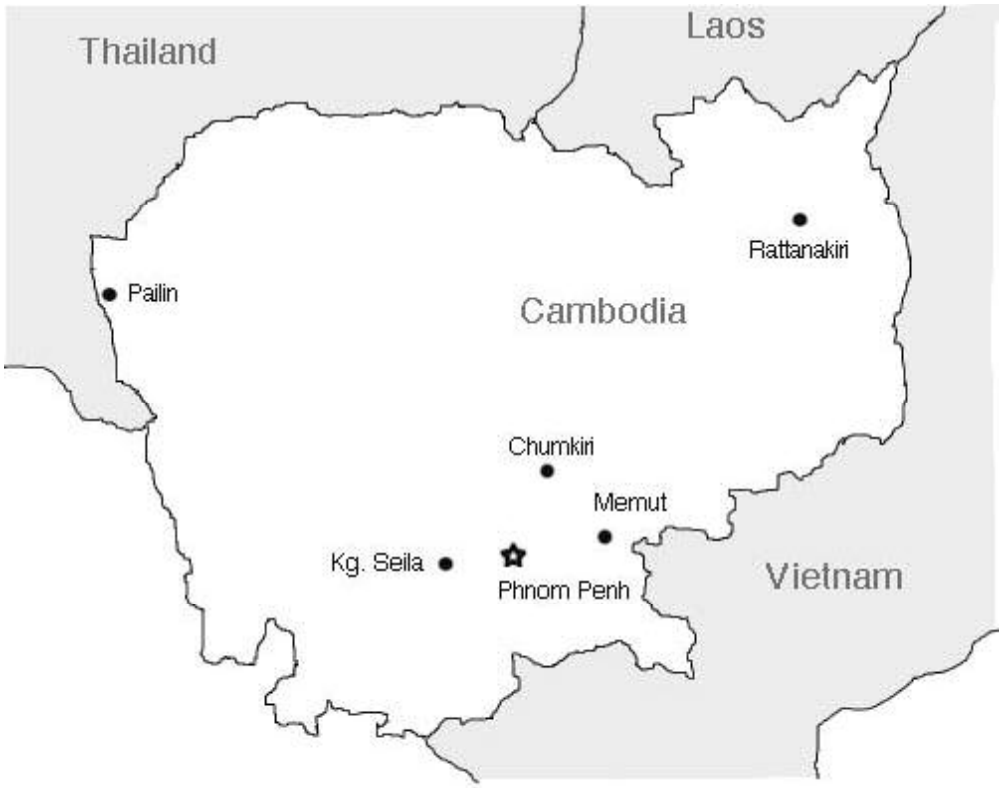


Figure 5.2 The median and 25th-75th percentile of pfmdr1 copy number at Pailin, Kg. Seila, Chumkiri, Memut, and Rattanakiri in Cambodia. The sample size at each site is: 111 at Pailin, 10 at Kg. Seila, 69 at Chumkiri, 160 at Memut, and 78 at Rattanakiri.

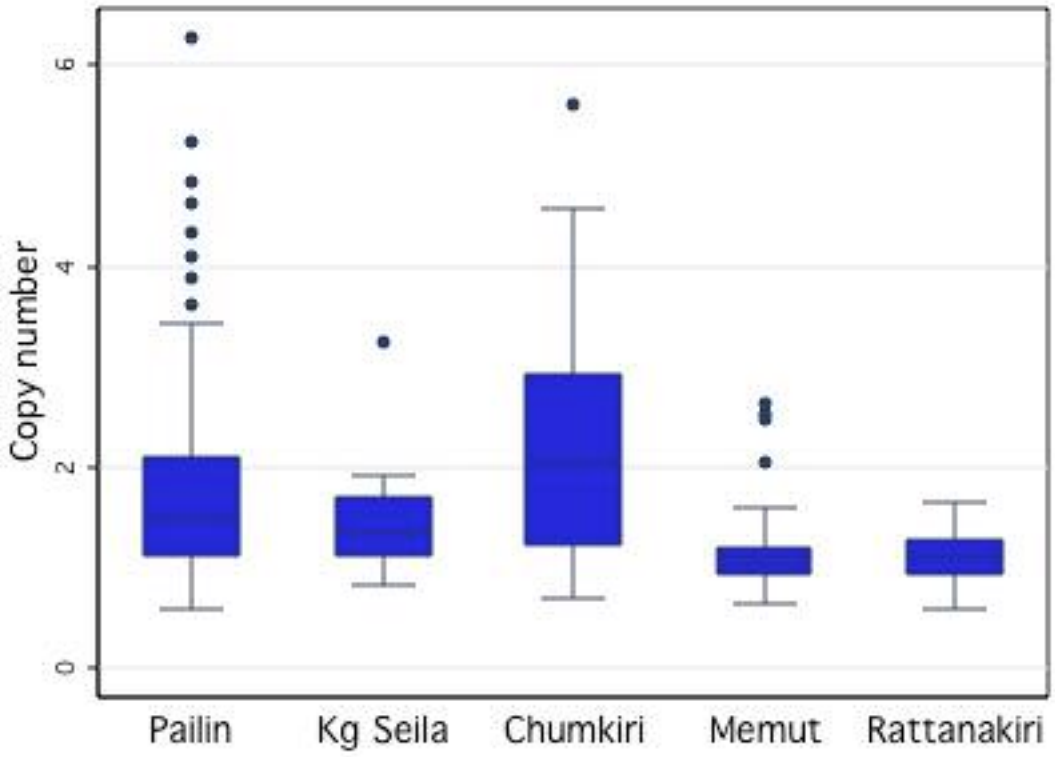
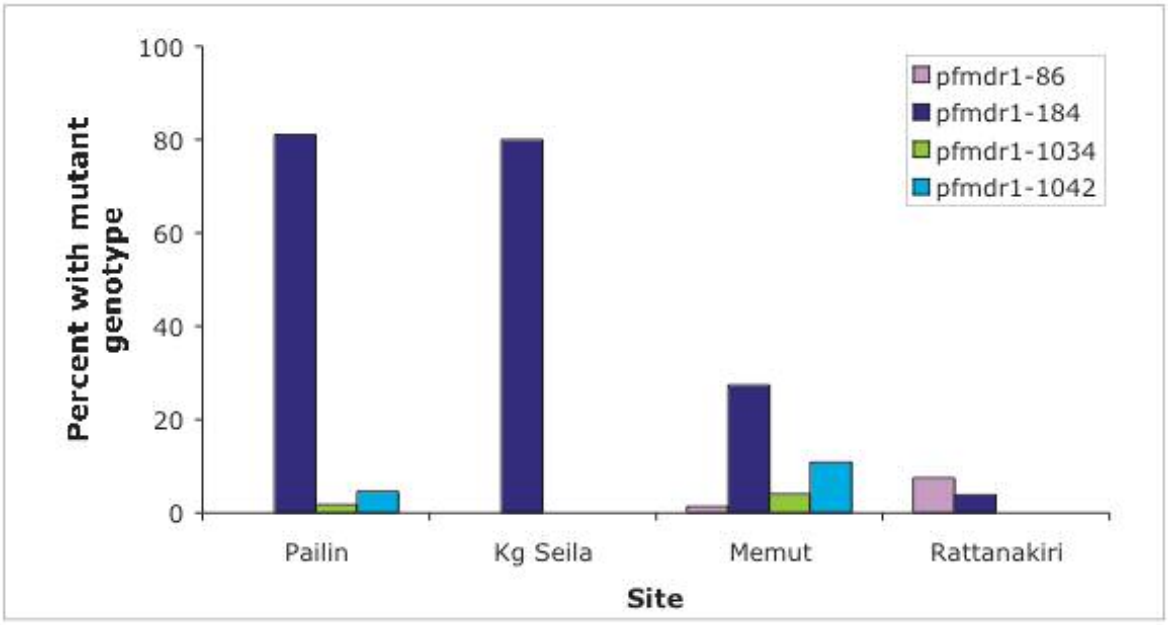


Figure 5.3 Distribution of mutant genotypes at *pfmdr1* codons 86, 184, 1034, and 1042



CHAPTER VI:

SUMMARY AND FUTURE DIRECTIONS

Falciparum malaria is a devastating illness that has a profound public health impact in the developing world. One of the challenges in the control of malaria is drug resistance. By having an effective and timely monitoring system of drug efficacy, policy makers can make educated decisions about what drug combinations to use and what populations to target for interventions. One method to increase the coverage of the current in vivo efficacy monitoring system is to use molecular markers of drug resistance. However, before molecular markers can be used, their association with clinical resistance should be established. To this end, we conducted two studies to estimate the effect of molecular markers and treatment failure. In addition, we conducted a cross-sectional study to determine the geographic distribution of molecular markers of mefloquine resistance.

RUTSHURU IN VIVO EFFICACY STUDY

The main purpose of this study was to estimate the relationship between *dhfr* and *dhps* mutations and SP treatment failure in Rutshuru, DRC. Clinical samples and data from an in vivo efficacy study were used to achieve this goal. *dhps* mutations 437 and 540 were found to be more strongly related to treatment failure than *dhfr*-59. In addition, there were significant interactions between the *dhps* mutations: a mutation at *dhps*-581 was preventive except in the presence of *dhps*-437 mutation, when it had no effect on treatment failure. Mutations at *dhps*-540 and *dhps*-437 appear to be antagonistic, as the effect of the two mutations together was less than each mutation alone. In addition, the interaction term between these two mutations was significant (p=0.023).

These results are consistent with many studies that have previously shown that *dhps* mutations are associated with SP treatment failure (80, 82, 84, 87-90). However, no other study has investigated the interaction between individual mutations. It would be interesting to see if the antagonism between *dhps*-437 and *dhps*-540 is present in other regions. This result should also be confirmed in transfection experiments.

The effect of *dhps*-437 and –540 on treatment failure differed by parasitemia. In children with parasitemia less than 45,000 parasites / μl, the presence of both *dhps*-437 and 540 mutations was associated with a 16% (95%CI: -3.5%, 36%) greater absolute risk of treatment failure than having neither mutation. In children with parasitemia greater than 45,000 parasites / μl, the presence of both mutation had a greater absolute risk of 51% (95%CI: 34%, 67%) compared to having neither mutation.

The greater effect of *dhps*-437 and -540 mutations in children with parasitemias greater than 45,000 parasites/μl suggests that drug resistance has a greater impact on people with more severe disease. By understanding what sub-group of people are hardest hit by drug resistance, interventions can be more accurately targeted. For example, if there are limited supplies of a certain second line antimalarial drug, then it might be most effective to use it only in people with severe disease. In addition, effect modification could be one reason why the effect of mutations on treatment failure appears to vary by region. Therefore, if molecular markers are going to be used for surveillance of resistance, a better understanding of the covariates that modify this relationship in the individual and at the population level is greatly needed.

PAILIN IN VIVO EFFICACY STUDY

The purpose of this study was to estimate the relationship between genetic changes in *pfmdr1* and recrudesesence after mefloquine-artesunate therapy. To achieve this goal, clinical samples and data were used from an in vivo efficacy study in Pailin, Cambodia. *pfmdr1* copy number was strongly associated with time to recrudescence (HR = 8.30, 95%CI: 2.60, 26.43). However, *pfmdr1* haplotype was not associated with time to recrudescence. *pfmdr1* copy number was higher when there was recurrence of disease than before mefloquine-artesunate treatment was given. This suggests that mefloquine-artesunate treatment is selecting for increased *pfmdr1* copy number. The parasite clearance time observed in this study was longer than reports from other areas. However, *pfmdr1* copy number and haplotype were not related to parasite clearance time. This suggests that other mechanisms of resistance other than *pfmdr1* are important in this area. Overall, these results are consistent with a previous study that investigated the role of *pfmdr1* copy number on recrudescence after mefloquine-artesunate and with another study on mefloquine monotherapy (104, 107). *pfmdr1* copy number, but not haplotype, might be an effective monitoring tool for mefloquine-artesunate resistance.

CAMBODIAN CROSS-SECTIONAL STUDY

The main purpose of this study was to determine the geographic variation in *pfmdr1* in Cambodia. *pfmdr1* copy number was found to vary substantially by region. *pfmdr1* copy number was higher in Pailin, which has a 82% cure rate, than in Rattanakiri, which has a 100% cure rate. High *pfmdr1* copy number was also found at Chumkiri, which is in an area not previously known for mefloquine resistance. Because of this finding, an in vivo efficacy

study has been scheduled for Chumkiri to confirm these results. This study demonstrates how using molecular makers for surveillance complements an in vivo efficacy monitoring system.

LIMITATIONS AND STRENGTHS

These studies had several limitations. One of the main limitations of all the studies is the small sample size. The Rutshuru and Pailin studies had 212 and 80 samples, respectively, available for genotyping. This small number translates into imprecise estimates of effect. In addition, these results are strongly influenced by chance. The small sample size restricted what comparisons could be made, such as how the effect of *pfmdr1* haplotype IV in the Pailin study could not be estimated because only four samples had this genotype. This small sample size also limits the power of finding effect measure modification. In the Cambodian cross-sectional study, the limited sample size affected the precision of the prevalence estimates of increased copy number. However, when compared to other studies in the field, the sample sizes of these studies are above average. Therefore, the problems of reduced precision and power and chance playing a large role are almost universal in this field.

Another limitation is the potential exposure misclassification. The relative proportion of genetic clones of *P. falciparum* present in the peripheral circulatory system varies (145). Therefore, it is possible that an infection contains a particular genetic variant yet it is not present in the peripheral blood when the sample is taken. Another issue is that these assays might not be sensitive enough to detect a mutation even when it is in the peripheral blood. *P. falciparum* infections do contain minor variants that are not detected by standard real-time PCR or nested PCR methods (188, 189). In HIV infection, minor variants have been shown to be clinically relevant (190, 191). However, it is unknown whether minor variants in *P.*

falciparum infections are involved in clinical resistance. These issues are also universal in this field.

Outcome misclassification is another possible source of bias, especially in the Rutshuru study. The current recommendation for in vivo efficacy studies involving SP is that subjects should be followed for at least 28 days to capture all treatment failures (32). The Rutshuru only had 14 days of follow-up. Therefore, it is very likely that some subjects classified as having an adequate clinical and parasitological response at day 14 had a recurrence of parasitemia later on. Another complication of the Rutshuru study is that the treatment failures were not PCR corrected for re-infections. The actual impact of this misclassification is probably small because re-infections within 14 days of treatment are rare. (The accuracy of assuming there are no re-infections within 14 days has not been assessed). Very few studies actually PCR correct treatment failures at or before 14 days. None of the studies that PCR corrected presented their results so that sensitivities and specificities can be calculated). Another complication of the Rutshuru study was the high number of exclusions, mostly due to loss to follow-up. However, sensitivity analysis showed that the impact of the exclusion of 17 children had a minimal impact on the results.

One last issue is whether these results are generalizable. In Rutshuru, 25% of the subjects were underweight and therefore it is not known whether these results are generalizable to healthier populations. Both in vivo studies had stringent enrollment criteria (such as certain age restrictions, and having no signs of severe disease or mixed infections). Therefore, it is not known whether these results would apply to the general population.

These studies also have several strengths. In all three studies, clinical samples were directly genotyped. In contrast, in many studies, the samples are cultured before genotyping

in order to have a more reliable yield of DNA. Genotyping cultured samples can result in exposure misclassification as *P. falciparum* genotypes has been shown to change when samples are adapted to culture (58). In addition, assays based on real-time PCR have less false positives and greater sensitivity than nested PCR (which is the standard method of genotyping) (134).

The Pailin study, the outcome was reliably assessed, as the 42 day follow-up most likely captured most instances of treatment failure. All recurrences of parasitemia were PCR corrected. While 10 people were lost to follow-up, everyone was followed for at least 14 days.

FUTURE DIRECTIONS

The Rutshuru and Pailin studies clearly show that molecular markers of resistance are related to clinical outcome. The Cambodian cross-sectional study demonstrated how the use of molecular markers for surveillance can be implemented. What are the other issues that need to be addressed before countries can implement surveillance of molecular markers?

One of the main questions that needs to be addressed is whether the effect of molecular markers is constant over a geographical area. While some studies have found that the effect of molecular markers is constant (192, 193), others have not (76, 133). Therefore, to ensure that the prevalence of increased *pfmdr1* copy number is truly reflective of the treatment failure rate in an area, the constancy of the effect of *pfmdr1* copy number of treatment failure should be established.

Another issue that needs to be addressed before molecular markers can be successfully implemented is that *dhfr/dhps* and *pfmdr1* do not have perfect sensitivity and specificity in

predicting outcome. One possible reason for this discrepancy is that we do not fully understand all the determinants of treatment failure (which can be used to better predict treatment failure). Another possibility is that we are not accurately measuring resistance. Other genetic changes might be involved in conferring resistance (such as modification of the transcription and translation of *dhfr/dhps* and *pfmdr1* or completely different genes). Also, the current assays are not sensitive enough to pick up minor variants of a clinical sample, which might be playing a role in resistance. Future studies on molecular markers and treatment failure should focus on increasing the accuracy of detection of molecular markers (either by taking more than one blood sample or by using techniques that are more sensitive to picking up minor variants).

In areas where in vivo studies are feasible, molecular markers should not replace the in vivo monitoring. The in vivo efficacy study produces results that are more easily interpretable, and therefore more likely to be used by policy makers. However, molecular markers have great potential to complement the current surveillance system for drug efficacy. Measuring the prevalence of molecular markers is much easier than conducting an in vivo efficacy trial (as no follow-up of subjects is needed). Therefore, molecular markers can be used to investigate drug efficacy in areas outside of sentinel surveillance sites. For example, in Cambodia, increased *pfmdr1* copy number was found in a site not previously known to have drug resistance. Molecular markers can help determine what areas need to be investigated further for drug resistance, and therefore lead to a more efficient and effective use of the in vivo efficacy study.

Molecular markers can be used to estimate the level of resistance in unstable areas where in vivo efficacy studies are unfeasible(153, 154). For example, the prevalence of *pfcrt, dhfr,*

and dhps mutations in an area of political unrest in Mali were used to determine what antimalarials should be used in this area (154). In DRC, measuring the prevalence of *dhfr* and *dhps* mutations might provide a way to measure resistance in the politically unstable areas of the country.

In addition, molecular markers can be used to track the emergence of resistance and to evaluate the effect of various interventions on the selection of resistance. For example, intermittent presumptive treatment with SP in Ghana was associated with an increase in *dhfr* and *dhps* mutations, which strongly suggests this preventive strategy facilitates the emergence of resistance(194). Molecular markers have also been used to evaluate the impact of antimalarials with different half-lives and the use of bednets on the selection of resistance (195, 196).

In summary, molecular markers have great potential to expand surveillance of resistance and to decrease the impact of malaria in the developing world.

APPENDIX A:

INCLUSION/EXCLUSION CHECKLIST AND SUBJECT INFORMATION FORM

FROM THE CAMBODIAN CROSS-SECTIONAL STUDY

Detection of Genetic Mutations Associated with Drug Resistance
in *P. falciparum* Isolates from Cambodia by Real-Time PCR

INCLUSION/EXCLUSION CRITERIA CHECK LIST

1. *P. falciparum* positive (Giemsa blood smear or rapid test*) □ Yes □ No

2. Age >=18 years old □ Yes □ No

3. No evidence of severe/complicated malaria □ Yes □ No
   requiring prompt management for life support

4. No history of bleeding tendency □ Yes □ No

5. The study has been explained to the volunteer □ Yes □ No

6. The consent form has been signed □ Yes □ No

If the answer is "No" to one or more of the above questions, the patient is ineligible for inclusion.
* To be confirmed by Giemsa blood smear.

_____ _____
Investigator's initials Date

Barcode **ID**
_____

Detection of Genetic Mutations Associated with Drug Resistance
in *P. falciparum* Isolates from Cambodia by Real-Time PCR

SUBJECT INFORMATION FORM

Site: I. _____

__ __ / __ __ / __ __
D D M M Y Y

Baseline Demographics

Age (in years): ____ ____ ne):

1. Male 2. Female

Current residence: Village/District:_____

Province:_____

Baseline Malaria Data

Number of illnesses believed due to malaria in past 12 months:
(Circle one)

0 1 2 3 >3 9. unknown

Number episodes of smear positive malaria in past 12 months:
(Circle one)

0 1 2 3 >3 9 unknown

Date of last drug therapy for malaria:
(Circle one)

<1 month	>=1 month	Never
1	2	3

Treatment received in
the last one month:
(circle all that apply)

1.chloroquine 2. SP (Fansidar) 3. quinine 4. mefloquine 5. primaquine
6. doxycycline/ tetracycline 7. artesunate 8. other_____ 9. unknown 10. None

Detection of Genetic Mutations Associated with Drug Resistance
in *P. falciparum* Isolates from Cambodia by Real-Time PCR

SUBJECT INFORMATION FORM (continued)

Feeling ill for how many days:
(Circle one)

0 (asymptomatic)

1 2 3 4 5 >5 9.unknown

Symptoms:
(Circle all reported)

1. Headache 2. Myalgias 3. Chills/Rigors 4. Malaise

5. Vomiting 6. Diarrhea 7. Cough 8. Fever or History of fever

Blood Smear Review (by NAMRU-2) Result:
P. falciparum confirmed

1. Yes 2. No

P. falciparum Asexual Parasite Count:
OR

_____ /500 WBC

_____ /5,000 RBC

P. falciparum Sexual Parasite Count:

_____ /500 WBC

Presence of Non-*P. falciparum*

1. Yes 2. No

If Yes:

Pv Pm Po*

Interviewer's Signature _____

(Name _____)

On-site Investigator's Signature _____

(Name _____)

*Pv=*P. vivax,* Pm= *P. malariae,* Po= *P. ovalae*

APPENDIX B:

RAPID REAL-TIME PCR GENOTYPING OF MUTATIONS ASSOCIATED WITH SULFADOXINE-PYRIMETHAMINE RESISTANCE IN *PLASMODIUM FALCIPARUM*

by

Alisa P. Alker, Victor Mwapasa, and Steven R. Meshnick

Published in Antimicrobial Agents and Chemotherapy 48(8): 2924-9.

ABSTRACT

Sulfadoxine-pyrimethamine (SP) -resistance of *Plasmodium falciparum* is an emerging public health threat. Resistance to these drugs is associated with point mutations in the genes encoding dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*). We describe an assay using real-time PCR and sequence specific probes that detects these mutations. Using DNA from plasmids, cultured strains, and clinical samples, real-time PCR could distinguish four *dhps* polymorphisms (codons 437, 540, 581, and 613) and 3 *dhfr* polymorphisms (codons 51, 59, and 108). This assay is rapid and sensitive, with a detection limit of 10 copies in most cases. This assay is amenable to large-scale studies of drug resistance.

INTRODUCTION

Malaria is a major public health threat, causing millions of deaths per year. One of the greatest challenges in the control of malaria is drug resistance, which has contributed to its re-emergence and spread (25). Currently, chloroquine resistance in *Plasmodium falciparum* is nearly universal; because of chloroquine resistance, five African countries have switched to sulfadoxine-pyrimethamine (SP) as their first-line antimalarial (25). However, resistance to SP is spreading.

Sulfadoxine and pyrimethamine inhibit dihydropteroate synthase (*dhps*) and dihydrofolate reductase (*dhfr*), respectively, which are two enzymes involved in folate biosynthesis. SP resistance is conferred by single nucleotide changes in either enzyme, and increases as mutations accumulate ((70) and references therein). For *dhfr*, the mutations 50-Arg, 51-Ile, 59-Arg and 108-Asn have been strongly associated with in vitro and in vivo resistance and are correlated with SP usage (75, 83, 85, 197). Mutations in *dhps* (436-Ala, 436-Phe, 437-

Gly, 540-Lys, 581-Gly, 613-Thr and 613-Ser) have also been associated with in vitro and in vivo resistance, though they have a weaker relationship with SP usage than *dhfr* mutations (74, 75, 83, 197-199). Other *dhfr* polymorphisms are more closely associated with cycloguanil resistance (16-Val and 108-Thr) (66).

Surveillance for antimalarial drug resistance is usually done using in vitro or in vivo methods. In vitro methods involve the culturing of malaria parasites, which is difficult and requires skilled technicians and tissue culture facilities. In vivo methods require patients to be followed up for at least 14 days, which is often challenging in field conditions. Thus, new surveillance tools are needed. Numerous studies, using standard PCR methods, have shown that *dhps* and *dhfr* mutations are closely associated with SP resistance. However, the usefulness of these assays is restricted due to difficulty, cost, high risk for contamination, and the inability to pick up low prevalent genotypes in a mixed sample (200). Real-time PCR, on the other hand, is simpler, and less prone to contamination. The purpose of this study is to develop a real-time PCR assay for *dhps* and *dhfr* mutations.

Real-time PCR Minor Groove Binding (MGB) probes provide a relatively inexpensive and sensitive way to detect single nucleotide polymorphisms (SNPs) in a large number of samples (144, 201). MGB probes are designed to hybridize to an internal region of the PCR amplicon, thereby providing another level of specificity beyond the site-specific primers. When the Taq DNA polymerase cleaves the probe from the 5' end, the fluorophore is released from the quencher, allowing it to fluoresce. The increase in fluorescence over time can be measured using a real-time PCR thermocycler. Single base differences between MGB probes and DNA cause the melting temperature to decrease significantly, which decreases the efficiency of probe hybridization (143, 144). Allelic discrimination is achieved by putting

in competition two probes complementary to wild type and mutant alleles, which are attached to different fluorophores (144). The risk for contamination is much lower in this technique, because there is no transfer of material between the addition of the DNA and the acquisition of the results (202). This technique has been used for allelic discrimination in humans, and a similar technique has been used to identify alleles that confer drug resistance in bacteria (85, 203, 204).

The impending loss of SP effectiveness in Africa, due to drug resistance, could have calamitous consequences. Partnering SP with another antimalarial like artesunate might preserve SP effectiveness by delaying the onset of SP resistance. In order to measure the effects of combination chemotherapy on the development of SP resistance in Africa, we attempted to develop real-time PCR assays for the 3 *dhfr* polymorphisms (51-Ile, 59-Arg, 108-Asn) and 5 *dhps* polymorphisms (436-Ala, 437-Gly, 540-Lys, 581-Gly, 613-Ser) reported from Africa.

MATERIALS AND METHODS

DNA

Six *dhps* plasmids (MRA-189 through MRA-194) and 5 *dhfr* plasmid (MRA-195 through MRA-199) contained in *Escherichia coli* were obtained from the Malaria Reagent Repository Resource (http://www.malaria.mr4.org/ and references therein), containing the wild type sequences and the most important mutations for each gene. The *E. coli* clones were grown on LB medium (1% tryptone, 0.5% yeast extract, 1.5% agarose in dI H_20) with 50 μg/ml ampicillin. The plasmids were extracted and purified using the Promega Wizard Plus minipreps DNA purification system (Promega, Madison, WI, USA). *P. falciparum* strain

3D7 parasites were kindly provided by Jesse Kwiek, Duke University. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA from six strains of *P. falciparum* (Dd2, HB3, W2, FCR3, K1,VI/S) was obtained from Malaria Reagent Repository (see citation above).

Clinical samples of malaria parasites were obtained from a subgroup of pregnant women enrolled in a study investigating the effect of maternal malarial infection on mother-to-child transmission of HIV. Details of the study, which commenced in December 2000, have been reported elsewhere (205). Briefly, consent was sought from women in their late third trimester of pregnancy before the onset of active labor. Peripheral blood samples were drawn to screen for malaria, using thick blood smear microscopy, and to detect HIV infection using Rapid Antibody Tests. All HIV-infected women and a subset of HIV-uninfected were enrolled in that study. For this current study, 24 clinical samples were genotyped: 21 from HIV and malaria-infected women, one from a parasitemic HIV-uninfected woman, and two controls samples from HIV-infected women who were not parasitemic. With the exception of one HIV-infected woman, all women reported taking at least one dose of sulfadoxine-pyrimethamine during the antenatal period.

Peripheral blood samples were centrifuged, and the plasma and packed cell fractions were stored at -80^0 Celsius. Subsequently, frozen packed cells were thawed and a few drops were transferred on filter paper. These were shipped to University of North Carolina at Chapel Hill, where DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany**).**

Primers and probes

All primers and probes in this study were designed using Primer Express software (Applied Biosystems, Courtaboeuf, France), using the defaults of the program, except that the minimum GC % was lowered to 20% due to the AT-rich nature of the *P. falciparum* genome. *P. falciparum* strain 3D7 sequences for *dhfr* (accession number:AF248537) and *dhps* (accession number: AF250167) were used in probe and primer design. When designing the primers and probes, it was assumed that all codons within the amplicon were wild type except for the SNP of interest. All primers were synthesized by Qiagen (Hilden, Germany). The probes were synthesized by Applied Biosystems (Foster City, CA) with the fluorophores FAM (6-carboxyfluorescein) linked to the 5' position of all wild type probes and VIC (chemical name not released by Applied Biosystems) linked the 5' position of all mutant probes. A non-fluorescent quencher and a minor groove binder were linked to the 3' ends of all probes (chemical names not released by Applied Biosystems).

Real-time PCR

PCR reactions were carried out in duplicate in 25 µl final volume containing 12.5 µl Universal PCR Master Mix, 5 µl DNA, forward and reverse primers at various concentrations, and both mutant and wild type probes at a final concentration of 200 nM. Optimal primer concentrations were determined by running reactions at all combinations of the forward and reverse probe at 300, 500, and 900 nM (Table B.1). All reactions were run on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the default settings; they were initially denatured at 95 ºC for 10 minutes and cycled 40 times, with each cycle consisting of 95 ºC for 15 s and 60 ºC for 60 s.

Data were transferred into Microsoft Excel ver 2002, where baseline (minimum) fluorescence was subtracted and replicates were averaged. To determine the detection limit for the wild type and mutant probes, ten-fold dilutions of the relevant plasmids were run in triplicate. The detection limit was taken as the lowest copy number where the standard deviation of the fluorescence of the wild type and mutant genotype did not overlap. To assess the compatibility of this assay with mixed samples, two primer -probe sets (*dhfr*-51, and *dhfr*-59) were run using DNA from different proportions of wild type and mutant plasmids (0:1, 1:9, 2:8, 5:5, 8:2, 9:1, 1:0). A total of 10,000 copies of plasmid DNA was used in each reaction.

To determine the specificity of the primer-probe sets, and to ensure their compatibility with genomic DNA, seven strains of *P. falciparum* (3D7, Dd2, FCR3, HB3, K1, VI/S, W2) were genotyped using real-time PCR. The *dhfr* and *dhps* genotypes of these strains, presented in Table B.2, have been previously reported (67, 69, 206, 207). Real-time PCR was performed as described above except that only 1 µl of DNA was used per reaction. The genotype was determined by visually comparing the change in fluorescence of the wild type and mutant probes.

Validation of Method by Genotyping Clinical Samples

The first step in genotyping the clinical samples involved using real-time PCR to detect the lactate dehydrogenase (*ldh*) gene in the conditions described previously (99). Serial dilutions of *P. falciparum* 3D7 DNA at known concentration were run on the same plate as the clinical samples. A standard curve of the 3D7 DNA was constructed and used to extrapolate the DNA concentration of the clinical samples. *P. falciparum* DNA was not detected in the two control samples from non-parasitemic women and therefore were not

processed further. The remaining 22 samples were genotyped by real-time PCR using the optimal primer and probe concentrations (Table B.1). The amount of DNA added per reaction varied (1-3 µl) depending on the detection limit of the primer-probe set and the concentration of the sample. Samples of low concentration were run for 45 cycles instead of 40 cycles. Each plate contained DNA from *P. falciparum* strains at relevant concentrations to act as wild type and mutant controls.

The genotype was determined by comparing the cycle threshold (CT) values of the wild type and mutant probe for each sample. The CT is the number of PCR cycles needed before the fluorescent signal surpasses a certain threshold (usually 20-40% of maximum).The more DNA of a specific genotype, the smaller the CT for that genotype-specific probe. For example, a sample was deemed to be wild type when the wild type probe CT was less than the mutant probe CT and the difference was similar to that seen with the wild type plasmid DNA. A sample was considered to be mixed when the CT's of both wild type and mutant probes were closer and intermediate between the patterns seen for pure and wild type plasmid DNA.

Five clinical samples were chosen to be sequenced based on the following criteria: 1. Real-time results were available for all seven SNP's 2. Both wild type and mutant genotypes were included, when possible and 3. If the sample was mixed, a dominant genotype could be distinguished from real-time PCR (which eliminated 3 samples from consideration).

Sequencing

The five clinical samples were amplified using the same primers as in the MGB real-time PCR assay. Reactions were carried out in 25 µl final volume containing 12.5 µl SYBR Green

Master Mix, 1 µl DNA, and forward and reverse primers at 300nM. All reactions were run on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the default settings with the dissociation step added at 60˚C. The dissociation curves were examined to ensure that each reaction contained only one product. The resulting amplicon was purified using Centri Spin-10 Columns (Princeton Separations, Adelphia, NJ). Sequencing was performed at the University of North Carolina Sequencing Core using the ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

RESULTS

Real-time PCR was both sensitive and specific for *P. falciparum dhfr* and *dhps* genetic polymorphisms. The reaction conditions were effective for a wide range of *P. falciparum* DNA concentrations and had detection limits ranging from 10 to 1000 copies (Table B.1).

For the 7 polymorphisms shown in Figure B.1, the wild type probe generated fluorescence with the wild type sequence but not the mutant sequence, while the mutant probe generated fluorescence with the mutant sequence but not the wild type sequence. No amplification was ever seen in the absence of template (data not shown).

Background fluorescence (wild type probe with mutant sequence and vice versa) was minimal, except in the case of the *dhfr* wild type (59-Cys) probe. However, this background reaction did not prevent genotype determination, since the wild type sequence still clearly amplified more rapidly than the mutant sequence. The original batch of the 59-Cys probe had a weak signal but provided sufficient discriminating ability. Subsequent batches of 59 wild type probe from Applied Biosystems had a much stronger signal and more background

binding. However, the discriminating ability was similar to the original batch (data not shown).

Attempts to distinguish polymorphisms at *dhps*-436 were unsuccessful

Real-time PCR was successful in determining the genotype of the *P. falciparum* strains for *dhfr*-51, *dhfr*-59, *dhps*-540, and *dhps*-581. For *dhfr*-108, the probe was designed to detect the asparagine mutant. Yet, the mutant probe also picked up the 108-Thr mutant, though the CT value was less than that for 108-Asn. Similarly, the *dhps*-613 mutant probe, designed to identify the 613-Ser mutation, also bound to the 613-Thr mutation. However, in this case the CT values for the mutant probe was similar for both 613-Ser and 613-Thr and therefore they could not be distinguished. In both cases, wild type could be distinguished from mutant sequences. The genotyping of *dhps*-437 was successful in the presence of 436-Ser. There was no binding of the wild type or mutant probe for *dhps*-437 in the presence of the 436-Ala and 436-Phe mutations.

For two of the loci (*dhfr*-51, *dhfr*-59), various mixtures of wild type and mutant plasmids were prepared and then PCR-amplified. In all cases, a mixture could be differentiated from a pure sample if the minor component was present at $\geq$ 10% (Figure B.2).

This method was validated using clinical samples. Complete *dhfr* genotypes were determined using real-time PCR for all 22 samples taken from parasitemic women. An example of the real-time amplification of a clinical sample is shown in Figure B.3. In regards to *dhps*, 21 samples were fully genotyped; the *dhps*-540 genotype could not be ascertained for one sample. The five selected clinical samples were successfully sequenced for all seven loci. For the 3 *dhfr* loci and 4 *dhps* loci, there was a 100% concordance of the dominant genotype between sequencing and real-time PCR (Table B.2). Sequencing was unable to

detect mixed infections that were identified using real-time PCR.

DISCUSSION

We describe a real-time PCR assay to detect polymorphisms associated with drug resistance in *P. falciparum*. We could successfully discriminate between wild type and resistant alleles in 7 of the 8 loci important in Africa.

As a surveillance tool, real-time PCR has certain advantages over classical PCR. First, the assay is rapid and can reliably distinguish between two alleles after a single 3-hour experiment. There is no need to run gels after the reaction. Second, the risk of contamination is far lower than with nested PCR because it is a closed tube reaction. Third, the assay can be automated and processed in a high-throughput fashion. The initial cost of real-time PCR (consisting of buying the actual machine and optimizing the assay) is higher than normal PCR and may not be available in all malaria-endemic countries. However, part of this cost could be made up if used for high throughput processing as reagents for a single reaction cost between $0.40 and $2.

Recently, a fluorogenic PCR assay using FRET probes was developed to genotype codons 50-60 of *dhfr* in *P. falciparum* (208). This technique has a similar advantage of low risk of contamination as the assay we describe. However, it has yet to be expanded to detect the other important mutations associated with drug resistance.

There are several limitations to the real-time PCR system described here. The most serious deficiency is its inability to distinguish alleles at position 436, and alleles at 437 when there is a mutation at the 436 position. However, the 436 mutations are rare in Africa and appear not to be involved in the "quintuple mutants" (108-Asn, 51-Ile, 59-Arg in *dhfr*, and

437-Gly and 540-Glu in *dhps*) that are especially important in Africa (70). Also, while real-time PCR can theoretically distinguish 3 or more alleles, we were not able to accomplish this at *dhfr* positions 108 and 613. While we can distinguish wild type from mutants at *dhfr* position 108, we cannot distinguish between 108-Asn, associated with SP resistance, and 108-Thr, associated with cycloguanil resistance. Thus, further work is needed to enable the detection of these other polymorphisms. In addition, it should be noted that the detection limit differs among the primer-probe sets. This could potentially lead to an under-representation of certain genotypes. Nevertheless, given the advantages of this method, real-time PCR measurement of 7 *dhfr* and *dhps* polymorphisms could serve as a useful tool for the surveillance of SP-resistant malaria.

TABLE B.1 Real-time primer and probe sequences and optimal concentrations*

Target	Oligonucleotides (5' → 3')	Final Conc. (nM)	Detection Limit (copies/rxn)
	F:TGAGGTTTTTAATAACTACACATTTAGAGGT		
dhfr-51	CT	F:300	
	R: TATCATTTACATTATCCACAGTTTCTTTGTT	R:300	
	WTP: AATGTAATTCCCTAGATATG		WT: 10
	MP: AAATGTATTTCCCTAGATATG		M: 10
dhfr-59	F: Same as *dhfr*-51	F:300	
	R: Same as *dhfr*-51	R:500	
	WTP: AATATTTTTGTGCAGTTACA		WT: 100
	MP: TGAAATATTTTCGTGCAGTTA		M: 10
dhfr-108	F:TGGATAATGTAAATGATATGCCTAATTCTAA	F:300	
	R:AATCTTCTTTTTTTAAGGTTCTAGACAATATA		
	ACA	R:300	
	WTP: AGAACAAGCTGGGAAA		WT: 1000
	MP: AGAACAAACTGGGAAAG		M: 10
dhps-437	F: TGAAATGATAAATGAAGGTGCTAGTGT	F:900	
	R:AATACAGGTACTACTAAATCTCTTTCACTAA		
	TTTTT	R:900	
	WTP: AGAATCCTCTGCTCCT		WT: 10
	MP: AATCCTCTGGTCCTTT		M: 10
dhps-436	Primers and WT probe same as *dhps*-437		
	MP: AGAATCCGCTGTC		Unsuccessful
dhps-540	F: AATGCATAAAAGAGGAAATCCACAT	F:300	
	R: TCGCAAATCCTAATCCAATATCAA	R:300	
	WTP: CAATGGATAAACTAACAAA		WT: 10
	MP: AATGGATGAACTAACAAA		M: 10
dhps-581	F: CCTCGTTATAGGATACTATTTGATATTGGAT	F:500	
	R: TGGGCAATAAATCTTTTTCTTGAATA	R:300	
	WTP: ATTTGCGAAGAAAC		WT: 10
	MP: ATTTGGGAAGAAACAT		M: 100
dhps-613	F: TGGATTAGGATTTGCGAAGAAAC	F:500	
	R:GTTGTGTATTTATTACAACATTTTGATCATTC	R:300	
	WTP: ATTTATTGCCCATTGCAT		WT: 10
	MP: AGATTTATTTCCCATTGCA (Serine)		M: 10
	MP: AGATTTATTACCCATTGCA (Threonine)		Unsuccessful

*The SNP is underlined in the probes. F= forward primer, R=reverse primer, WTP=wild type probe, MP=mutant probe.

TABLE B.2 *dhfr* and *dhps* genotypes for *P. falciparum* strains and clinical samples, as determined by sequencing*

		dhfr			*dhps*				
	Strain/Sample	**51**	**59**	**108**	**436**	**437**	**540**	**581**	**613**
Strains	3D7	Asn	Cys	Ser	Ser	Gly	Lys	Ala	Ala
	Dd2	Ile	Arg	Asn	Phe	Gly	Lys	Ala	Ser
	HB3	Asn	Cys	Asn	Ser	Ala	Lys	Ala	Ala
	W2	Ile	Arg	Asn	Phe	Gly	Lys	Ala	Ser
	FCR3	Asn	Cys	Thr	Ser	Ala	Lys	Ala	Ala
	K1	Asn	Arg	Asn	Ser	Gly	Lys	Gly	Ala
	VI/S	Ile	Arg	Asn	Phe	Gly	Lys	Ala	Thr
Clinical Samples	CS1318	Ile	Arg	Asn	Ser	Ala	Lys*	Ala	Ala
	CS1338	Ile*	Arg	Asn	Ser	Gly	Glu	Ala*	Ala
	CS1452	Ile	Arg	Asn	Ser	Ala*	Glu	Gly*	Ala
	CS1648	Ile	Cys	Asn	Ser	Gly	Glu	Ala	Ala
	CS1915	Ile*	Arg	Asn	Ser	Gly	Glu	Ala	Ala
Reference	WT	Asn	Cys	Ser	Ser	Ala	Lys	Ala	Ala
	M	Ile	Arg	Asn/Thr	Ala/Phe	Gly	Glu	Gly	Ser/Thr

The last two rows portray the wild type (WT) and mutant (M) genotypes for each polymorphism. See text for citations. Clinical samples that were identified as mixed with real-time PCR are indicated with a "".

Figure B.1 Real-time PCR detection of *P. falciparum* genotypes. Plasmid DNA containing wild type and mutant template DNA (as labeled) were amplified with mixtures of wild type probe (FAM labeled, solid line) and mutant probe (VIC-labeled, broken line). Relative fluorescence was calculated by subtracting the minimal fluorescence from each value.

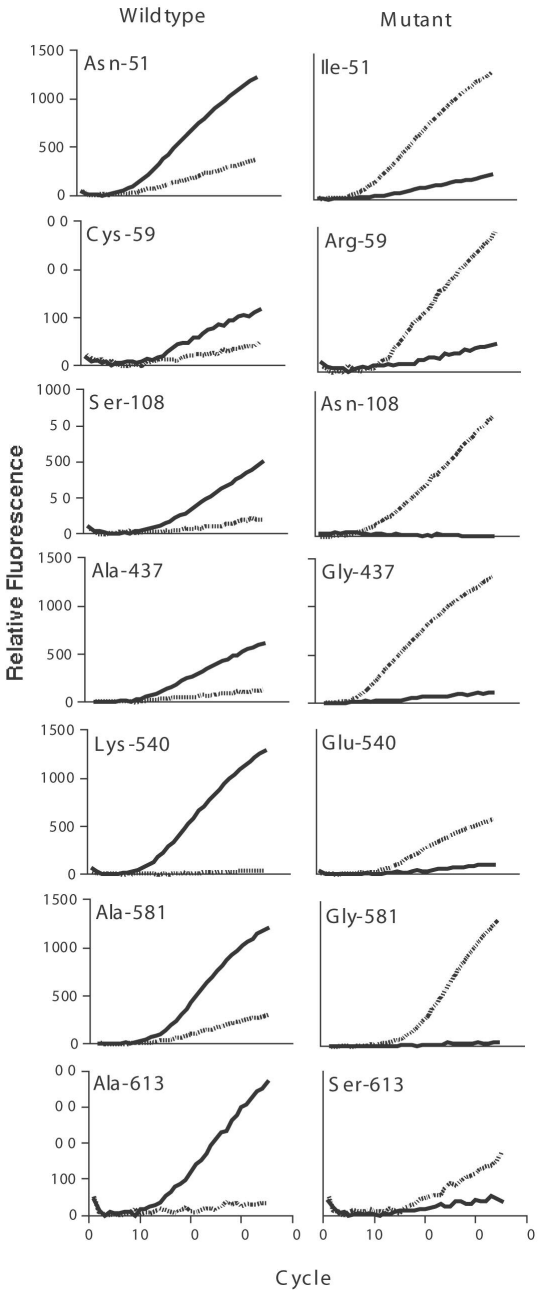


Figure B.2 Real-time PCR genotyping of a mixed genotype sample. Various mixtures of wild type and mutant plasmid DNA were amplified. The results for the 51 VIC probe are presented as averages of three replicates from which the minimal fluorescence has been subtracted. The solid line represents a 100% mutant sample. The gray line represents a 10% mutant and 90% wild type sample. The broken line represents a 100% wild type sample.

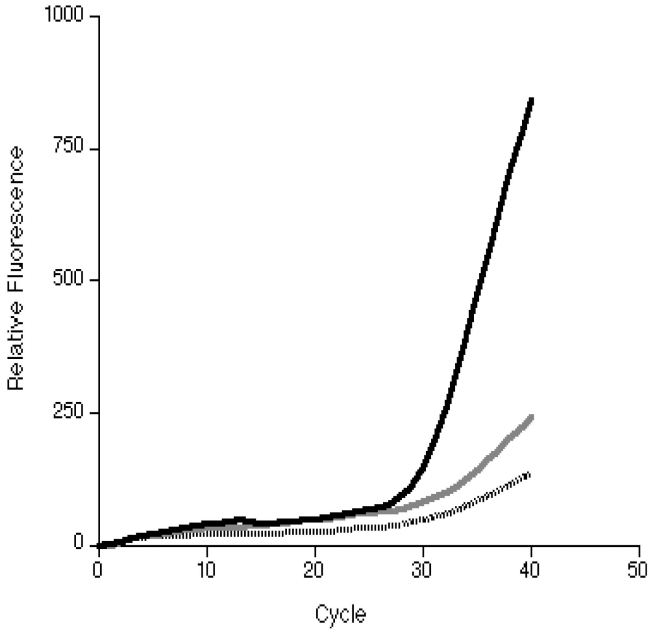
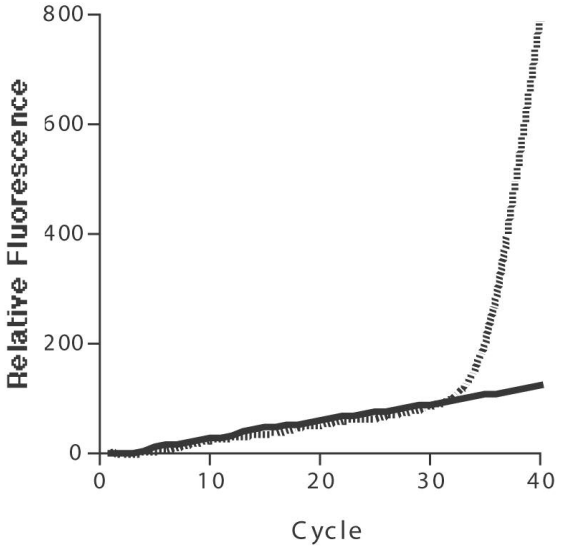


Figure B.3 Real-time PCR genotyping of 108 polymorphism in clinical sample CS1338. The FAM labeled probe (solid line) is designed to detect the wild type sequence. The VIC labeled probe, (broken line) is designed to detect the mutant sequence. The change of fluorescence of the two probes was compared to controls ran at similar concentrations. The genotype was determined to be mutant (Asn/Thr) with no wild type component. Relative fluorescence was calculated by subtracting the minimal fluorescence from each value.



APPENDIX C:

MUTATIONS ASSOCIATED WITH SULFADOXINE-PYRIMETHAMINE AND CHLORPROGUANIL RESISTANCE IN PLASMODIUM FACLIPARUM FROM BLANTYRE, MALAWI

by

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ABSTRACT

We conducted a prevalence study of mutations in *Plasmodium falciparum* that are associated with antifolate resistance in Blantyre, Malawi. The dihydrofolate reductase (*dhfr*) 164-leu mutation, which confirs resistance to both pyrimethamine and chlorproguanil, was found in 4.7% of the samples. Previously unreported mutations in dihydropteroate synthase (*dhps*) were also found.

INTRODUCTION

Resistance to sulfadoxine-pyrimethamine (SP) in *Plasmodium falciparum* is conferred by mutations in the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes. The five main mutations ("quintuple mutant": *dhfr*-51, 59, 108 and *dhps*-437 and 540) strongly predict clinical outcome (70, 82). Mutations in *dhfr*-164, *dhps*-581 and *dhps*-613 develop later in a population, and are associated with increased SP resistance (75).

One proposed alternative to SP is chlorproguanil-dapsone (LapDap). Both chlorproguanil and pyrimethamine target *dhfr*, and changes at codon 164 confer resistance to both drugs (69). This cross-resistance might limit the usefulness of LapDap in areas where SP has been used heavily (209). Currently in Africa, the *dhfr*-164 mutation is rare. Nevertheless, the prevalence of this mutation should be monitored where LapDap is being considered as a candidate therapy.

In Malawi, SP has been the first-line agent for malaria since 1993. The quintuple mutation is highly prevalent: 78% of samples contained the quintuplet mutation in the Salima district in 2001 (210). However, subsequent mutations (*dhps*-613, *dhps*-581 and *dhfr*-164) have not

been previously reported. The purpose of this study is to determine the prevalence of mutations in *dhfr* and *dhps* in Blantyre, Malawi.

METHODS

Clinical samples of *Plasmodium falciparum* were acquired from pregnant women at Queen Elizabeth Hospital (Blantyre, Malawi) who were enrolled in a study investigating mother-to-child transmission of HIV (205). Venous blood was taken at enrollment, and the plasma and packed-cell fractions were frozen at –80 °C. Malaria was screened for using thick-blood-smear microscopy. A random sample of the *Plasmodium falciparum* -positive samples were included in this study. 22 samples were transferred to filterpaper and processed as previously described (142). 68 additional samples were shipped frozen to UNC Chapel Hill, where DNA was extracted using the Qiamp DNA mini kit (Qiagen). The samples for this study were collected between March 2001 and May 2003. In this subgroup, 15.7% report taking SP within the last two weeks while 85.4% report taking SP at some point during their pregnancy. No one reported using LapDap.

The 22 samples from bloodspots were previously genotyped for codons 51, 59, and 108 in *dhfr* and codons 437, 540, 581 and 613 in *dhps* (142). However, the *dhfr*/*dhps* genotype was reported only for 5 samples. The prevalence of the mutations in all 22 samples will be reported here.

dhfr-164 was genotyped using a new real-time PCR assay. Two minor groove binder (MGB) probes were developed: one that detects the isoleucine (wild-type) sequence and another that detects the leucine (mutant) sequence (Table C.1). The primers and probes were synthesized by MWG Biotech (High Point, NC) and Applied Biosystems (ABI),

respecitively. PCR reactions were carried out in duplicate in 25 µl reactions containing 12.5 µl Universal PCR Master Mix (ABI), 2 µl DNA, forward primer at 300nM, reverse primer at 500 nM, and both probes at 200 nM. All reactions were run on an ABI PRISM 7000 (ABI); they were initially denatured at 95 ºC for 10 minutes and cycled 45 times, with each cycle consisting of 92 ºC for 14 s and 60 ºC for 60 s. 21 samples from bloodspots and 68 samples from peripheral blood were tested using this assay. The results were analyzed as previously described (142).

To validate this assay, genomic DNA (Dd2, HB3, W2, FCR3, K1, and VI/S from the Malaria Reagent Repository Resource, http://www.malaria.mr4.org/) was genotyped for *dhfr*-164. The assay correctly identified VI/S as mutant and the rest as wild-type. Clinical samples were sequenced for further validation. The amplified product from the real-time reaction was purified using CentriSpin-10 columns (Princeton Separations) then sequenced as previously described (142).

RESULTS

Of the samples from blood spots, 90.1% contained the quintuple mutation (Figure C.1). One sample (4.6%) contained a *dhps*-613 mutant component while three different samples (13.6%) contained a *dhps*-581 mutant component. These mutations were only observed in mixed infections.

Because of its potential pubic health implications, *dhfr*-164 was genotyped in a larger number of samples. Genotyping was successful for 18 out of 21 blood spot samples and 67 out of 68 samples from stored blood. In the resulting 85 samples, four contained the *dhfr*-164-leu mutation, resulting in a prevalence of 4.7% (95% confidence interval: 0, 9.2%). One

sample contained only the mutant *dhfr*-164 genotype while the rest contained a mixture of wild-type and mutant genotypes. The genotypes of the fully mutant sample and two wild-type samples were confirmed with sequencing. The four samples with the *dhfr*-164 mutation also contained the quintuple mutation but were wild-type at *dhps*-581 and *dhps*-613.

DISCUSSION

In Malawi, the 14 day clinical efficacy of SP appears to have stabilized at ~80% in children under 5 between 1998-2002, which has been attributed to delay in emergence of the *dhfr*-164 and *dhps*-581 mutations (211). This is the first report of the *dhfr*-164, *dhps*-581, and *dhps*-613 mutations in clinical samples from Malawi. The presence of these mutations suggests resistance to SP may be increasing.

The presence of the *dhfr*-164-leu mutation is also troubling because it confers cross-resistance to chlorproguanil (LapDap). In Africa, the *dhfr*-164 mutation is rarely found: previous reports of this mutation were from travelers, people treated with LapDap, and as a rare component of samples (88, 188, 189, 212, 213). In contrast, we report the *dhfr*-164 mutation as a major component of samples in African women who report not taking LapDap.

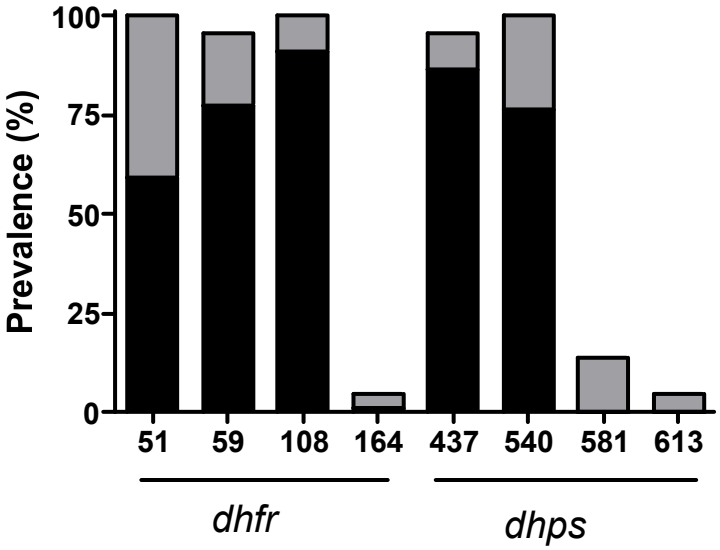
Our screening for mutations in *dhfr* and *dhps* was restricted to pregnant women in Blantyre with previous SP exposure, and therefore the prevalence of these mutations in the general population is uncertain. In fact, recent clinical trials of LapDap in children in Blantyre proved effective (214, 215), suggesting that the *dhfr*-164 mutation remains rare. Surveillance for these mutations in the general population is needed, and results must be related to those of *in vivo* efficacy studies to confirm these mutations are associated with high levels of clinical SP or LapDap resistance. New technologies for SNP detection, such as

real-time PCR, enable the rapid screening of a large number of samples and can be employed for the monitoring of parasite mutations associated with drug resistance.

Table C.1 Sequences of primers and probes used in real-time PCR for the *dhfr*-164 genotyping assay

Type	Sequence (5'→3')
Forward primer	ATC ATT AAC AAA GTT GAA GAT CTA ATA GTT TTA
Reverse primer	TCG CTA ACA GAA ATA ATT TGA TAC TCA
Wild-type probe	6FAM-ATG TTT TAT TAT AGG AGG TTC CGT T-MGB
Mutant probe	VIC-ATG TTT TAT TTT AGG AGG TTC CGT T-MGB

Figure C.1 The prevalence of mutant, and mixed genotypes in samples from pregnant women with malaria in Blanytre, Malawi. A mutant genotype is defined as: *dhfr*-51-Ile, *dhfr*-59-Arg, *dhfr*-108-Asn/Thr, *dhfr*-164-Leu, *dhps*-437-Gly, *dhps*-540-Glu, *dhps*-581-Gly, *dhps*-613-Ser/Thr. The sample size is 22 for all codons except for *dhps*-540 (n=21) and *dhfr*-164 (n=85). Black = mutant and gray = mixed.



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